The role of Natural Killer cells in adaptive immune responses

Amir Horowitz

Thesis submitted to the University of London in fulfilment of the requirements for the Doctorate of Philosophy

August 2010

Faculty of Infectious and Tropical Diseases
Department of Immunology and Infection
London School of Hygiene and Tropical Medicine
Keppel Street
London WC1E 7HT

I, Amir Horowitz, confirm that the work presented in this thesis is my own.

Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed.....[redacted].....
August 2010

Abbreviations

APCs	antigen presenting cells
BCG	*Mycobacterium bovis* Bacillus-Calmette Guerin
CBA	cytometric bead array
CFSE	carboxyfluorescein diacetate, succinimidyl ester
CHS	contact hypersensitivity
CI	confidence interval
CMV	cytomegalovirus
CS	circumsporozoite
CSA	chondroitin sulfate A
CTL	cytotoxix lymphocyte
DMSO	dimethyl sulfoxide
DV	Dengue virus
E	Envelope protein
ELISA	enzyme-linked immunosorbant assay
GM	growth medium
HBs	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HI-FCS	heat-inactivated fetal calf serum
HLA	human leukocyte antigen
ICAM-1	intercellular adhesion molecule-1
IFN	Interferon
Ig	immunoglobulin
IL	Interleuken
IPT	intermittent preventive anti-malarial treatments
ILTs	immunoglobulin-like transcripts

ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
KIR	Killer Ig-like receptor
LAMP	lysosomal associated membrane protein
LPS	Lipopolysaccharide
mDCs	myeloid lineage dendritic cells
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MNC	mononuclear cell
MOI	multiplicity of infection
nAchR	nicotinic acetylcholine receptor
NCAM	neural cell adhesion molecule
NCP	nucleocapsid protein
NK	Natural Killer
PAMPs	pathogen associated molecular patterns
PBMC	peripheral blood mononuclear cells
PEP	post-exposure prophylaxis
PfEMP-1	*Pf*-erythrocytic membrane protein-1
***Pf*-RBC**	*Plasmodium falciparum*-infected erythrocytes
P/I	PMA/ionomycin
PRRs	pattern recognition receptors
RIG	rabies immunoglobulin
RNP	ribonucleoprotein
RV	rabies virus
SE	standard error
TCR	T cell receptor
TLRs	Toll-like receptors
TSP	thrombospondin
WNV	West Nile virus

Abstract

Human natural killer (NK) cells have been shown to respond to numerous pathogenic stimuli, producing IFN-γ as well as cytolytic effector molecules such as perforin and granzymes. Previous work on NK cell responses to *Plasmodium falciparum*-infected erythrocytes (*Pf*-RBC) has shown that these responses are dependent on contact with accessory cells, such as macrophages and myeloid-lineage dendritic cells (mDCs), as well as on soluble mediators such as IL-2, IL-12, IL-15, IL-18 and IFN-α. It has also been observed that these responses are heterogeneous between donors; part of this heterogeneity seems to be genetically encoded, depending for example on killer immunoglobulin-like receptor (KIR) genotype, but heterogeneity in accessory cell stimuli has also been observed. The work described in this thesis further dissects the NK cell response to *Pf*-RBC in malaria naïve donors, with a focus on describing the role of T cells in NK cell responses to pathogens. My data demonstrate that while NK cells are dependent upon accessory cell signals, they are also highly dependent upon signals emanating from antigen-specific CD4+ T cells; T cell help for NK cell responses is MHC class II-dependent, IL-2-dependent and contact-independent. I have observed significant production of IL-2 from CD4+ T cells in the very early hours of co-incubation of peripheral blood mononuclear cells (PBMC) and *Pf*-RBC, preceding NK cell-derived IFN-γ.

Having documented T cell-dependent NK cell activation by *Pf*-RBC in malaria naïve donors, I next explored whether similar T cell-dependent NK cell responses were observed in African children undergoing vaccination with the candidate pre-erythrocytic malaria vaccine, RTS,S. I characterized expression of CD69 and

production of IFN-γ in NK cells and T cells and expression of CD25 in T cells. My data demonstrate that robust recall NK cell and T cell responses are mounted during in vitro re-stimulation with the RTS,S vaccine antigen, Hepatitis B surface antigen (HBs) peptides and while circumsporozoite (CS)-induced IFN-γ responses were not as strong, expression of CD25 in T cells and CD69 in NK cells and in T cells were significantly higher in RTS,S vaccinated children than in rabies vaccinated controls. Nearly half of the IFN-γ was derived from NK cells. I also measured secreted levels of IFN-γ, IL-2 and IL-10 in culture supernatants. I observed high levels of IFN-γ in culture supernatants of RTS,S vaccinated PBMC only in response to HBs peptides, however, there was strong vaccine antigen-specific IL-2 production to both HBs and CS peptides, which was significantly more robust in RTS,S vaccinees than in the rabies vaccinated controls. Finally, my data demonstrate that the IL-2 secretion in response to HBs and CS peptides was highly correlated with the early activation of NK cells (expression of CD69).

Finally, to formally test the hypothesis that antigen-specific CD4+ T cells can enhance NK cell responses to pathogens, I carried out a study of the T cell and NK cell response to heat-killed rabies virus in individuals undergoing rabies vaccination. The results of this study demonstrate that vaccine antigen-specific CD4+ T cells induced by vaccination can recruit NK cells to secrete IFN-γ, to degranulate, to release perforin and to proliferate. The post-vaccination NK cell response is detected within 6-12 hours after re-exposure to rabies virus and, somewhat unexpectedly, is sustained for at least 7 days, well after the T cell response is underway. Importantly, in the first 24 hrs after re-exposure to virus, NK cells represent more than 70% of the IFN-γ secreting effector cells, indicating a potentially very important role for NK cells in the early phase of the post-vaccination effector response.

Ever since the immune system was dichotomized into innate and adaptive arms, vaccine-induced immunity has been explained solely in terms of priming of effector and memory B and T lymphocytes. The potential for innate immune cells to contribute to enhanced cytotoxicity or cytokine production post-vaccination has been almost completely overlooked. My data suggest that IL-2 secreting CD4+ T cells and NK cell activation markers, such as IFN-γ production, expression of CD69 and CD25, upregulation of the lysosome-associated membrane protein (LAMP)-1 and release of perforin, may prove to be more reliable indicators of vaccine efficacy than simply counting the numbers of IFN-γ-secreting PBMCs and that these parameters need to be considered for inclusion of future protocols for evaluating vaccine immunogenicity and efficacy in clinical trials.

Declaration

Laboratory work

The data presented in **Chapter 3** of this thesis were performed mostly by myself with some help, early on, from Dr. Kirsty Newman (LSHTM, London, UK) as well as a collaboration with Prof. Daniel Davis (Imperial College, London, UK) and his PhD student J. Henry Evans (Imperial College, London, UK). The data presented in **Chapter 4** of this thesis were produced in partnership with Dr. Julius Hafalla (LSHTM, London, UK). The pooled peptides, representative of Hepatitis B surface antigen (HBs) and of the circumsporozoite (CS) proteins, were provided by GSK biologicals (Belgium). All vaccinations (RTS,S/ASO1E and rabies virus vaccine) were administered before my involvement in the phase IIb trial. The data presented in **Chapter 5** of this thesis were produced exclusively by myself. All vaccines were administered at LSHTM by Dr. Ron Behrens (Hospital for Tropical DEiseases (HTD)/LSHTM, London, UK) and Bernadette Carroll (HTD, London, UK). The data presented in Figure 5.12 used a recombinant nucleocapsid protein, derived from rabies virus that was produced and kindly provided by Dr. M. Juozapaitis (The Institute of Biotechnology, Vilnius, Lithuania).

Phase IIb trial of the malaria vaccine RTS,S/ASO1E

The phase IIb trial was part of an ongoing study designed and implemented by Glaxo Smith Kline (GSK). The RTS,S malaria vaccine trial was designed and sponsored by GlaxoSmithKline Biologicals (Rixensaart, Belgium), funded by the Malaria Vaccine Initiative (Bethesda, Maryland, USA) and implemented by the Joint Malaria

Programme (Korogwe, Tanzania), Tanzania under the leadership of the trial co-ordinators Dr Lorenz von Seidlein and Dr John Lusingu.

Analysis

I undertook all analyses unless stated otherwise. The statistical analyses for all data presented in **Chapter 4** of this thesis were performed by Dr. Philip Bejon (Nuffield Department of Medicine, Oxford University, Oxford, UK). Statistical analyses for most of the data presented in **Chapter 5** of this thesis were performed by myself, with assistance from Dr. Patrick Corran and Dr. Lucy Okell. The statistical analyses for data in **Figures 5.9c** and **5.11** were performed by Dr. Lucy Okell.

Publications

Chapter 3 has been adapted from an article published in *Journal of Immunology* by myself, Kirsty Newman, J. Henry Evans, Daniel Korbel, Daniel Davis and Eleanor Riley (Horowitz, Newman et al. 2010) as well as from an article published in Methods of Molecular Biology by myself and Eleanor Riley (Horowitz and Riley 2010).

Chapter 4 has been adapted from a manuscript, which is in preparation for submission. **Chapter 5** has been adapted from an article published in *Journal of Immunology* by myself, Ron Behrens, Lucy Okell, Anthony Fooks and Eleanor Riley (Horowitz, Behrens et al. 2010).

Acknowledgments

I would like to thank my supervisor Eleanor Riley for giving me the opportunity to take on this project. I have learned a great deal from her on how to think and write like a scientist over these last 3 years. She has put a great deal of faith and trust in my ability to get the job done. Consequently, she gave me a great deal of latitude to run in many directions that I chose to. Thanks so much for everything you've done for me over these last 3 years, Eleanor. You have been a wonderful mentor!

I would also like to thank the entire Riley group: Patrick Corran has helped me with everything from statistics to scientific writing and presentation; Brian de Souza has been there during the 7 AM shifts for the last 3 years and I have enjoyed our scientific discussions very much. He has always been there to provide me with guidance when I just can't wait for another 2 hours before others start coming into work; Kevin Couper has been an enormous help with respect to murine immunology and there's truly very little that ever escapes him; Chris Drakeley has mostly been a pain in the ass...running in the opposite direction every time I have a question for him, but he's been lots of fun and extremely helpful when I trap him in his office; Julius Hafalla has been an amazing colleague and friend to me over the last 3 years. He has always made himself available to me for both work and non-work matters. Thanks so much, Jules!; Jackie Cook has also been amazing to have in the lab. Without Jackie I would have been lost on statistics...makes it look really difficult (better than impossible!). Emily Findlay and Ana Villegas Mendez have been around the shortest amount of time but they are both great desk mates. Thanks for letting me bounce questions off you guys. As we share the office/lab with the Dockrell group, I must express many thanks to Jackie Cliff and Steve Smith. They are both brilliant immunologists and have been incredibly kind to me over the years. Thank you both very much for making my experience so enjoyable.

I would like to Thank Henry Evans and Dan Davis as well as Ron Behrens and Anthony Fooks. It was a wonderful experience getting to collaborate with you all and I hope that these projects continue to produce lots of meaningful advances in the future.

I would like to especially thank Carolynne Stanley and Liz King. Without and doubt, you both are the glue that holds this place together. Thanks also to all of my blood donors and study subjects, both in London and overseas.

I would like to thank Siobhan Renihan and Higina Fernandes. You both have taken amazing care and concern for my well-being while here in London. Siobhan, there's always a job for you on the ranch in Texas! Of course, you'll have to drop your British accent and step into the women that you know you are.

To Cleetus and Babs (my surrogate work family), you both have been my very best friends here, both at LSHTM and in London! I know that we'll always remain as close no matter where we are. I will always know what situations will remind me of you both. Which situations will remind you of me? I would also like to thank Vanessa Yardley. She has also been an amazing friend both in and out of the office. Thanks for making London so bearable, Vanessa.

I would like to especially thank all of my friends back home and especially my family: Sharon, Tom, Ayelet, Nim, Matt, Jenn, Janet, Lenny, Mateo and Phin. You all have been so incredibly supportive of me over the years. You make me feel very proud of who I have become! Thank you.

And finally to my fiancé, Andrea. THANK YOU SO MUCH! I love you with all of my heart and am grateful for your putting up with everything that you've had to deal with during this experience!!! I have always looked up to you both personally and professionally, Andrea. I hope that you will be proud of calling me Dr. Amir!!

Table of Contents

LIST OF ABREVIATIONS ..... **(I)**

ABSTRACT **2**

DECLARATION **5**

ACKNOWLEDGMENTS **7**

LIST OF TABLES & FIGURES **8**

CHAPTER 1: INTRODUCTION **11**

1.1 INNATE AND ADAPTIVE IMMUNE RESPONSES 12
1.2 INNATE IMMUNE RESPONSE TO INFECTION 13
1.3 NK CELL RESPONSES 13
1.4 CLASSICAL OR DIRECT ACTIVATION OF NK CELLS 14
1.5 INDIRECT ACTIVATION OF NK CELLS 18
1.6 NK CELLS IN ADAPTIVE IMMUNE RESPONSES 22
1.7 AIM OF THE PROJECT 25

CHAPTER 2: GENERAL MATERIALS AND METHODS **27**

2.1 ISOLATION OF PBMC 28
2.2 CRYOPRESERVATION OF PBMC 28
2.3 *PLASMODIUM FALCIPARUM* CULTURE 29
2.4 PREPARATION OF ANTIGENS FOR IN VITRO STIMULATION ASSAYS 30
2.4.1 PURIFICATION OF LIVE SCHIZONTS 30
2.4.2 *MYCOBACTERIUM BOVIS* BACILLUS CALMETTE-GUERIN CULTURE 30
2.4.3 PREPARATION OF RTS,S MALARIA VACCINE ANTIGENS 31
2.4.4 PREPARATION OF RABIES VACCINE ANTIGENS 31
2.5 CELL CULTURE 31
2.6 DEPLETION OF SPECIFIC SUBSETS OF T CELLS 32
2.6.1 CD3 DEPLETION 32
2.6.2 CD4+, CD8+, TCR-$\gamma\delta$+ AND TCR-$\alpha\beta$+ T CELL POPULATIONS 33
2.7 CELL PHENOTYPING USING FLOW CYTOMETRY 34

CHAPTER 3: CROSS-TALK BETWEEN T CELLS AND NK CELLS GENERATES RAPID EFFECTOR RESPONSES TO P. FALCIPARUM-INFECTED ERYTHROCYTES **37**

3.1 INTRODUCTION 38
3.1.1 THE BURDEN OF MALARIA 38
3.1.2 MALARIA PARASITES AND PATHOGENESIS 42
3.1.3 INNATE IMMUNE RESPONSES TO *PLASMODIUM* INFECTION 44
3.1.4 CELLULAR SOURCES OF IFN-γ 44
3.1.5 NK CELLS IN *P. FALCIPARUM* INFECTION 45
3.2 MATERIALS AND METHODS 49
3.2.1 BLOOD DONORS 50
3.2.2 *P. FALCIUPARUM* PARASITES 50
3.2.3 *M. BOVIS* BCG 50
3.2.4 DEPLETION OF T CELL SUBSETS 51
3.2.5 BLOCKING EXPERIMENTS 51
3.2.6 CELL SURFACE AND INTRACELLULAR STAINING FOR FLOW CYTOMETRY 52
3.2.7 CYTOKINE ASSAYS 52
3.2.8 STATISTICAL ANALYSIS 52
3.3 RESULTS 54
3.3.1 T CELLS AND NK CELLS BOTH CONTRIBUTE TO THE EARLY IFN-γ RESPONSE TO P. FALCIPARUM 54

3.3.2 NK CELL AND T CELL RESPONSES TO *PF*-RBC ARE BOTH CYTOKINE AND MHC CLASS II DEPENDENT 60
3.3.3 NK CELL AND T CELL RESPONSES TO PF-RBC ARE DEPENDENT UPON T CELL HELP 68
3.3.4 CD4+ T CELLS PRODUCE IL-2 WITHIN 12 HOURS OF CO-CULTURE WITH *PF*-RBC 73
3.3.5 γδ T CELLS PRODUCE TNF IN RESPONSE TO *PF*-RBC BUT TNF IS NOT REQUIRED FOR NK CELL OR T CELL IFN-γ RESPONSES 75
3.3.6 T CELL HELP FOR NK CELL ACTIVATION BY *PF*-RBC DOES NOT REQUIRE NK CELL-T CELL CONTACT 77
3.4 DISCUSSION 79

CHAPTER 4: MARKERS OF T CELL AND NK CELL RECALL RESPONSES TO VACCINE ANTIGENS IN A PHASE IIB TRIAL OF THE MALARIA VACCINE RTS,S/ASO1E IN TANZANIAN CHILDREN 84

4.1 INTRODUCTION 85
4.2 MATERIALS AND METHODS 89
4.2.1 STUDY SUBJECTS AND VACCINATIONS 89
4.2.2 PBMC PREPARATION AND CULTURE 91
4.2.3 ANTIGENS: HBs AND CS PEPTIDES 91
4.2.4 CELL SURFACE AND INTRACELLULAR STAINING BY FLOW CYTOMETRY 92
4.2.5 CYTOKINE SECRETION ASSAYS 92
4.2.6 SEROLOGY 92
4.2.7 HEPATITIS B VIRUS ANTIBODY MEASUREMENT 93
4.2.8 STATISTICAL ANALYSIS 93
4.3 RESULTS 94
4.3.1 T CELL RESPONSES TO HBs AND CS PEPTIDES IN RTS,S/ASO1E VACCINATED SUBJECTS 94
4.3.2 NK CELLS ARE ALSO ACTIVATED BY HBs AND CS PEPTIDES IN RTS,S/ASO1E VACCINATED CHILDREN 96
4.3.3 NK CELLS AND T CELLS CONTRIBUTE EQUALLY TO RTS,S-VACCINE ANTIGEN RECALL RESPONSES 99
4.3.4 DETECTION OF SOLUBLE CYTOKINES IN SUPERNATANTS FROM PBMC IN RESPONSE TO HBs AND CS PEPTIDES 102
4.3.5 PRODUCTION OF IL-2 IS CORRELATED WITH ACTIVATION OF NK CELLS IN RTS,S VACCINEES IN RESPONSE TO HBs AND CS PEPTIDES 105
4.4 DISCUSSION 109

CHAPTER 5: NK CELLS AS EFFECTORS OF ACQUIRED IMMUNERESPONSES: EFFECTOR CD4+ T CELL-DEPENDENT ACTIVATION OF NK CELLS FOLLOWING RABIES VIRTUS VACCINATION 114

5.1 INTRODUCTION 115
5.1.1 THE BURDEN OF RABIES VIRUS 116
5.1.2 RABIES VIRUS AND PATHOGENESIS 120
5.1.3 IMMUNOPRPHYLAXIS 124
5.2 MATERIALS AND METHODS 126
5.2.1 VACCINATION OF STUDY PARTICIPANTS 126
5.2.2 BLOOD SAMPLING, PBMC PREPARATION AND CELL CULTURE 126
5.2.3 CELL SURFACE AND INTRACELLULAR STAINING BY FLOW CYTOMETRY 127
5.2.4 STATISTICAL ANALYSIS 127
5.3 RESULTS 128
5.3.1 T CELLS AND NK CELLS UPREGULATE CD69 AND PRTODUCE IFN-γ IN RESPONSE TO RABIES ANTIGEN, AFTER RABIES VACCINATION 128
5.3.2 POST-VACCINATION, NK CELLS MAKE BOTH EARLY AND SUSTAINED IFN-γ RESPONSES TO VACCINE ANTIGENS 131
5.3.3 NK CELLS DEGRANULATE AND RELEASE PERFORIN IN RESPONSE TO RABIES ANTIGEN AFTER RABIES VACCINATION 133
5.3.4 ANTIGEN-SPECIFIC IL-2 PRODUCTION FROM CD45RO+ CD4+ T CELLS IS REQUIRED FOR NK CELL RECALL RESPONSES 138

5.3.5 EXTENSIVE NK CELL PROLIFERATION DURING POST-VACCINATION RECASLL RESPONSE .. 142
5.3.6 IL-12 AND IL-18 SYNERGIZE WITH IL-2 TO ACTIVATE NK CELLS AFTER VACCINATION 144
5.3.7 THE RECALL NK CELL RESPONSE TO RABIES VIRUS IS AUGMENTED IN THE PRESENCE OF RABIES IMMUNE SERUM 146
5.3.8 PRE-VACCINATION NK CELLS RESPOND TO RABIES VIRUS 07/162 WHEN CULTURED WITH PRIMED (POST-VACCINATION) T CELLS 149
5.4 DISCUSSION 151

CHAPTER 6: FINAL DISCUSSION 160

6.1 STUDY SIGNIFICANCE 161
6.2 KEY FINDINGS AND FUTURE PERSPECTIVES 163
6.2 (i) MALARIA 163
6.2 (ii) VACCINATION 166
6.3 CONCLUDING REMARKS 170

BIBLIOGRAPHY 172

APPENDIX 1: CROSS-TALK BETWEEN T CELLS AND NK CELLS GENERATES RAPID EFFECTOR RESPONSES TO PLASMODIUM FALCIPARUM-INFECTED ERYTHROCYTES .....

APPENDIX 2: NK CELLS AS EFFECTORS OF ACQUIRED IMMUNERESPONSES: EFFECTOR CD4+ T CELL-DEPENDENT ACTIVATION OF NK CELLS FOLLOWING VACCINATION.....

List of tables and Figures

List of Tables

CHAPTER 1

1.1 NK CELL RECEPTORS INVOLVED IN CLASSICAL ACTIVATION OR INHIBITION PATHWAYS 16

CHAPTER 2

2.1 ANTIBODY COCKTAILS USED FOR 4 COLOUR FLOW CYTOMETRY 35
2.2 ANTIBODY COCKTAIL USED FOR 8 COLOUR FLOW CYTOMETRY 36
2.3 ANTIBODY COCKTAIL USED FOR 9 COLOUR FLOW CYTOMETRY 36

CHAPTER 4

4.1 BASELINE CHARACTERISTICS OF SUBJECTS ACCORDING TO VACCINE GROUP 90
4.2 THE PROPORTION OF VACCINATED CHILDREN WHOSE PBMCS MADE AN NK OR T CELL RESPONSE TO VACCINE 102
4.3 THE PROPORTION OF VACCINATED CHILDREN WHOSE PBMCS SECRETED CYTOKINES IN RESPONSE TO RTS,S-VACCINE ANTIGENS 105

CHAPTER 5

5.1 RABIES VACCINES LICENSED AND MARKETED IN THE UNITED STATES, 2008 118
5.2 STATISTICAL ANALYSIS OF DATA PRESENTED IN FIGURE 5.10C 141

List of Figures

CHAPTER 1

1.1 DIRECT ACTIVATION OF NK CELLS 15
1.2 INDIRECT ACTIVATION OF NK CELLS UPON INTERACTION WITH ACCESSORY CELLS AND PATHOGENS 20
1.3 SIGNALS PASSED FROM ACCESSORY CELLS TO NK CELLS 21

CHAPTER 3

3.1 THE BURDEN OF MALARIA 38
3.2 MALARIA LIFE CYCLE 41
3.3 POTENTIAL PATHWAYS OF NK CELL ACTIVATION BY *P. FALCIPARUM*-INFECTED ERYTHROCYTES 45
3.4 TITRATING *PF*-RBC INPUT DOSE TO DEFINE OPTIMAL MULTIPLICITY OF INFECTION 53
3.5 NK CELLS AND T CELLS CONTRIBUTE TO THE EARLY IFN-γ RESPONSE TO *PF*-RBC 55
3.6 IFN-γ RESPONSE OF NK CELLS AND T CELL SUBSETS 57
3.7 REPRESENTATIVE FLOW CYTOMETRY PLOTS TO SHOW THE EFFECTS OF BLOCKING ANTIBODIES ON NK CELL AND T CELL IFN-γ RESPONSES TO *PF*-RBC 59
3.8 NK CELL AND T CELL RESPONSES TO *PF-RBC* ARE BOTH CYTOKINE AND MHC-CLASS II DEPENDENT 61

3.9 TITRATING *M. BOVIS* BCG INPUT DOSE TO DEFINE OPTIMAL MULTIPLICITY OF INFECTION FOR NK CELL ACTIVATION 62
3.10 PRIOR BCG VACCINATION HAS NO SIGNIFICANT EFFECT ON THENK CELL IFN-γ RESPONSE TO BCG 64
3.11 NK CELL AND T CELL RESPONSES TO BCG ARE ALSO MHC-CLASS II DEPENDENT 64
3.12 NK CELL AND T CELL RESPONSES TO LPS ARE INDEPENDENT OF MHC CLASS I AND CLASS II PRESENTATION 66
3.13 REPRESENTATIVE FLOW CYTOMTERY PLOTS DEMONSTRATING EFFECTS OF DEPLETING T CELL SUBSETS ON IFN-γ PRODUCTION BY NK CELLS IN RESPONSE TO *PF*-RBC 67
3.14 NK CELL RESPONSES TO *PF*-RBC ARE DEPENDENT UPON $CD4^+$ T CELL HELP 69
3.15 NK CELL RESPONSES TO *M. BOVIS* BCG ARE DEPENDENT UPON $CD4^+$ T CELL HELP 70
3.16 NK CELL RESPONSES TO LPS ARE INDEPENDENT OF T CELL HELP 70
3.17 RAPID IL-2 PRODUCTION BY $CD4^+$ T CELLS AFTER CO-CULTURE WITH *PF-RBC* 72
3.18 EARLY IL-2 PRODUCTION IS HIGHLY CORRELATED WITH NK CELL IFN-γ PRODUCTION IN RESPONSE TO *PF-RBC* 72
3.19 RAPID TNF PRODUCTION FROM MACROPHAGES AND γδ+ T CELLS IN RESPONSE TO *PF*-RBC 74
3.20 T CELL HELP FOR NK CELL ACTIVATION BY *PF-RBC* DOES NOT REQUIRE NK CELL-T CELL CONTACT 76

CHAPTER 4

4.1 SCHEMATIC OF RTS,S MALARIA VACCINE 86
4.2 AFTER RTS,S/ASO1E VACCINATION, T CELLS UPREGULATE EXPRESSION OF CD69 AND CD25 AND PRODUCE IFN-γ IN RESPONSE TO VACCINE ANTIGENS 95
4.3 NK CELLS UPREGULATE EXPRESSION OF CD69 AND PRODUCE IFN-γ IN RESPONSE TO RTS,S-VACCINE ANTIGENS 97
4.4 NK CELLS AND T CELLS CONTRIBUTE EQUALLY TO THE IFN-γ RECALL RESPONSE TO RTS,S VACCINE ANTIGENS 100
4.5 CYTOKINES SECRETED IN RESPONSE TO RTS,S VACCINE ANTIGENS 103
4.6 CORRELATIONS BETWEEN EXPRESSION LEVELS OF CD69 ON NK CELLS AND LEVELS OF SECRETED IL-2 IN RTS,S/ASO1E-VACCINATED CHILDREN IN RESPONSE TO HBS PEPTIDES 106
4.7 CORRELATIONS BETWEEN EXPRESSION LEVELS OF CD69 ON NK CELLS AND LEVELS OF SECRETED IL-2 IN RTS,S/ASO1E-VACCINATED CHILDREN IN RESPONSE TO CS PEPTIDES 108

CHAPTER 5

5.1 COUNTRIES AT RISK OF SPREAD OF RABIES VIRUS 119
5.2 A SCHEMATIC OF A FULLY ASSEMBLED RABIES VIRUS VIRION 120
5.3 A SCHEMATIC OF THE RABIES VIRUS LIFE CYCLE 121
5.4 A SCHEMATIC OF RABIES VIRUS INFECTING A MOTOR NEURON 122
5.5 AFTER RABIES VACCINATION, T CELLS AND NK CELLS UPREGULATE CD69 AND PRODUCE IFN-γ IN RESPONSE TO RABIES VIRUS 129
5.6 COMPARISON OF IFN-γ PRODUCTION BETWEEN $CD56^{BRIGHT}$ AND $CD56^{DIM}$ NK CELL SUBSETS 130
5.7 POST-VACCINATION, NK CELLS MAKE BOTH EARLY AND SUSTAINED IFN-γ RESPONSES TO VACCINE ANTIGENS 132
5.8 REPRESENTATIVE STAINING OF LAMP-1 AND PERFORIN EXPRESSION IN CD8+ T CELLS AND NK CELLS 135
5.9 POST-VACCINATION, NK CELLS DEGRANULATE AND RELEASE PERFORIN IN RESPONSE TO RABIES VIRUS 136
5.10 ANTIGEN-SPECIFIC IL-2 PRODUCTION FROM $CD45RO^+$ $CD4^+$ T CELLS IS REQUIRED FOR NK CELL RECALL RESPONSES 140
5.11 EXTENSIVE NK CELL PROLIFERATION DURING POST-VACCINATION RECALL RESPONSE 143
5.12 IL-12 AND IL-18 SYNERGIZE WITH IL-2 TO ACTIVATE NK CELLS AFTER VACCINATION 145
5.13 IMMUNE SERUM ENHANCES THE RECALL NK CELL RESPONSE TO RABIES VIRUS 146

5.14 MEASURING THE EFFECTS OF IMMUNE SERUM ON NK CELLS IN RESPONSE TO RABIES VIRUS OVER TIME 147
5.15 PRE-VACCINATION NK CELLS RESPOND TO RABIES VIRUS 07/162 WHEN CULTURED WITH POST-VACCINATION T CELLS 150
5.16 SCHEMATIC REPRESENTATION OF SUGGESTED PATHWAY FOR "RECALL" NK CELL RESPONSES 153
5.17 SCHEMATIC REPRESENTATION OF PROPOSED INTERACTIONS BETWEEN NK CELLS AND T CELLS AFTER VACCINATION 157

Chapter 1: Introduction

1. Introduction

1.1 *Innate and adaptive immune responses*

The innate immune system, comprising large populations of cells expressing non-rearranging receptors for non-self molecules, is designed to provide rapid but generic responses to foreign organisms. Conversely, the adaptive immune system comprises very many small populations of cells expressing highly diverse, clonally rearranged receptors for foreign antigens; adaptive responses are thus slow (since clonal expansion is required to generate sufficiently large populations of effector and memory cells) but highly specific. Immune memory is an intrinsic feature of adaptive responses and underpins the concept of vaccination but has been assumed to be lacking in the innate response.

Bi-directional interactions between the innate and adaptive immune systems are essential for effective immunity to infection as well as for successful vaccination. Innate effector cells amplify and direct the subsequent adaptive response, a function, which is deliberately imitated by vaccine adjuvants (Schijns 2000; Pashine, Valiante et al. 2005; Kanzler, Barrat et al. 2007). Conversely, a key mechanism by which antigen-specific T cells mediate anti-microbial immunity is by releasing cytokines (such as IFN-γ) that activate innate cells to kill pathogens or pathogen-infected cells. The cells that are commonly described in orchestrating the innate immune response include monocytes (which differentiate into macrophages), dendritic cells (DCs), $\gamma\delta$+ T cells, Natural Killer (NK) T cells and NK cells. Through contact-dependent as well as contact-independent (secretion of cytokines and chemokines) mechanisms, the innate immune response is largely responsible for directing/polarizing the downstream adaptive immune response, which is primarily credited to B and T lymphocytes.

1.2 *Innate immune response to infection*

While the innate immune response is viewed as having very little pathogen specificity, there are groups of pattern recognition receptors (PRRs), such as the toll-like receptors (TLRs),that are triggered by receptor engagement of pathogen associated molecular patterns (PAMPs) during phagocytosis of infected or non-self cells. These receptor-ligand interactions occur both at the surface of, as well as inside, the phagocytes. These phagocytes - most notably monocytes/macrophages, neutrophils and DCs - produce a wide array of cytokines (IL-12, IL-15, IL-18, IFN-α/β, IL-10 and TGF-β) following exposure to and recognition of pathogens, and the spectrum of the cytokine array will vary greatly depending on the specific PRRs engaged and will in turn influence the nature of the developing T cells. This cytokine cascade manifests in the form of inflammation, triggering microbicidal activity and priming the downstream B and T cell responses. Infection-induced immunopathology is commonly due to the poor regulation of these inflammatory cytokines.

1.3 *NK cell responses*

NK cells are classified as large, granular lymphocytes that are derived from bone marrow. NK cells are innate immune effectors that, by cytokine production or cytotoxicity, help to contain an infection until an effective adaptive T and B cell response is mounted. NK cells become activated either in the absence of potent inhibitory signalling via their cell surface receptors for self-MHC molecules (the "missing-self" phenomenon; direct/classical activation) and/or in the presence of activating signals provided by virally infected cells or by myeloid accessory cells responding to microbial ligation of pattern recognition receptors (indirect activation) (Newman and Riley 2007). To date, no antigen-specific receptors have been identified on human NK cells, although the some activating receptors have been shown to directly recognize pathogen-derived ligands, e.g. haemagglutinins on influenza-infected cells (Mandelboim, Lieberman et al. 2001).

1.4 *Classical or direct activation of NK cells*

NK cells were first defined by their spontaneous ability to kill transformed cells (Kiessling, Klein et al. 1975). In 1986, Kärre demonstrated in vivo that NK cells were able to target and kill tumour cells that had down-regulated expression of MHC class I molecules (Karre, Ljunggren et al. 1986; Ljunggren and Karre 1990). Later in 1990, Ljunggren and Kärre coined the term, "missing-self hypothesis" to describe the general phenomenon whereby cells that did not express markers of "self" would be killed by NK cells. More recently, it has become clear that the "classical" or "direct" pathways of activating NK cells are actually a balance between numerous activating and inhibitory receptors. A schematic representation of this balance of activating and inhibitory receptors is shown in **Figure 1.1**.

Table 1 summarizes our current understanding of NK activating and inhibitory receptors, with their respective ligands. To date, there are 3 known families of inhibitory receptors: Killer Ig-like receptors (KIRs) in humans or the equivalent Ly49 homodimers in mice, Immunoglobulin like transcripts (ILTs) and CD94/NKG2 heterodimers. All these families of inhibitory receptors recognize and bind to various regions of MHC class I molecules (classical HLA-A, -B, -C or HLA-E). Inhibition of NK cells is regulated via an immunoreceptor tyrosine-based inhibition motif (ITIM), which contains a conserved sequence of amino acids (S/I/V/LxYxxI/V/L) located in the cytoplasmic tails of these proteins. Upon triggering via binding to its respective ligand, the ITIM becomes phosphorylated by Src kinase, providing a docking site for recruitment of the phosphotyrosine phosphatases SHP-1 and SHP-2, or the inositol-phosphatase called SHIP. Once bound to ITIMs, these phosphatases then transduce signals to couple into distinct signaling pathways, which result in varying degrees of inhibition (Ono, Okada et al. 1997; Ravetch and Lanier 2000).

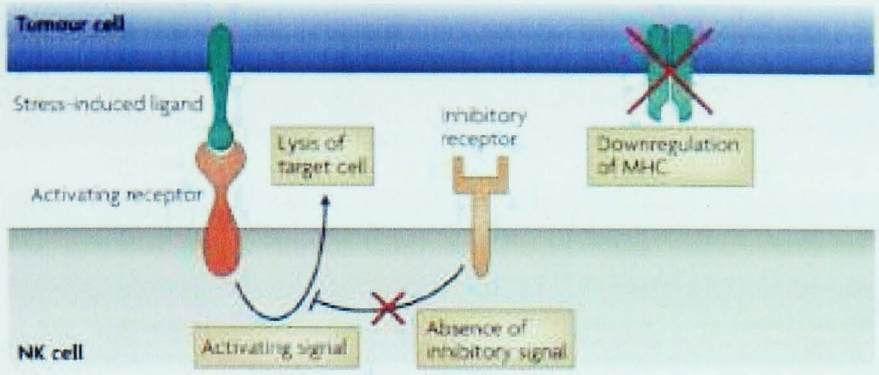


Figure 1.1 Direct activation of NK cells (Newman and Riley 2007).

The "classical" or direct model of activation of NK cells involves a balance of signals between inhibitory and activating NK cell receptors. Inhibitory receptors bind to MHC class I molecules and prevent NK cell-mediated killing of "self"-cells, whereas activating receptors bind to stress-induced ligands that are upregulated on infected or transformed cells. Numerous infections cause down-regulation of MHC class I molecules which skews the balance of strength between activating and inhibitory receptors allowing for effective activation of NK cells.

Receptor	Species	Class	Motif/Adaptor	Ligand
Activating receptor				
CD16 (FcγRIII)	**H, M**	**IgSF**	**ITAM/FcγR**	**Immune complexes**
CD94/NKG2C, E	H, M	C-lectin	ITAM/DAP12	HLA-E (H), Qa-1b (M)
NKG2D	H, M	C-lectin	YINM/DAP10, PI3K	MICA, B, ULBs (H), Rae1s(H, M), H60 (M)
KIR2S, KIR3S	H	IgSF	ITAM/DAP12	HLA class I
Ly49D, H, P	M	C-lectin	ITAM/DAP12	H-2 class I, MCMV m157 (Ly49H)
NCR (NKp30, 44, 46)	H, M (NKp46)	IgSF	ITAM/FcγR, CD3ζ (H), DAP12	Viral hemagglutinins (?)
ILT-1 (Ig-like transcript 1)	H	IgSF	ITAM/FcγR, DAP12	?
CD28	H, M	IgSF	YXXM/P13K	CD80,CD86
CD69	H,M	C-lectin	?	?
CD226 (DNAM-1)	H	IgSF	?	CD112 (Nectin-2),CD155
CD244 (2B4)	H,M	SLAM	TXYXXV-I/SAP,Fyn	CD48
Inhibitory Receptor				
CD85 (ILT-2)	H	IgSF	ITIM/SHP-1	HLA-A, -B, -G
CD94/NKG2A	H, M	C-lectin	ITIM/SHP-1, -2	HLA-E (H), Qa-1b (M)
KIR2DL, KIR3DL	H	IgSF	ITIM/SHP-1, -2	HLA class I
Ly49 A-C, E-G, I-O	M	C-lectin	ITIM/SHP-1, -2	H-2 class I

Table 1.1 NK cell receptors involved in classical activation or inhibition pathways (adapted from)(Di Santo 2006).
H = human
M = mouse
? = unknown

There also exist activating KIR and NKG2 molecules that lack ITIMs but instead associate non-covalently via a charged residue in the receptor transmembrane domain with the immunoreceptor tyrosine-based activating motif (ITAM)-containing adaptors DAP12, FCεRIγ, or CD3ζ. Natural cytotoxicity receptors, such as NKp30, 44 and 46 as well as the FcγRIIIA (CD16) also have charged residues, which associate with ITAMs. ITAMs differ from ITIMs in that they contain a conserved sequence of 4 amino acids that is repeated twice in the cytoplasmic tails (YxxL), which are commonly separated by 7-12 amino acids ($YxxL_{(7-12)}YxxL$). Upon phosphorylation of the tyrosine residues, these ITAMs form docking sites for the above mentioned adaptor proteins which recruit and activate the Syk or ZAP70 tyrosine kinases (or in

some cases involve the adaptor protein DAP10) and initiate the cell signaling pathways (Di Santo 2006, Orr and Lanier 2010). This mechanistic overview of classifying activating and inhibitory receptors has recently become somewhat confused in that numerous monovalent ligands have been described to induce inhibitory signaling when ITAM-phosphorylation occurs (Pinheiro da Silva, Aloulou et al. 2008).

There are also several co-activating/co-stimulatory receptors that are expressed on NK cells, such as CD2, LFA-1, CD244 (2B4) and CD226 (DYNAM-1). These receptors recognize self-derived ligands which are naturally found to be expressed on many tissues in the host (Lanier 2008).

It seems increasingly clear that the degree of direct activation of NK cells in response to pathogens, transformed or non-self cells is the result of skewing the balance between engagement of inhibitory and activating receptors. We know that, by engaging inhibitory receptors, MHC class I molecules expressed on normal cells prevent the activation of NK cells, but it is far less clear which activating receptors are required for NK cell activation when MHC class I molecules are down-regulated. NK cell-mediated killing of cytomegalovirus (CMV)-infected cells has been noted for *in vivo* (Karre, Ljunggren et al. 1986) in mice as well as *in vitro* (Nishimura, Stroynowski et al. 1988; Leiden, Karpinski et al. 1989; Pena, Alonso et al. 1990; Litwin, Gumperz et al. 1993). In numerous studies, down-regulation of MHC class I molecules has been documented in response to CMV infection, possibly as a mode of immune evasion from CTL-mediated killing of infected cells (Fletcher, Prentice et al. 1998; Biron, Nguyen et al. 1999; Tortorella, Gewurz et al. 2000).

Direct activation of NK cells has also been well characterized in Hepatitis C virus (HCV) infection. It has recently become understood that KIR2DL2 and KIR2DL3 molecules compete for binding to HLA-C (Khakoo, Thio et al. 2004; Ahlenstiel, Martin et al. 2008; Romero, Azocar et al. 2008). Because the binding affinity for KIR2DL3 interactions with HLA-C is significantly lower than for KIR2DL1-HLA-C interactions, NK cells from these individuals become more easily activated via the NKG2D-mediated pathway presumably by binding stress-inducible class I-like molecules (MICA/B) and ULBPs as has been demonstrated by numerous cancer models. This direct pathway of NKG2D-mediated activation of NK cells has been associated with protection from HCV persistence. The role of NK cells and KIR-MHC interactions in acute HCV infection is much less understood. Very recently, Amadei *et al.* demonstrated that NKG2D expression was upregulated in patients with acute HCV as compared to healthy controls (Amadei, Urbani et al. 2010). They also observed significantly higher levels of activation of NK cells in the patients with acute HCV as measured by IFN-γ production and degranulation (CD107a). Interestingly, subset analyses revealed that both $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets had elevated expression of NKG2D as well as both subsets carried HLA-C group 1 and 2 ligand-specific KIR receptors. These data strongly support the notion that balance between inhibitory and activating signaling will largely determine the whether the NK cells become activated.

1.5 *Indirect activation of NK cells*

Indirect activation of NK cells results secondarily from activation of accessory cells such as monocytes/macrophages and myeloid DCs (mDCs), following ligation of pathogen ligands by PRRs. While there are certainly scenarios of pathogens or pathogen-derived ligands that directly activate NK cells, most pathogens seem to activate NK cells indirectly, by way of accessory cells. Activation of antigen presenting cells (APCs) leads to secretion of soluble cytokines (e.g. IL-12, IL-15, IL-

18 and IFN-α) (Newman, Korbel et al. 2006; Newman and Riley 2007), which bind to cytokine receptors on NK cells. Contact-dependent interactions, (e.g. ICAM-1/LFA-1) (Baratin, Roetynck et al. 2007) also lead to NK cell activation. These observations have been documented in numerous viral [e.g. Influenza (Monteiro, Harvey et al. 1998; He, Draghi et al. 2004; Siren, Sareneva et al. 2004; He, Holmes et al. 2008)], bacterial [e.g. *Lactobacillus casei (Takeda, Suzuki et al. 2006)*] and protozoal [e.g. *Plasmodium falciparum* and *Leishmania major (Akuffo, Alexis et al. 1999; Newman, Korbel et al. 2006)*] models of infection.

There are numerous other viral and bacterial infections where NK cells have played significant roles in controlling acute infection. For example, a recent study characterizing flaviviruses (West Nile virus (WNV) and Dengue virus (DV)) demonstrated direct recognition of envelope (E) protein by NKp44 activating receptor and the data strongly suggested that this was a direct route for activation of NK cells (Hershkovitz, Rosental et al. 2009). However, NKp44 is only expressed on NK cells upon activation (Vitale, Bottino et al. 1998), which suggests that these cells must have received other signals (indirect activation) upstream to the E protein, e.g. soluble signals from accessory cells (IL-12, IL-18, IFN-α) and from T cells (IL-2). In another study, activation of NK cells was observed in response to *Mycobacterium bovis* BCG, *Nocardia farcinica* and *Pseudomonas aeruginosa (*Esin, Batoni et al. 2008). Esin *et al*. observed that NK cells were activated via NKp44 recognition of cell wall-derived proteins. These proteins, however, were not identified, and again NKp44-mediated signaling implies some prior upstream NK cell activation.

Figures 1.2 and 1.3 are schematic representations demonstrating the complex interactions between NK cells, APCs and pathogens.

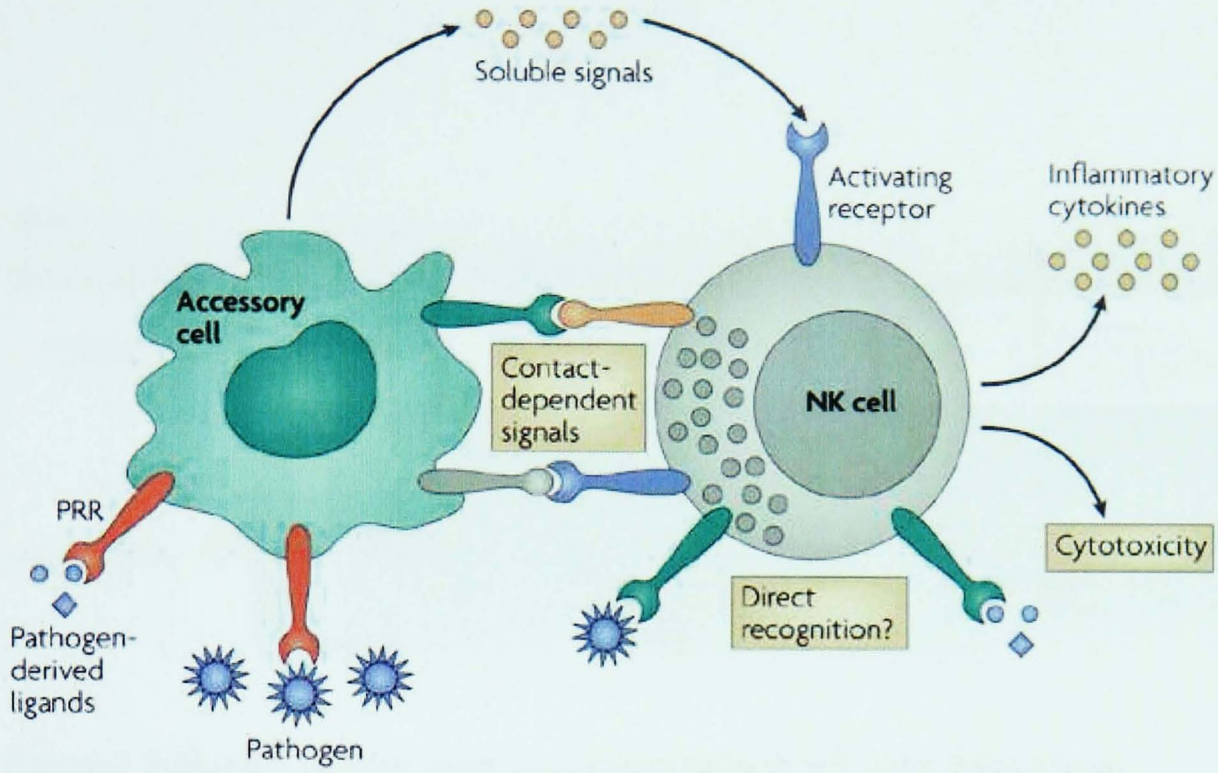


Figure 1.2 Indirect activation of NK cells upon interaction with accessory cells and pathogens (taken from (Newman and Riley 2007)). APCs directly recognize pathogens via PRRs and upon activation secrete cytokines, such as IL-12, IL-12, IL-15 and IFN-α/β. APCs also make direct contact with NK cells, possibly through ICAM-1/LFA-1 interaction. NK cells respond to soluble and contact-dependent stimuli and quickly become activated (as early as 3-6 hours), resulting in robust production of IFN-γ as well as the release of cytolytic granules, such as perforin and granzymes.

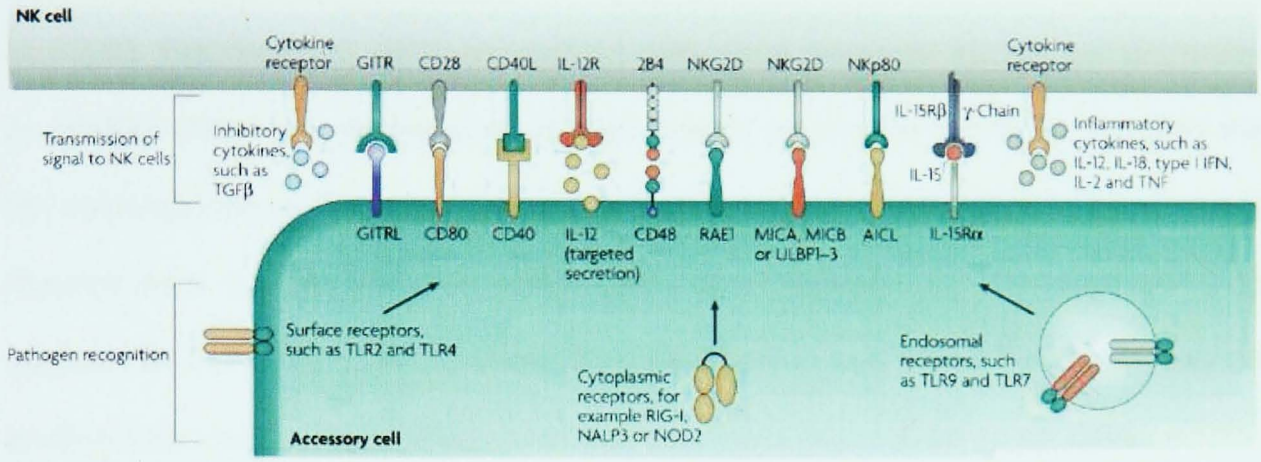


Figure 1.3 Signals passed from accessory cells to NK cells (taken from (Newman and Riley 2007)). Pathogens are recognized by PRRs located on the plasma membrane, in the cytoplasm and in endosomal compartments. APCs will pass a wide array of signals, both membrane-bound as well as soluble, to NK cells. The nature of the specific NK cell response will vary greatly depending on the signals, which are passed from the APCs.

Our lab has had a long-standing interest in the indirect activation of NK cells in response to malaria-infected erythrocytes (*Pf*-RBC), and we have previously demonstrated that NK cell activation is also dependent on IL-2 (Newman, Korbel et al. 2006). Furthermore, although myeloid cells were essential for NK cell activation by *Pf*-RBC (Newman, Korbel et al. 2006) we were never able to fully recapitulate the NK cell response of mixed peripheral blood mononuclear cells (PBMC) by addition of myeloid cells (plastic adherent monocytes, macrophages and dendritic cells) to purified NK cells (*unpublished data*). This observation suggested that there may be another important cell type involved in providing essential signals to NK cells.

These observations, bolstered by reports of T cell and IL-2-dependent activation of human NK cells by influenza A virus (He, Draghi et al. 2004) and of an increase in the frequency of IFN-γ-producing NK cells among re-stimulated PBMCs after influenza vaccination (Long, Michaelsson et al. 2008) led us to speculate that IL-2 from antigen-specific T cells might allow NK cells to contribute to the effector arm of adaptive immune responses. Testing this hypothesis was one aim of my PhD research (**Chapter 3**).

1.6 *NK cells in adaptive immune responses*

It is now known that appropriately activated murine NK cells can assume some functional characteristics of memory cells (O'Leary, Goodarzi et al. 2006; Cooper, Elliott et al. 2009; Sun, Beilke et al. 2009), raising the possibility that NK cells may also contribute to adaptive immune responses. This idea that NK cells share features of the adaptive immune system such as memory is very new. In 2006, O'Leary and colleagues, using the hapten-induced contact hypersensitivity (CHS) response model in $Rag^{-/-}$ mice, demonstrated clear NK cell responses to the sensitizing agent that

persisted for at least 4 weeks, whereas no response was seen in unsensitized animals (O'Leary, Goodarzi et al. 2006). Using adoptive transfer of hapten-sensitized NK cells into naïve mice, they demonstrated 'transferable' hapten-specific memory, which was shown to be localised within a Ly49C-I+ population of NK cells residing in the liver. These findings suggested for the first time a specific role for sustained "antigen-specific" memory recall responses by NK cells, a feature previously only credited to B cells and T cells in the adaptive arm of immunity.

In 2009, two further landmark studies were published characterising 'memory-like' NK cells in mice (Cooper, Elliott et al. 2009; Sun, Beilke et al. 2009). In one of the studies, Sun and colleagues demonstrate that a population of NK cells bearing the murine cytomegalovirus (MCMV)-binding Ly49H receptor expanded by 100-fold and 1000-fold in the spleen and liver, respectively, within 7 days following infection. Sun and colleagues also showed that although these cells undergo a contraction phase in the months after resolution of infection Ly49H+ cells persist at higher than normal levels in both lymphoid and non-lymphoid tissues and that these self-regenerating NK cells can respond rapidly to re-infection by degranulating and producing effector molecules. Finally they demonstrated that upon adoptively transferring these 'memory-like' NK cells into naïve mice and then challenging with MCMV, a rapid secondary expansion ensued leading to protective immunity. Furthermore, at least ten-fold more naïve NK cells than 'memory' NK cells were required in order to achieve similar levels of protection against MCMV infection.

The other study by Cooper and colleagues demonstrated that by adoptively transferring cytokine-activated (IL-12, IL-18, and low-dose IL-15) NK cells (from $Rag^{-/-}$ mice) into naïve mice, these cells could be easily detected more than 20 days later when they no longer show any phenotypic differences from the naïve host cells. They observed significantly greater levels of IFN-γ production by these cells when re-

stimulated as compared to naïve host NK cells or to the transferred control NK cells (preactivated with low dose IL-15 alone), suggesting the ability of retention of memory to prior activation.

NK cells have also been documented to play significant roles in vaccine-induced immune responses. In 2008, Long and colleagues investigated the effects of the routinely used trivalent influenza virus vaccine on the cellular immune response in healthy adult volunteers (Long, Michaelsson et al. 2008). They demonstrated that immunization with influenza vaccine caused significant increases in the frequency of IFN-γ+ NK cells in the majority of the vaccinees. These increases were shown to be as high as 70-fold greater than seen among the pre-vaccinated PBMCs. These findings were complemented by another study demonstrating that NK cell activity to influenza virus following vaccination with the inactivated whole virus vaccine depended on IL-2 from influenza-specific memory T cells (He, Draghi et al. 2004).

Interestingly, another study characterising yellow fever vaccination (YF17D), which is a highly protective single injection of live, attenuated yellow fever virus (YFV), demonstrated significantly increased NK cell expression of TLR3 and TLR9 which peaked by 7 days following vaccination. This increased expression of TLRs 3 and 9 correlated positively with increased CD69 expression as well as increased IFN-γ (reaching statistically significant levels by 2 days and 15 days following vaccination, respectively) (Neves, Matos et al. 2009). This study implicates a role for TLRs in the activation of NK cells following vaccination. These findings are supported by another study looking at *in vivo* recall activation of YF vaccinated NK cells, mediated by TLR3 and TLR9 in the presence of IL-12, following YF vaccine virus infection of murine DCs (Sivori, Falco et al. 2004).

1.7 *Aim of the project*

Despite recent evidence from murine models strongly suggesting a capacity for NK cells to differentiate into a 'memory' phenotype, there have been no studies in human NK cells to explore the potential for NK cell memory. NK cell activation during infection depends upon a fine-tuned orchestration of signals (both contact-dependent as well as contact-independent) in response to pathogens or pathogen-infected cells that derives from activation of accessory cells. I hypothesize that NK cell activation also depends upon rapid secretion of IL-2 from effector memory CD4+ T cells. This antigen-specific T cell compartment is extremely small in the immediate hours after primary exposure to a foreign antigen but will be significantly greater at secondary exposure. I hypothesize that these antigen-specific CD4+ T cells recruit IL-2 responsive NK cells to the adaptive immune response, allowing them to amplify the inflammatory milieu until the B and T cell responses are fully realized. Conventional dogma of the innate and adaptive immune responses to primary and secondary infection has overlooked the possibility that NK cells may respond in a "recall"-like manner during secondary exposure to antigen during infection or as a result of vaccination.

I further hypothesize, therefore, that NK cell responses should be stronger in the presence of pathogen-specific T cells, i.e. during secondary responses (after vaccination), than in the absence of such cells (i.e. before vaccination).

The major objective of my thesis is to describe the sequence of events leading to optimal "indirect" activation of NK cells during "recall" responses to infection or following vaccination. In order to achieve this objective, the specific aims of this thesis are:

- To investigate the role of T cells and the interactions with NK cells in response to *Plasmodium falciparum*-infected red blood cells (*Pf*-RBC) as well

as other non-viral pathogens such as *Mycobacterium bovis* BCG (BCG) and LPS, by way of identifying the key signals that lead to activation of NK cells as well as the precise sequence of these events (**Chapter 3**).

- To determine if NK cells play a role during vaccine-induced recall responses by way of characterising NK and T cell responses of children in a phase IIb vaccine trial of the RTS,S/ASO1E-adjuvanted malaria vaccine (**Chapter 4**).
- To further dissect the "recall" NK cell response following vaccination using rabies virus vaccine as a model (**Chapter 5**).

Chapter 2: Materials and Methods

2.1 Isolation of PBMC

Venous blood was collected into heparinised tubes (CP Pharmaceuticals, Wrexham, UK), and diluted 1:1 in RPMI-1640 (Gibco BRL, UK). Up to 35ml of the cell suspension was carefully layered onto 15 ml of a Histopaque 1077 gradient (Sigma, Dorset, UK) and spun at 400 x g for 30 minutes with the centrifuge brake turned off. The PBMC formed a distinct buffy coat layer at the top of the gradient and were carefully transferred into a fresh tube with a sterile, disposable 3ml pastette and washed twice in RPMI 1640 (500 g, 10 minutes). The cell pellet was resuspended in a small volume of complete growth medium (GM; RPMI 1640, 10% autologous serum or heat-inactivated Fetal Calf Serum (HI-FCS), 100 IU/ml penicillin/streptomycin (Gibco), 2 mM L-glutamine (Gibco)) and the cells were counted in a haemocytometer using a 1:10 dilution of Trypan blue (Sigma) to determine the cell viability.

2.2 Cryopreservation of PBMC

Freshly isolated PBMC were resuspended at 2 x 10^7 PBMC/ml ice cold heat-inactivated (56°C water bath; 30 minutes – gently invert every 10-15 minutes) fetal calf serum (HI-FCS; Gibco) and incubated on ice for 30 minutes. An equal volume of HI-FCS (20% DMSO (Sigma)) was added for a final concentration of 10^7 PBMC/ml in HI-FCS (10% DMSO). Cryovials were stored in a Mr. Frosty® container (Nalgene) at -80°C overnight and then transferred to liquid nitrogen for long-term storage.

Individual vials were thawed in 37°C water bath (until only small amount of frozen suspension remaining) and then immediately transferred to ice to maintain temperature of 0°C. Using sterile disposable 3ml transfer pastette, cell suspension was transferred to a new sterile 15 ml Falcon tube containing RPMI (10% HI-FCS) with special attention to transferring solution in a drop-wise manner in order to ensure very thorough washing of PBMC. Cells were centrifuged at 300 x g for 5 minutes at

4°C with brakes on low setting. Supernatant was discarded with special attention not to disturb cell pellet. Washes were repeated 3 times. Cell pellets were resuspended at a concentration of 5 x 10^6 cells/ml in GM and then were allowed to recover overnight at 37°C with 5% CO_2. The following morning, cells were transferred from culture plates to new 15ml Falcon tubes and washed 2 times at 300 x g for 5 minutes at 4°C with brakes on low setting. Cells were counted immediately before final wash. After final wash, cells were resuspended at a concentration of 2 x 10^6 cells/ml in GM and set on ice until ready for use.

2.3 *Plasmodium falciparum* culture

P. falciparum parasites (strain 3D7) were grown as previously described (Horowitz and Riley 2010) in O^{Rh-} human erythrocytes (National Blood Service, London, UK) in RPMI 1640 (Gibco) supplemented with 25 mM HEPES (Sigma), 28 mM sodium bicarbonate (BDH), 20 µg/l hypoxanthine (Sigma) and 10% normal human AB serum (National Blood Service). Cultures were gassed with 3% O_2, 4% CO_2 and 93% N_2 and incubated at 37℃. Parasite cultures were routi nely shown to be free from *mycoplasma/acholeplasma* species contamination using an elisa based Mycoplasma Detection Kit (Roche). O^{Rh-} human erythrocytes were aliquoted into 50ml conical tubes upon arrival and stored at 4°C for up to 3 weeks.

2.4 Preparation of antigens for in vitro stimulation assays

2.4.1 Purification of live schizonts

Highly pure (>95%) mature schizonts (*Pf*-RBC) were harvested from cultures of 5-15% parasitaemia by adherence to a LD separation column (Miltenyi Biotec). Columns were washed thoroughly with RPMI (0.1% BSA) to remove uninfected erythrocytes before elution. Purified *Pf*-RBC were washed in chilled RPMI-1640 and then resuspended at 10^9 *Pf*-RBC/ml in RPMI-1640.

2.4.2 *Mycobacterium bovis* Bacillus Calmette-Guerin culture

Mycobacterium bovis Bacillus Calmette-Guérin (BCG, Pasteur strain; kind gift from U. Schaible) was grown in Difco Middlebrook 7H9 Broth (Becton Dickinson) complemented with 0.05% Polysorbate 80 (v/v) BDH, UK) and 10% (v/v) BBL oleic acid, bovine albumin, dextrose, and catalase (OADC, Becton Dickinson). BCG cultures were incubated at 30°C with gentle shaking to avoid cell clumping. Cultures were typically grown until the medium appeared very murky. Cultures were then allowed to settle and then passed through a 25-gauge needle syringe by aspirating up and down 15-20 times. Finally the suspension was aliquoted into 1ml cryovials and frozen at -80°C. Three vials from randomly selected positions in each frozen box were grown on agar plates in a dilution series to allow enumeration of viable bacteria/ml. Numbers of viable BCG were enumerated from the average count from all vials, and the multiplicity of infection (MOI) could then be established.

2.4.3 Preparation of RTS, S malaria vaccine antigens

Peptide pools representing the circumsporozoite protein (CS) and the Hepatitis B surface antigen (HBs) were provided by Glaxo-Smith-Kline (GSK). Peptides were frozen at a concentration of 1mg/ml, and used at a final concentration of 1μg/ml.

2.4.4 Preparation of rabies vaccine antigens

Ampoules of rabies vaccine standard were purchased from the National Institute of Biological Standards and Controls (NIBSC, UK). Each ampoule contained the freeze dried residue of 0.5 ml aliquots of a commercial rabies vaccine containing inactivated Pitman Moore virus grown in Vero cells and freeze dried at NIBSC. Each ampoule was resuspended in 0.5ml of sterile water and allowed to sit for 30 minutes at RT. The concentration of each ampoule was 6.6 IU/ml of rabies virus glycoprotein content. 1:10 and 1:100 dilutions were used for *in vitro* assays.

2.5 Cell culture

Cells were resuspended at 2 x 10^6 PBMC/ml and transferred in 200μl volumes into 96-well U-bottom plates (400,000 cells/well) for overnight (24 hour) assays. For CFSE-labelled proliferation assays, PBMC were resuspended at 5 x 10^5 PBMC/ml and transferred in 200μl volumes into 96-well U-bottom plates (100,000 cells/well) for up to 7 days. Overnight assays were run in duplicates and proliferation assays were run in triplicates. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. For cultures lasting longer than 3 days, half of the volume in each well was replaced with fresh medium on Day 4 in order to replenish depleted nutrients and balance the pH of the medium. Phorbol myristate acetate (PMA) and Ionomycin (P/I) were used at a concentration of 50ng/ml PMA and 1μg/ml of ionomycin. P/I was typically added to cultures for the last 5 hours. Recombinant human cytokines: IL-12 (Peprotech) and IL-18 (MBL) (rhIL-12+IL-18) were used at a concentration of 0.1 mg/ml each. rhIL-12+IL-18 were added to cultures at the start of culture.

Lipopolysaccharide (LPS) purified from *Salmonella typhimurium* (Sigma) was used at a concentration of 1 µg/ml as an irrelevant antigen control; GM served as a negative control for all assays. Finally, Brefeldin A (10µg/ml, eBioscience) and monensin (BD bioscience; golgistop) were added to cultures for the last 5 hours of incubation.

2.6 Depletion of Specific Subsets of T cells

2.6.1 CD3 depletion

PBMC were selected for CD3 expressing cells using one of 2 kits:

1. EasySep CD3 T cell selection kit (Stemcell technologies):

Briefly, PBMC were incubated with EasySep anti-CD3 antibody cocktail for 15 minutes at RT. Then, EasySep magnetic nanoparticles were added and the cell suspensions were incubated for another 10 minutes at RT. Finally, the tube containing the cells was placed in the EasySep magnet and allowed to sit for 5 minutes. The CD3-depleted PBMC were then decanted into a new tube. The original tube was washed 2 times in PBS and allowed to sit in the magnet in order to retrieve a sufficiently high yield of CD3-depleted PBMC. The cells were resuspended at 2 x 10^6 CD3-depleted PBMC/ml in GM and set on ice until ready for use. Cells were checked by flow cytometry to determine the purity and quality of depletions.

2. CD3 FlowComp Dynalbeads (Dynal/Invitrogen):

This kit was not yet available commercially and was kindly provided by Karoline Schjetne at Dynal/Invitrogen. Briefly, PBMC were incubated in the presence of the CD3 FlowComp antibody cocktail for 10 minutes. The cells were then washed and resuspended in Isolation buffer (Mg^{2+} and Ca^{2+} free PBS containing 0.1% BSA and 2mM EDTA) followed by addition of FlowComp dynalbeads. The cell suspension was incubated for an additional 15 minutes at RT with gentle rotation. After the incubation was complete, the tubes were placed in a magnetic particle separator (Dynal MPC™-

L) for 5 minutes at RT. The cell supernatant was then transferred to a new tube and washed. The cells were resuspended at 2 x 10^6 CD3-depleted PBMC/ml in GM and set on ice until ready for use. Cells were checked by flow cytometry to determine the purity and quality of depletions.

2.6.2 CD4+, CD8+, TCR-γδ+, and TCR-αβ+ T cell depletions

These depletions were carried out using MACS beads and column separation (Miltenyi Biotec). Briefly, cells were labelled with antibodies specific for either CD4+, CD8+, TCR-γδ+, or TCR-αβ+ T cells. All the stains were added separately in their own tubes and all antibodies were conjugated to Phycoerythrin (PE) and cell suspensions were incubated on ice for 30 minutes. Cells were then washed and supernatant was discarded. Cells were resuspended in MACS buffer (PBS containing 0.1% BSA and degassed), and then incubated with magnetic beads labelled with anti-PE antibodies on ice for 20 minutes. Cells were then washed and resuspended in 500µl buffer/10^7 cells. Cell suspensions were carefully pipetted onto magnetic columns and allowed to filter through via gravity. Columns were then washed 3 times using MACS buffer. Columns were removed from magnets and set onto 15ml falcon tubes. Five ml of MACS buffer was then plunged through the column with sufficient force to dislodge the bound cells using plunger provided in kit. Cells were washed and then resuspended at 2 x 10^6 PBMC/ml in GM and set on ice until ready for use.

All cell suspensions were then stained for respective T cell subsets to confirm purity and quality of depletions. Only preparations giving at least 97% purity were used in subsequent experiments.

2.7 Cell phenotyping using Flow Cytometry

After the relevant culture period, the cells were washed twice in FACS buffer (1x PBS, 0.1% sodium azide (Sigma), 2% HI-FCS) and centrifuged in 96-well plates for 2 minutes at 500 x g. Fluorochrome conjugated antibodies were added in the dark at 4℃ and at concentrations previously determined by titration. Panels of antibody cocktails can be found in Table 4. The plates were incubated in the dark at 4℃ for 30 minutes. The cells were then washed twice in 200µl of FACS buffer. The cells were then fixed at room temperature for 15-30 minutes in 100µl fixation buffer (1x PBS, 2% paraformaldehyde or BD cytofix solution (1x; BD Pharmingen)). The cells were then washed once in FACS buffer. The cells were then resuspended in 100µl permeabilization buffer (BD Pharmingen, UK). Intracellular antibody cocktails were added, and the plates were incubated in the dark at 4℃ for 30 minutes. Details of cell surface and intracellular antibody cocktails can be found in **Table 2.1** (FACS Caliber), **Table 2.2** (FACS Canto II) and **Table 2.3** (Becton Dickenson Cyan ADP). Cells were then washed twice in permeabilization buffer. Finally, the cells were resuspended in 200µl of fixation buffer and stored at 4℃ until ready for acquisition on the flow cytometer. All antibodies were mouse monoclonals with the exception of anti-IL2 APC, which was a monoclonal rat antibody. Flow cytometric analyses were performed using a Becton Dickinson FACSCalibur or FACSCanto II flow cytometer or Becton Dickenson Cyan ADP flow cytometer and FlowJo analysis software (TreeStar). All statistical analyses were performed using Prism 5 software (Graph Pad Software Inc., San Diego, USA) and STATA 10 software (Stata Corp.).

FACS Caliber:	FL1- FITC	FL2- PE	FL3- PerCP	FL4- APC
NK cell Panel 1	IFN-γ 1.5μl Serotec	CD69 2.5μl BD Biosciences	CD3 2.5μl BD Biosciences	CD56 2.5μl Beckman Coulter
NK cell Panel 2	LAMP-1 10μl BD Biosciences	Perforin 5μl BD Biosciences	CD3 2.5μl BD Biosciences	CD56 2.5μl Beckman Coulter
CD8+ T cell Panel	LAMP-1 10μl BD Biosciences	Perforin 5μl BD Biosciences	CD3 2.5μl BD Biosciences	CD8 2.5μl BD Biosciences
CD4+ T cell Panel	IFN-γ 1.5μl Serotec	CD4 2.5μl BD Biosciences	CD3 2.5μl BD Biosciences	IL-2 0.5μl BD Biosciences
TCR-γδ T cell Panel	IFN-γ 1.5μl Serotec	CD3 2.5μl BD Biosciences	TCR γδ-b/s 2.5μl BD Biosciences	TNF-a 5μl BD Biosciences
Treg Panel	CD4 2.5μl eBioscience	FoxP3 5μl BD Biosciences	CD127 2.5μl BD Biosciences	CD25 2.5μl eBioscience
Macrophage Panel	CD14 5μl BD Biosciences	CD68 5μl BD Biosciences		TNF-α 5μl BD Biosciences
b/s - biotin/strepvidin-PerCP				

Table 2.1. Antibody cocktails used for 4 colour flow cytometry

FACS Canto II:	FL1 - FITC	FL2 - AmCyan	FL3 - Pe	FL4 - PerCP	FL5 - PE Cy7	FL6 - Pacific Blue	FL7 - APC	FL8 - APC Cy7
Panel 1	IFN-γ 1.5µl	CD3 5µl	TCR αβ 2.5µl	TCR γδ-b/s 2.5µl	CD56 5µl	CD8 5µl	IL-2 5µl	CD4 5ml
	Serotec	BD Biosciences	BD Biosciences	BD Biosciences	BD Biosciences	BD Biosciences	BD Biosciences	BD Biosciences
b/s - biotin/strepvidin-PerCP								

Table 2.2 Antibody cocktail used for 8 colour flow cytometry

Becton Dickenson Cyan	FL1 - FITC	FL2 - PE Texas Red	FL3 - Pe	FL4 - PerCP	FL5 - PE Cy7	FL6 - Pacific Blue	FL8 - APC	FL9 - APC Cy7
Panel 1	IFN-γ 1.5µl	CD3 5µl	CD69 2.5µl	TCR γδ-b/s 2.5µl	CD56 5µl	CD8 5µl	IL-2 5µl	CD4 5ml
	Serotec	BD Biosciences	BD Biosciences	BD Biosciences	BD Biosciences	BD Biosciences	BD Biosciences	BD Biosciences
Panel 2	IFN-γ 1.5µl	CD3 5µl	Perforin 5µl	CD69 2.5µl	CD56 5µl	CD8 5µl	LAMP-1 10µl	CD4 5ml
	Serotec	BD Biosciences	BD Biosciences	BD Biosciences	BD Biosciences	BD Biosciences	BD Biosciences	BD Biosciences
b/s - biotin/strepvidin-PerCP								

Table 2.3 Antibody cocktail used for 9 colour flow cytometry

Chapter 3: Cross-talk between T cells and NK cells generates rapid effector responses to *P. falciparum*-infected erythrocytes

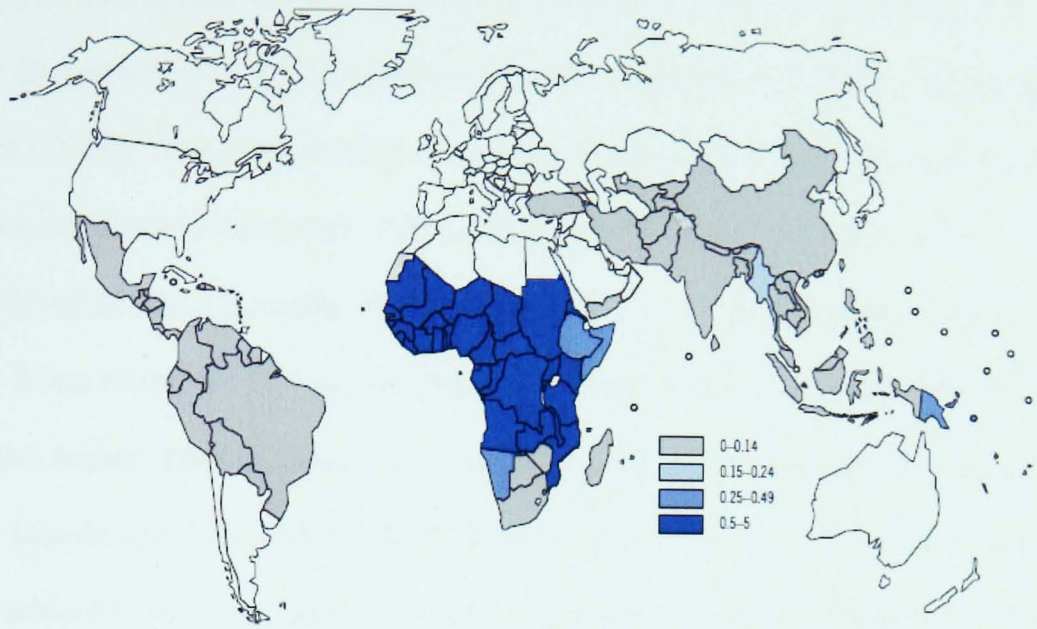
---

Adapted from (Horowitz, Newman *et al.* 2010)

3.1 *Introduction*

3.1.1 The burden of malaria

A.



B.

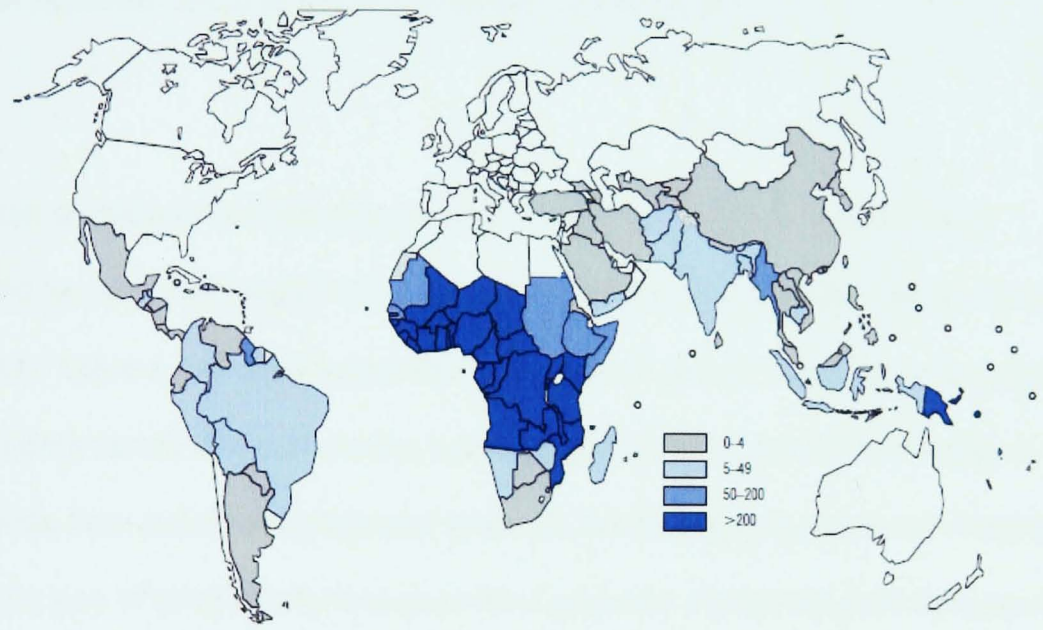


Fig. 3.1: The burden of malaria

(**A**) Estimated number of malaria-related fatalities (per 1000) as defined as fatalities due to low blood pressure, severe anaemia (iron deficiency), pulmonary oedema, kidney failure, internal bleeding (haemorrhaging), jaundice, state of shock, coma, convulsions and paralysis.; (**B**) Estimated incidence of clinical malaria as defined by the number of cases (per 1000). World Malaria Report, 2008 (WHO 2008).

While malaria has officially been eliminated from temporate zones such as Europe and North America during the last century, it remains an overwhelming problem for public health throughout the tropics (Hay, Guerra et al. 2004; WHO 2008). It is currently documented in 109 countries, potentially affecting 3.3 billion people (**Figure 3.1**) (WHO 2008). Malaria parasites are transmitted to vertebrates through the bite of the female anopheline mosquito. There are 4 well characterized species of *Plasmodium* that infect humans: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*, although it has recently been shown that many diagnosed *P. malariae* infections are actually the simian-derived species, *P. knowlesi (*Garnham 1996; Singh, Kim Sung et al. 2004; Cox-Singh, Davis et al. 2008). *P. falciparum*, which is the most common species, accounts for the highest mortality rates and is responsible for more than 90% of all reported cases of malaria infection (WHO 2008).

The burden of malaria is most pronounced in young children (< 5 years) and primigravid women (McGregor 1984; Snow, Korenromp et al. 2004; Desai, ter Kuile et al. 2007). Malaria has also been shown to have severe impacts on socio-economic indices at both family and community levels (Breman 2001; Sachs and Malaney 2002). Aside from public and personal costs for treatment and prevention measures, it leads to a loss of productivity due to chronic physical disabilities and disrupted education (Fernando, Gunawardena et al. 2003; Mung'Ala-Odera, Snow et al. 2004; Carter, Ross et al. 2005).

Public health initiatives such as indoor residual spraying (Sadasivaiah, Tozan et al. 2007) and the use of insecticide treated bednets (Diallo, Cousens et al. 2004; Lengeler 2004; Lindblade, Eisele et al. 2004) have all proven very effective at reducing the global burden of malaria. Studies characterizing the efficacy of

intermittent preventive anti-malarial treatments (IPT) (van Eijk, Ayisi et al. 2004; Schellenberg, Cisse et al. 2006) suggested much promise as well, however, more recent meta-analysis suggested that IPT is less effective than originally envisaged (Aponte, Schellenberg et al. 2009). More recently, the use of artemisinin combination therapies (ACT), shown to have high efficacy at killing both erythrocytic as well as sexual stages of the plasmodium parasite, has been linked to a significant reduction in transmission (Greenwood and Mutabingwa 2002; White 2004; Okell, Drakeley et al. 2008); however, cases of drug resistance have already been reported (Eastman and Fidock 2009). While all of these initiatives have been demonstrated as invaluable tools in the fight against malaria, they all share a common obstacle, i.e. they require continuous applications raising concerns regarding their long-term financial sustainability. In order to achieve true global eradication, a cheap and sustainable vaccine is needed.

Unexpectedly, we have witnessed a sudden decline in malaria prevalence in many regions of Africa in recent years (Barnes, Durrheim et al. 2005; Nyarango, Gebremeskel et al. 2006; Bhattarai, Ali et al. 2007; Ceesay, Casals-Pascual et al. 2008; O'Meara, Bejon et al. 2008; Rodrigues, Schellenberg et al. 2008; WHO 2008). Recent large-scale initiatives such as the Roll Back Malaria Programme (Mufunda, Nyarango et al. 2007) and The US President's Malaria Initiative (Loewenberg 2007) have been credited as having an effect on the prevention and treatment of malaria by implementing transparency mechanisms to ensure that medications and bed nets are effectively delivered to those in need; however, factors such as climate change may also be influencing transmission rates by affecting the amount of rain fall and temperatures and their consequent effects on mosquitoes. However, much more rigorous scientific approaches will need to be taken in order to assess the true causes and likely duration of this decline.

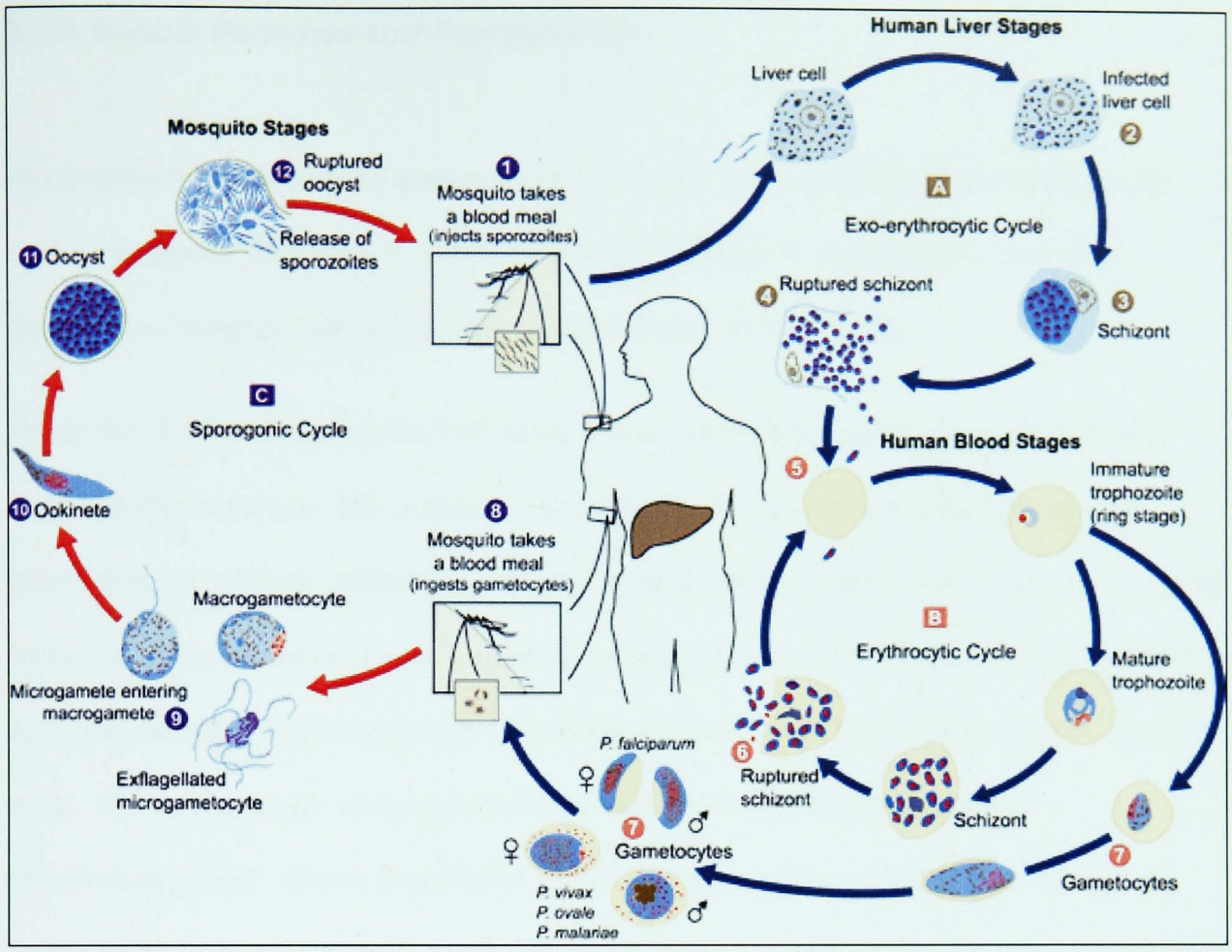


Figure 3.2 – Malaria Life Cycle (CDC 2006)

3.1.2 *Malaria Parasites and Pathogenesis*

Plasmodium has 3 distinct stages of its life cycle: pre-erythrocytic and erythrocytic and sporogonic. **Figure 3.2** illustrates the life cycle of *P. falciparum* and in the description listed below numbers and letters refer to this figure.

When the female anophelene mosquito transmits haploid sporozoites (**1**) into the host sub-dermal layer, the parasite travels through the capillary vessels into the blood stream leading to the liver where it infects hepatocytes. This process is thought to occur within minutes of sporozoite inoculation. Up to 20 sporozoites are released from the mosquito with each bite (Rosenberg, Wirtz et al. 1990; Ponnudurai, Lensen et al. 1991). However, some sporozoites remain in the skin (Sidjanski and Vanderberg 1997; Sinnis and Coppi 2007; Ejigiri and Sinnis 2009) where they are targeted by phagocytic cells, such as macrophages or traverse to the lymphatics and drain to the lymph nodes where they can prime CD8+ T cells (Chakravarty, Cockburn et al. 2007; Yamauchi, Coppi et al. 2007; Amino, Giovannini et al. 2008). The sporozoites that do reach the liver pass through kupffer cells (Baer, Roosevelt et al. 2007) and hepatocytes (Mota, Pradel et al. 2001; Frevert 2004), which is thought to be a necessary step in activation of sporozoites to initiate stable infection. Once inside the hepatocyte, they form parasitophorous vacuoles (**2**) and develop into exo-erythrocytic schizonts (**3**) through a process known as liver schizogony, which requires several rounds of asexual reproduction. This process is referred to as the exo-erythrocytic (asexual) cycle (**A**) and can take more than 5 days before being able to initiate stable blood-stage infection by releasing merozoites, although the timing can differ significantly for other *Plasmodium* species (**4**). Merozoites then infect erythrocytes (*Pf*-RBC) (**5**), and this stage represents the erythrocytic stage of malaria infection (**B**). The erythrocytic stage of infection involves the maturation of trophozoites into schizonts leading to the lysis of RBC and release of 16-32 new

merozoites (**6**), which infect new RBC. Rather than undergoing schizogony, some merozoites develop into male or female gametocytes (**7**); male and female gametocytes are taken up by the mosquito (**8**) and fertilize in the gut to form a zygote (**9**), which initiates the sporogonic cycle (**C**). Depending on ambient temperature, this process (**9-11**) can require up to 3 weeks, culminating in migration of thousands of new sporozoites into the salivary glands (**12**), ready for inoculation into a new host (Sinden 1999; Mota and Rodriguez 2004).

The symptoms of malaria infection are often times very mild, in the form of headaches, myalgia, fever, vomiting, diarrhea and malaise. These symptoms are highly correlated with the onset of erythrocytic stage infection and become more and more debilitating as blood stage parasitaemia increases. The degree of severity of malaria-associated pathology varies. While the majority of infections present as mild illness, a small subset of non-immune individuals develop more severe symptoms, such as anaemia, which is most commonly seen in young children and pregnant women (Duffy and Fried 2005). A percentage of individuals progress to renal failure and liver dysfunctions. Other symptoms include cerebral malaria (most common in slightly older children and non-immune adults (Reyburn, Mbatia et al. 2005), respiratory dysfunctions, hypoglycaemia, metabolic acidosis, and pulmonary oedema (Miller, Baruch et al. 2002).

3.1.3 Innate immune responses to Plasmodium infection

Without the intervention of anti-malarial treatments or immunity, blood stage parasitaemia can increase at an exponential rate, leading to death. The innate, and adaptive, immune responses are capable of containing peak parasitaemia by limiting the number of infected erythrocytes, and subsequently preventing onset of severe pathology. There is a systemic Th1 response to the lysis of *Pf*-RBC, however, and this can contribute to pathology. In the context of malaria infection, the cells that are thought to play a role in the innate immune response include, macrophages, monocytes, and dendritic cells (DCs), γδ+ T cells, Natural Killer (NK) T cells, and Natural Killer (NK) cells.

3.1.4 Cellular Sources of IFN-γ

The optimal immune response to a malaria infection likely comprises rapid induction of inflammatory anti-parasitic responses followed by equally rapid resolution of inflammation (mediated by anti-inflammatory cytokines) to prevent immunopathology (Artavanis-Tsakonas, Tongren et al. 2003). Rapid and robust cell-mediated immune responses can inhibit intra-erythrocytic replication of malaria parasites and thereby prevent onset of clinical malaria (Stevenson and Riley 2004). This process can be primed by ultra-low dose infection/vaccination (Pombo, Lawrence et al. 2002; Roestenberg, McCall et al. 2009) but has yet to be mimicked by subunit vaccines. Understanding the cellular and molecular pathways of this very early cellular response may allow the design of new approaches to vaccination but there is still considerable debate over the precise sequence of events. In particular, the timing and magnitude of IFN-γ secretion are thought to be pivotal in determining the outcome of disease and it is thus of importance to identify the major cellular sources of Interferon (IFN)-γ, the kinetics of its production and the pathways by which it is induced and regulated.

3.1.5 *NK cells in P. falciparum infection*

Data from murine models as well as from clinical studies have suggested that early production of IFN-γ, i.e. within the first 24 hours during blood stage malaria infection can significantly reduce the degree of pathology, especially in non-immune individuals (De Souza, Williamson et al. 1997; Mohan, Moulin et al. 1997; Stevenson and Riley 2004). Subsequently, studies have shown that human NK cells can be a very early source of IFN-γ when cultured *in vitro* with *Pf*-RBC for 24 hours, although the response is quite heterogenous (Artavanis-Tsakonas and Riley 2002; Artavanis-Tsakonas, Eleme et al. 2003; Korbel, Newman et al. 2005; Korbel 2006; Newman, Korbel et al. 2006) and the underlying mechanisms leading to the activation of NK cells is only beginning to unfold. It is known that NK cells can be activated or inhibited via numerous pathways, however the nature and sequence of events by which the bi-directional interactions between APCs and T cells interact with NK cells remain unclear and will be discussed in detail later in this section. Some potential pathways of NK cell activation can be seen in **Figure 3.3**.

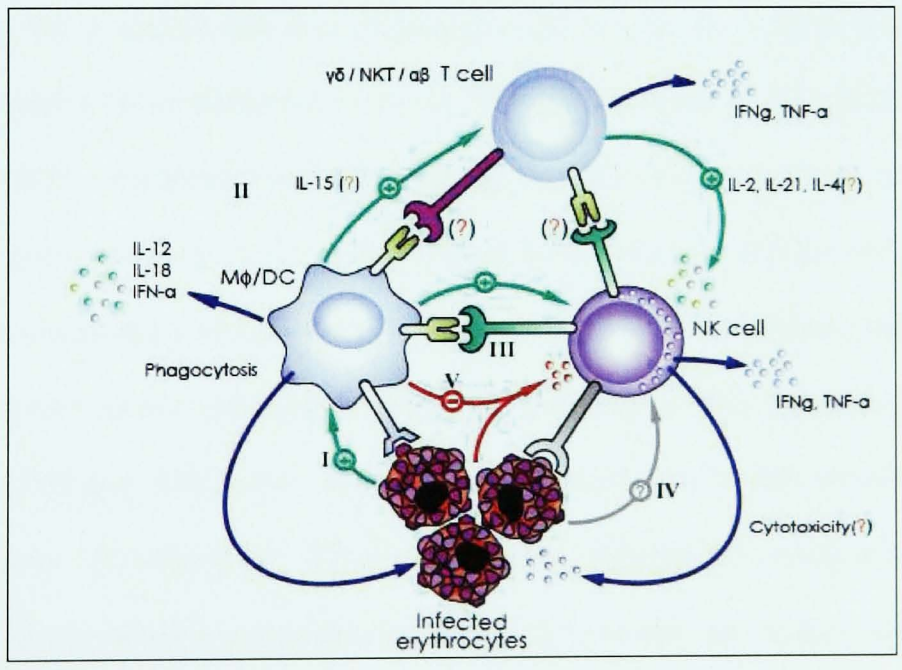


Figure 3.3 – Potential pathways of NK cell activation by *P. falciparum*-infected erythrocytes – Adapted from (Korbel 2006)

Figure 3.3 illustrates the potential pathways of NK cell activation in response to *Pf*-RBC, and in the description below, numbers refer to the figure.

Direct recognition of schizont infected RBC by accessory cells (**I**) (monocytes/macrophages and mDCs) likely occurs via pattern recognition receptors (PRRs) such as TLR4 (Seixas, Moura Nunes et al. 2009) and TLR9 (Franklin, Parroche et al. 2009; Seixas, Moura Nunes et al. 2009; Coban, Igari et al. 2010; Wu, Gowda et al. 2010) in mice and TLR1, TLR2 and TLR4 (McCall, Netea et al. 2007) in humans. Interestingly, one study demonstrated direct sensing of *Pf*-RBC by NK cells (**IV**) via binding of the *Pf* erythrocytic membrane protein-1 (*Pf*EMP-1) to chondroitin sulfate A, (CSA; host receptor on NK cells) along with binding of Intracellular adhesion molecule-1 (ICAM-1; expressed on NK cells) with Lymphocyte function-associated antigen (LFA)-1 (expressed on APC) (Baratin, Roetynck et al. 2007).

Upon recognition, the schizonts are then phagocytosed, and Ag processing ensues. This process leads to activation of accessory cells (**II**) but can also lead to DC inhibition (**V**) (Urban, Ferguson et al. 1999). Acccessory cell activation by *Pf*-RBC has been shown to lead to secretion of Th1 cytokines (e.g. IL-12, IL-18, IFN-α). IL-15, which mediates peripheral T cell maturation, is also released and has been reported to drive T cell-mediated production of IL-21, which has been shown to act synergistically with IL-15 and/or IL-18 in the activation of human NK cells *in vitro (*Strengell, Matikainen et al. 2003). Activated T cells also secrete IL-2, which subsequently acts on NK cells. Both, IFN-γ and TNF-α, produced by T cells and NK cells can act in a feedback cycle with the accessory cells leading to further secondary activation as well as priming of the adaptive immune response.

NK cells make contact with accessory cells (**III**), possibly via CD40:CD40L and/or ICAM-1 with its host ligand complex, CD11a/CD18 (LFA-1) (Korbel 2006; Baratin, Roetynck et al. 2007). It is also possible that accessory cells are delivering cytokines such as IL-12 in a polarized manner, as observed by confocal image based microscopy (Newman, Korbel et al. 2006). This same report has shown that without this interaction between accessory cells and NK cells, there is sub-optimal expression of CD25 (IL2Rα), Lysosome Associated Membrane Protein-1 (LAMP-1), and IFN-γ production.

NK cells interact directly with *Pf*-RBC (Artavanis-Tsakonas, Eleme et al. 2003; Korbel, Newman et al. 2005; Korbel 2006). The pathway for this interaction remains unknown but the interaction has been documented via confocal microscopy which demonstrated conjugate formation between NK cells and *Pf*-RBC in 8 of 9 donors tested and in more than 40% of the NK cells imaged as well as cytoskeletal reorganization in a proportion of the conjugates (Korbel, Newman et al. 2005).

NK cell activation may be negatively regulated via anti-inflammatory cytokines, e.g. TGF-β and IL-10 (Omer, Kurtzhals et al. 2000). These cytokines are extremely important at turning off pro-inflammatory pathways and preventing downstream tissue damage and seem likely produced by accessory cells in response to *Pf*-RBC, although recent reports suggest that subsets of NK cells can also play a regulatory role by producing IL-10 (Maroof, Beattie et al. 2008; Perona-Wright, Mohrs et al. 2009; Yoshida, Akbar et al. 2010). TGF-β and IL-10 can also be produced prematurely by malaria parasites as a mode of immune evasion. A previous report demonstrated that latent TGF-β could be directly activated by *Pf*-RBC to modulate the innate

Th1 response by malaria parasites in a process involving thrombospondin (TSP)-like molecules and metalloproteinase activity (Omer, de Souza et al. 2003; Newman, Korbel et al. 2006).

Several features of NK cell responses to *Pf*-RBC suggest that there are genetic factors influencing their responses. For example: 1) There is a large degree of heterogeneity between individuals' NK cell IFN-γ production, as well as expression of activation markers CD69, CD25, and LAMP-1; 2) For any individual, the NK cell IFN-γ response to *Pf*-RBC over time is very stable; 3) Different sub-populations of NK cells within an individual will respond differently, and the response of each subset is consistent over time, suggesting that there exist stable differences between NK clones (Korbel, Norman et al. 2009).

It has been firmly established that NK cells are very early responders to infection with malaria, *in vivo* and *in vitro* with respect to IFN-γ production. It still remains unclear, however, what the actual triggers are, which cause these cells to become activated or inhibited. In 2009, we demonstrated a highly significant correlation between the relative activation responses of $CD56^{dim}$ and $CD56^{bright}$ NK cells with KIR genotype (Korbel, Norman et al. 2009), demonstrating that these 2 subsets of NK cells are independently regulated. Furthermore our study demonstrated that the anti-microbial response by $CD56^{bright}$, but not $CD56^{dim}$, NK cells is highly correlated with the degree of activation in myeloid accessory cells and that $CD56^{dim}$ cells tend to be relatively inhibited. Finally, the data also revealed that the ratios of $CD56^{dim}$ to $CD56^{bright}$ NK cells producing IFN-γ varies between individuals, however, they remain constant within an individual over time. Taken together, these data demonstrate that there exist at least 2 independent regulatory mechanisms responsible for modulating NK cell activation/inhibition. It

remains unclear if KIR genotypes regulate the specific NK cell subsets in a similar manner in response to other pathogens or if this is a unique effect in response to *Pf*-RBC.

To briefly summarize the sequence of events taking place during the innate immune response to malaria, *Pf*-RBC are recognized by accessory cells via various potential PRRs, such as TLRs 4 and 9 in mice and TLRs 1,2 and 4 in humans. This leads to accessory cell activation and the release of type-1 cytokines, such as IL-12, IL-18, and IFN-α. These cytokines have been shown, *in vitro (Currier, Sattabongkot et al. 1992; Fell, Currier et al. 1994; Dick, Waterfall et al. 1996; Artavanis-Tsakonas and Riley 2002; Newman, Korbel et al. 2006)*, to be required for optimal NK cell IFN-γ production, however, it is important to note that the PBMC response to malaria is not recapitulated, simply by adding activated DC and macrophages to purified NK cells, suggesting that the presence of other cell types are involved in this response. We hypothesized that T cells may contribute to NK cell activation.

In this chapter, I have carefully compared the magnitude and the timing of T cell and NK cell responses to *Pf*-RBC in a large cohort of malaria-naïve donors.

3.2 *Materials and methods*

3.2.1 Blood donors

Adult blood donors were recruited at the London School of Hygiene and Tropical Medicine through an anonymous blood donation system. All donors were malaria naïve and healthy and gave fully informed consent for their blood to be used in this study. Ethical approval was given by the LSHTM Ethics Committee, application #805.

3.2.2 P. falciparum parasites

P. falciparum parasites (strain 3D7) were grown in O^{Rh-} human erythrocytes (National Blood Service, London, UK) in RPMI 1640 (Gibco) supplemented with 25 mM HEPES (Sigma), 28 mM sodium bicarbonate (BDH), 20 µg/l hypoxanthine (Sigma) and 10% normal human AB serum (National Blood Service). Cultures were gassed with 3% O_2, 4% CO_2 and 93% N_2 and incubated at 37℃. Parasite cultures were routinely shown to be free from *mycoplasma/acholeplasma* species contamination using an ELISA-based Mycoplasma Detection Kit (Roche) incorporating polyclonal antibodies against *M. arginini*, *M. hyorhinis*, *A. laidlawii* and *M. orale*. Highly pure (>95%) mature schizonts were harvested from cultures of 5-15% parasitaemia by adherence to a LD separation column (Miltenyi Biotec). Columns were washed thoroughly with PBS to remove uninfected erythrocytes before elution. Schizont-infected (*Pf*-RBC) or uninfected (uRBC) erythrocytes were added at a ratio of 3 RBC per mononuclear cell.

3.2.3 M. bovis BCG bacteria

Mycobacterium bovis Bacillus Calmette-Guérin (BCG, Pasteur strain; kind gift from U. Schaible) was grown in Difco Middlebrook 7H9 Broth (Becton Dickinson) complemented with 0.05% Polysorbate 80 (v/v) BDH, UK) and 10% (v/v) BBL oleic acid, bovine albumin, dextrose, and catalase (OADC, Becton Dickinson). BCG cultures were incubated at 30°C with gentle shaking

to avoid cell clumping. Cultures were grown typically until the culture appeared very murky. Cultures were then allowed to settle and then solution was passed through a 25-gauge needle syringe by aspirating up and down 15-20 times. Finally solution was aliquotted into 1ml cryovials and frozen at -80°C. Three vials from randomly selected positions in each frozen box were grown on agar plates in a dilution series to allow for enumeration of viable bacteria/ml. Numbers of viable BCG were enumerated from the average count from all vials, and the multiplicity of infection (MOI) could then be established.

3.2.4 Depletion of T cell subsets

PBMC were depleted of specific T cell subset populations by methods described in **Chapter 2** (Materials and Methods) but after depletion, the cell concentration was readjusted to 2 x 10^6/ml and cells were cultured with *Pf*-RBCs at a ratio of 1:3 (as previously) and also at a ratio of 1:10 to ensure that changing the ratio of NK cells and accessory cells to *Pf*-RBCs did not adversely affect the outcome of the experiments. No significant differences were seen between the 1:3 and the 1:10 cultures, so only data from 1:3 cultures are presented.

3.2.5 Blocking experiments

Two million PBMC were cultured overnight in 24 well flat bottom plates in the presence of purified blocking/neutralising antibodies or appropriate isotype control antibodies for the following cytokines, cytokine receptors, and MHC molecules (all BD Bioscience): anti-IL-12 (p40/p70; clone C11.5), anti-IL-18-αchain (clone H44), anti-IL-2 (clone MQ1 17H12), anti-IFNR-α[2b] (clone 7N4-1), anti-TNF (clone MAb1), anti-MHC class I (HLA-A, B, C; clone W6/32), and anti-MHC class II (HLA-DR, DP, DQ; clone Tü-39).

3.2.6 Cell surface and intracellular staining for flow cytometry

Surface and intracellular staining was performed as described previously in Chapter 2 (Materials and Methods). The antibodies/reagents used were: anti-CD3 PerCP, anti-αβ TCR-FITC, anti-γδ TCR (biotinylated), Strepdavidin PerCP, anti-IFN-γ APC, anti-CD4 APC-Cy7, anti-CD8 Pacific Blue and anti-CD3 PE-Texas Red (BD Bioscience); anti-CD56 APC (Beckman Coulter), anti-IFN-γ FITC, anti-IL-2 APC (BD biosciences), anti-CD56 PE-Cy7 (BD biosciences). All antibodies were mouse monoclonals with exception of anti-IL-2 APC (rat monoclonal). Anti-CD3 antibodies were included in the intracellular staining mixes to ensure that T cells that down-regulated CD3 upon stimulation were not misclassified.

3.2.7 Cytokine assays

Secreted IL-2 and TNF-α were measured, according to the manufacturer's instructions, by cytometric bead array (CBA Th1/TH2/TH17 kit; BD Biosciences). All samples were acquired using FACScaliber flow cytometer and analysis was performed using FCAP Array software (Soft Flow Inc.).

3.2.8 Statistical analysis

All statistical analyses were performed using Prism 5 software (Graph Pad Software Inc., San Diego, USA) and STATA10 (StataCorp.).

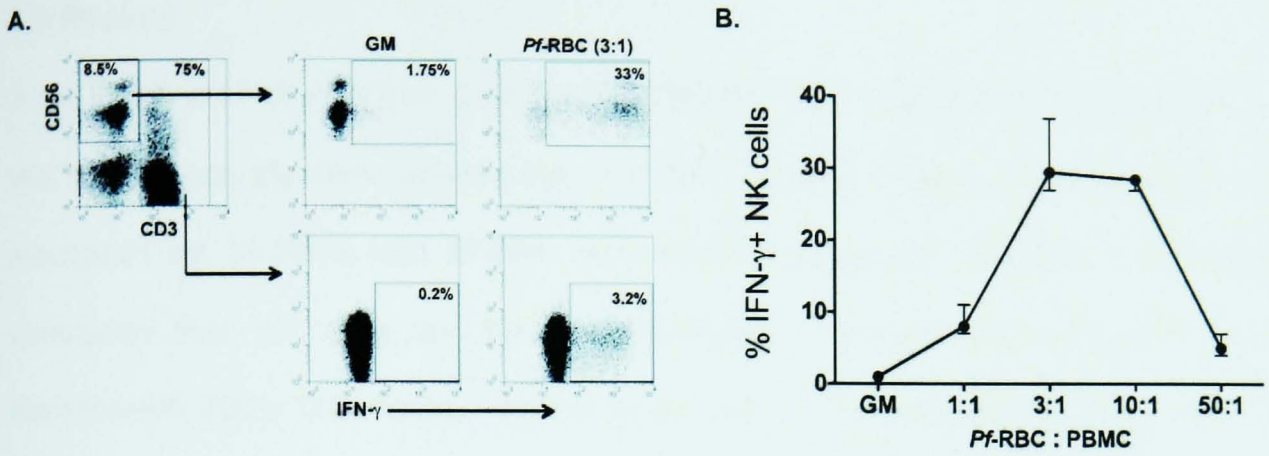


Figure 3.4 Titrating *Pf*-RBC input dose to define optimal multiplicity of infection.
(**A**) Representative flow cytometry data from 1 donor demonstrating gating strategy for NK cells ($CD56^+$ $CD3^-$ lymphocytes) and T cells (all $CD3^+$ lymphocytes) and for detection of intracellular IFN-γ after 24 hrs co-culture with (right column) or without (left column; GM) *Pf*-RBC (MOI = 3:1). (**B**) The proportion of all NK cells expressing intracellular IFN-γ after 21 hrs co-culture with *Pf*-RBC at varying MOI. N= 5 malaria naïve donors. Data are representative of median values with error bars representing range.

3.3 *Results*

3.3.1 *T cells and NK cells both contribute to the early IFN-γ response to P. falciparum*

We have previously demonstrated that both NK cells and T cells can produce IFN-γ following incubation for 18-24hrs with *Pf*-RBC (Artavanis-Tsakonas and Riley 2002) but others have concluded that γδT cells are the only significant source of early IFN-γ (Hensmann and Kwiatkowski 2001; D'Ombrain, Hansen et al. 2007). This discrepancy may reflect, in part, heterogeneity between human blood donors in the timing and magnitude of the response and in the source of IFN-γ (Korbel, Newman et al. 2005). I sought, therefore, to characterise more systematically the early IFN-γ response to *Pf*-RBC.

I first titrated the concentration of *Pf*-RBC in the cultures to identify the optimum Pf-RBC:monconuclear cell (MNC) ratio for induction of IFN-γ. (**Figure 3.4**). PBMC were cultured for 21 hours either without stimulus (GM) or in the presence of varying amounts of *Pf*-RBC (ranging from 1:1 to 50:1 *Pf*-RBC:PBMC). Lymphocytes were identified as $CD3^+$ T cells or as $CD3^-CD56^+$ NK cells (**Fig. 3.4A**). I found that a multiplicity of infection (MOI) = 3:1 *Pf*-RBC:PBMC was the optimal dose input, confirming previous studies in our laboratory (**Fig.3.4B**) (Artavanis-Tsakonas and Riley 2002).

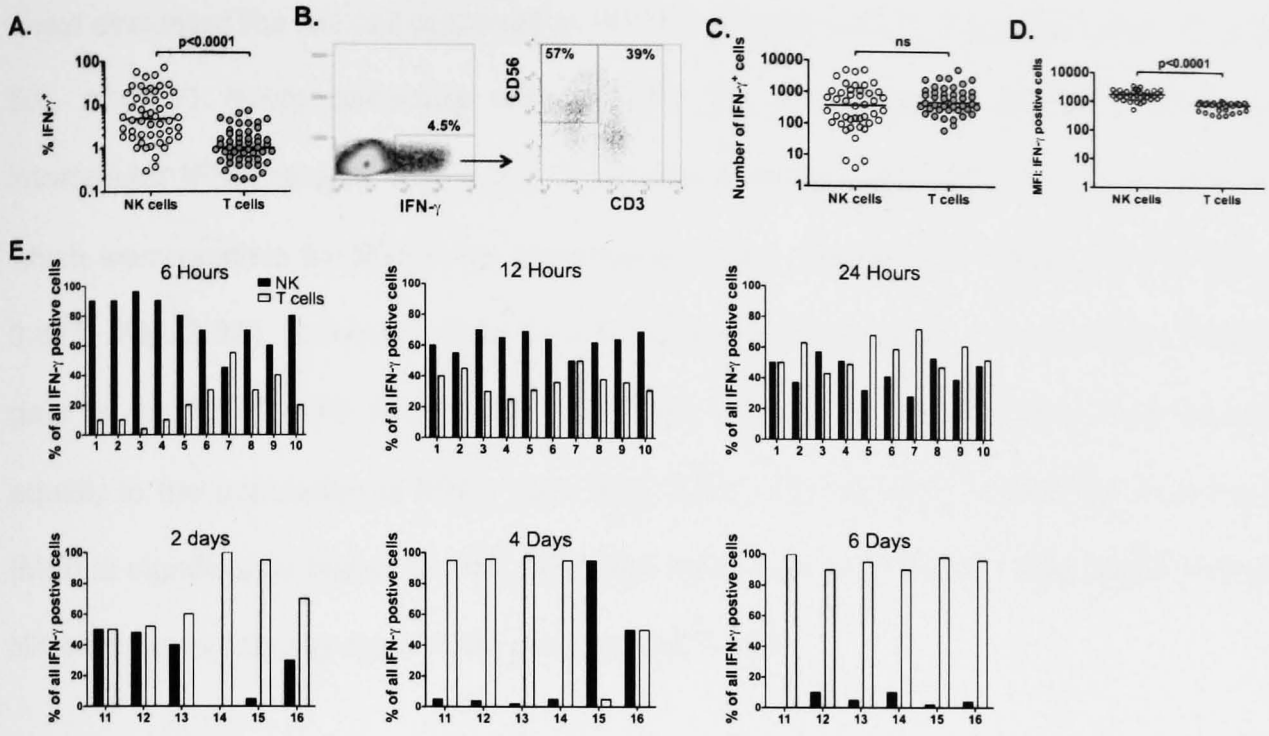


Figure 3.5 NK cells and T cells contribute to the early IFN-γ response to *Pf*-RBC
PBMC were cultured overnight, either without stimulus (GM) or with *Pf*-RBC at an MOI of 1 PBMC : 3 *Pf*-RBC. (**A**) The proportion of all NK cells or all T cells expressing intracellular IFN-γ after 24 hrs co-culture with *Pf*-RBC. N= 50 malaria naïve donors. (**B**) Gating strategy for identification of all IFN-γ+ lymphocytes and their subsequent identification as either NK cells or T cells. (**C**) The number of IFN-γ^+ NK cells or T cells per 100,000 lymphocytes after 24 hrs co-culture with *Pf*-RBC. N= 50 naïve donors. (**D**) The MFI of staining for intracellular IFN-γ in NK cells and T cells after 24 hrs co-culture with *Pf*-RBC. N= 50 naïve donors. (**E**) The percentage of all IFN-γ+ cells that are either NK cells (black bars) or T cells (white bars) after 6, 12 or 24hrs or 2, 4 or 6 days co-culture with *Pf*-RBC. Numbers on X axis identify individual blood donors. B,D,E: Horizontal lines represent medians. P-values are for 2 tailed paired Wilcoxon tests with 95% CI comparing NK cells with T cells from the same donor.

I next evaluated the NK cell response to Pf-RBC at a MOI of 3:1 in a large cohort of donors (N = 50). After 21 hours co-culture with *Pf*-RBC, the proportion of NK cells which contained intracellular IFN-γ ranged from 0% to 81% with a median of 4.65%. The proportion of T cells, which were positive for IFN-γ was significantly lower, ranging from 0% to 7% with a median of 0.82% (**Fig. 3.5A**). However, since T cells outnumber NK cells by ~10:1 among PBMCs, when I gate on all IFN-γ^+ cells (**Fig. 3.5B**) I find that T cells and NK cells contribute approximately equally to the population of IFN-γ^+ cells (**Fig. 3.5C**). On the other hand, IFN-γ staining intensity (MFI) is significantly higher for NK cells than for T cells ($p < 0.0001$) (**Fig. 3.5D**) indicating that NK cells are producing more IFN-γ per cell than T cells.

To determine whether NK and T cells responded to *Pf*-RBC with similar kinetics, I determined the proportion of all IFN-γ+ cells that were either NK cells or T cells after co-culturing for periods from 6 hrs to 6 days (**Fig. 3.5E**). Although there is heterogeneity in the response among donors, the distinct trend was for IFN-γ+ cells to be almost exclusively NK cells after 6 hrs of co-culture and for NK cells to continue to dominate the IFN-γ response at 12 hrs. However, by 24 hrs the response was evenly split between NK cells and T cells and T cell responses gradually came to dominate the IFN-γ response over the next 5 days. This observation, that the NK cell IFN-γ response is very rapid but transient, is not unexpected but does likely explain many of the apparent discrepancies in the literature where "innate" responses tend to have been assayed at a single point in time (Hensmann and Kwiatkowski 2001; D'Ombrain, Hansen et al. 2007).

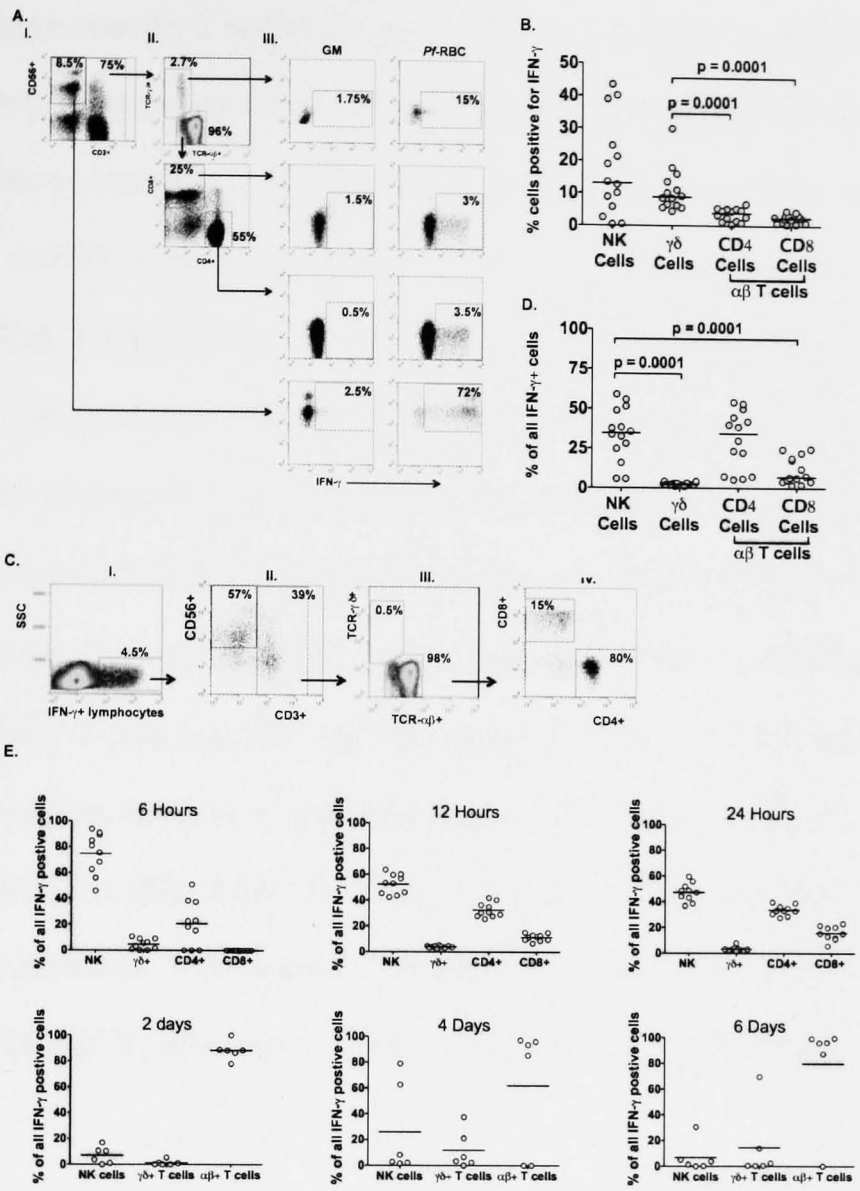


Figure 3.6 IFN-γ response of NK cells and T cell subsets.

PBMC were incubated with *Pf*-RBC for periods of 6hrs to 6 days and analysed by flow cytometry. (**A**) Representative flow cytometry data from 1 donor demonstrating gating strategy for (I) NK cells ($CD56^+$ $CD3^-$ lymphocytes) and T cells (all $CD3^+$ lymphocytes), which are then further classified as (II) TCR-$\gamma\delta^+$ or TCR-$\alpha\beta^+$ T cells and then all TCR-$\alpha\beta^+$ T cells are identified as being $CD4^+$ or $CD8^+$ T cells. Each population of lymphocytes can then be gated (III) for detection of intracellular IFN-γ after 24 hrs co-culture with *Pf*-RBC (right column) or without (left column; GM).(**B**) The percentage of all NK cells or all $\gamma\delta^+$ T cells, $CD4^+$ T cells or $CD8^+$ T cells expressing intracellular IFN-γ after 24 hrs co-culture with *Pf*-RBC. N= 14 naïve donors. (**C**) Gating strategy for identification of all IFN-γ+ lymphocytes and their subsequent identification as either NK cells or $\gamma\delta^+$ T cells, $CD4^+$ T cells or $CD8^+$ T cells. (**D**) The percentage of IFN-γ^+ cells that are NK cells or $\gamma\delta^+$ T cells, $CD4^+$ T cells or $CD8^+$ T cells per 100,000 lymphocytes after 24 hrs co-culture with *Pf*-RBC. N= 14 naïve donors. (**E**) The percentage of all IFN-γ+ cells that are either NK cells or T cells after 6, 12 or 24 hrs or 2, 4 or 6 days co-culture with *Pf*-RBC. Numbers on X axis identify individual blood donors. B,D,E: Horizontal lines represent medians. P-values are for 2 tailed paired Wilcoxon tests with 95% CI comparing NK cells with T cells from the same donor.

Since potent antigen-specific T cell responses to *Pf*-RBC might not be expected among malaria naïve donors I next sought to identify which subsets of T cells were producing IFN-γ. PBMCs from 14 donors were incubated with *Pf*-RBC for 21 hours and gated as NK cells, as $CD3^+$ $\gamma\delta TCR^+$ or $CD3^+$ $\alpha\beta TCR^+$ T cells; at some key time points, αβ T cells were gated as either $CD4^+$ or $CD8^+$ T cells (**Fig. 3.6A**). When each lymphocyte population was separately analysed for IFN-γ expression we again found that - on average - a higher proportion of NK cells than T cells produce IFN-γ, but among the T cell populations, a significantly higher proportion of $\gamma\delta TCR^+$ T cells were IFN-γ^+ than either of the $\alpha\beta TCR^+$ T cell subsets (**Fig. 3.6B**). However, because the majority of circulating T cells are $\alpha\beta TCR^+$, when I gate on all IFN-γ^+ cells (**Fig. 3.6C**) I find that the majority of IFN-γ^+ T cells are $CD4^+$ $\alpha\beta TCR^+$ and that $CD4^+$ $\alpha\beta TCR^+$ T cells and NK cells contribute equally to the total IFN-γ^+ population with $CD8^+$ $\alpha\beta TCR^+$ and $\gamma\delta TCR^+$ cells making only a minor contribution (**Fig. 3.6D**). To make sure that I had not missed the optimal timing for responses of any particular T cell subset, I repeated the kinetic analysis over 6 days (**Fig. 3.6E**) and found that TCR-$\alpha\beta^+$ T cells were the major contributing T cell population throughout the response.

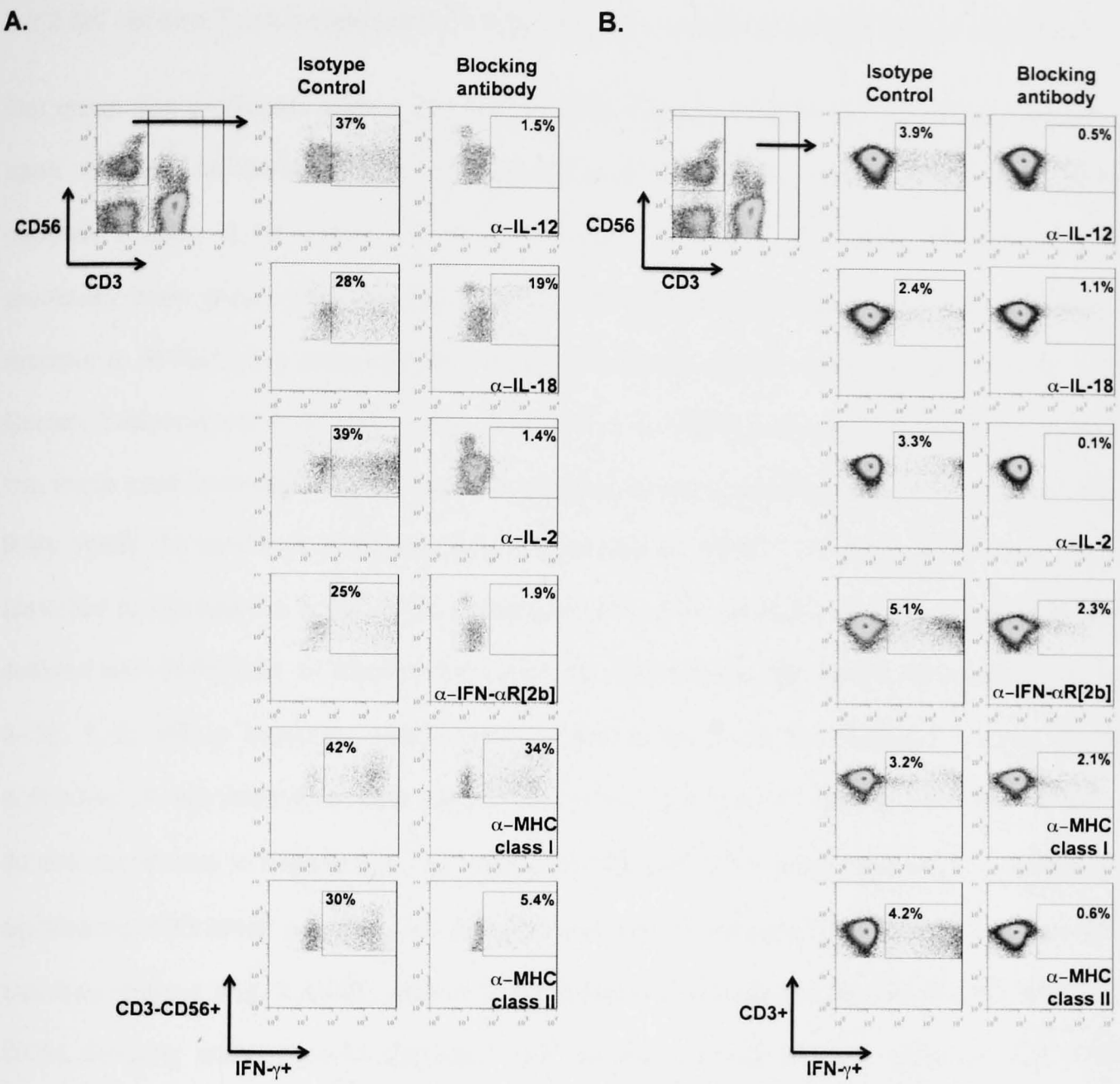


Figure 3.7 Representative flow cytometry plots to show the effects of blocking antibodies on NK cell and T cell IFN-γ responses to *Pf*-RBC.

PBMC were cultured in with *Pf*-RBC in the presence of blocking antibodies to IL-12, IL-18, IL-2, IFN-αR[2b], MHC Class 1 or MHC Class II, or their respective isotype matched controls. Representative flow cytometry data are shown for 1 donor demonstrating the gating strategy for (**A**) NK cells ($CD56^+$ $CD3^-$ lymphocytes) and (**B**) T cells (all $CD3^+$ lymphocytes), which are then further classified as IFN-γ^+ (% shown) or IFN-γ^-.

3.3.2 *NK cell and T cell responses to Pf-RBC are both cytokine and MHC-class II dependent*

Our group has previously shown that NK cell responses to *Pf*-RBC are absolutely dependent upon myeloid cell-derived IL-12 (Artavanis-Tsakonas, Eleme et al. 2003) and partially dependent upon IL-18, IFN-α and IL-2 (Newman, Korbel et al. 2006). Moreover, it has previously been shown that memory $CD4^+$ T cells from malaria naïve individuals are able to respond to *Pf*-RBC in a classical MHC-class II-restricted manner (Jones, Hickling et al. 1990; Currier, Sattabongkot et al. 1992; Dick, Waterfall et al. 1996) and it is now generally accepted that these cells have been primed by cross-reacting antigens (Currier, Sattabongkot et al. 1992; Riley 1999). To confirm and extend these observations, PBMC from 10 malaria naïve donors (selected to represent a range of NK responses from non-responders to high responders) were cultured with *Pf*-RBC for 24 hours in the presence of blocking or neutralising antibodies to IL-12, IL-18, IL-2, IFN-α receptor, MHC-Class I, MHC-Class II or the relevant isotype control antibodies. A representative data set for one donor is shown in **Figure 3.7**; data for all 10 donors are shown in **Figure 3.8**. As expected, NK cell IFN-γ responses were markedly and significantly diminished by anti-IL-12, anti-IL-2 and anti-IFN-αR although the effect of anti-IL-18 was less obvious (**Fig. 3.8A-D**). Also in confirmation of a previous study (Korbel, Norman et al. 2009), blocking antibody to MHC-Class I had no effect on the NK cell response (**Fig. 3.8E**). Somewhat unexpectedly however, NK cell responses to *Pf*-RBC were markedly reduced in the presence of MHC-Class II blocking antibody (**Fig. 3.8F**).

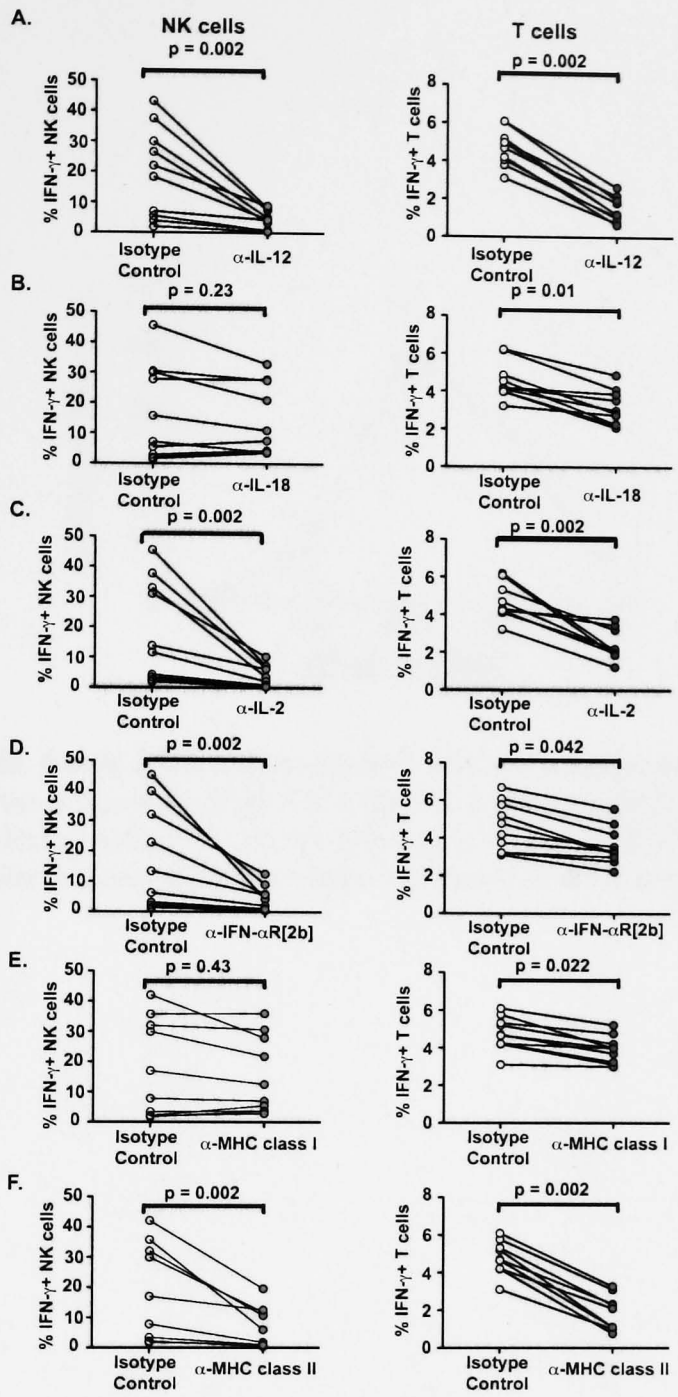


Figure 3.8 NK cell and T cell responses to *Pf*-RBC are both cytokine and MHC-class II dependent.

PBMC from 10 donors were cultured with *Pf*-RBC for 24 hours in the presence of blocking antibodies to cytokines, cytokine receptors or MHC molecules or with isotype matched control antibodies and the percentages of NK cells (left columns) and T cells (right columns) expressing intracellular IFN-γ determined by flow cytometry. P-values are for 2-tailed paired Wilcoxon test with 95% CI. (**A**) anti-IL-12; (**B**) anti-IL-18; (**C**) anti-IL-2; (**D**) anti-IFN-α receptor; (**E**) anti-MHC Class I; (**F**) anti-MHC Class II.

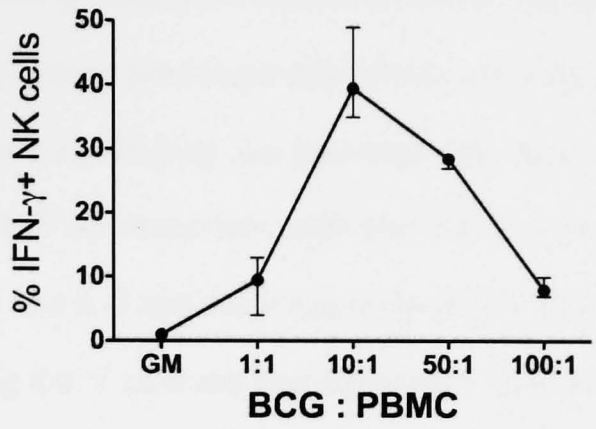


Figure 3.9 Titrating *M. bovis* BCG input dose to define optimal multiplicity of infection for NK cell activation. The proportion of all NK cells expressing intracellular IFN-γafter 21 hrs co-culture with BCG at varying MOI in 5 donors who have received BCG vaccination. Data are representative of median values with error bars representing the range.

As expected, given the previously published data (Jones, Hickling et al. 1990; Currier, Sattabongkot et al. 1992; Dick, Waterfall et al. 1996; Riley 1999; Artavanis-Tsakonas, Eleme et al. 2003; Newman, Korbel et al. 2006; Korbel, Norman et al. 2009), the preponderance of $CD4^+$ T cells in the IFN-γ responding population and the small but noticeable contribution of $CD8^+$ T cells, total T cell IFN-γ responses were markedly diminished in the presence of anti-MHC-Class II antibodies (**Fig. 3.8F**) and significantly, but less markedly, diminished by MHC-Class I blockade (**Fig. 3.8E**). Total T cell responses were also significantly inhibited by neutralising antibodies to IL-12, IL-18 and IL-2 and there was a marginally significant effect of anti-IFN-αR (**Figure 3.8A-D**) indicating that T cells are also dependent upon accessory cell-derived cytokines.

The observation that blocking MHC class II molecules inhibited the production of IFN-γ, led me to question if this was unique to NK cell and T cell responses to *Pf*-RBC or if responses to other pathogens or pathogen-related stimuli are similarly regulated by MHC class II presentation. I used *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG, Pasteur strain; kind gift from U. Schaible) as a model organism as well as LPS and recombinant human IL-12 and IL-18 (rhIL-12 + rhIL-18) to represent pathogen-derived proteins and exogenous stimuli. I first co-incubated PBMC with varying doses of BCG, in order to establish the most effective MOI (**Figure 3.9**).

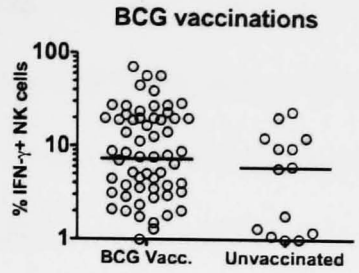


Figure 3.10. Prior BCG vaccination has no significant effect on the NK cell IFN-γ response to BCG. PBMC were cultured overnight with BCG at an MOI of 1 PBMC : 10 BCG. The proportion of all NK cells expressing intracellular IFN-γ after 24 hrs co-culture with BCG. N= 50 malaria naïve donors. 2-tailed paired Mann-Whitney test was performed to measure statistical significance.

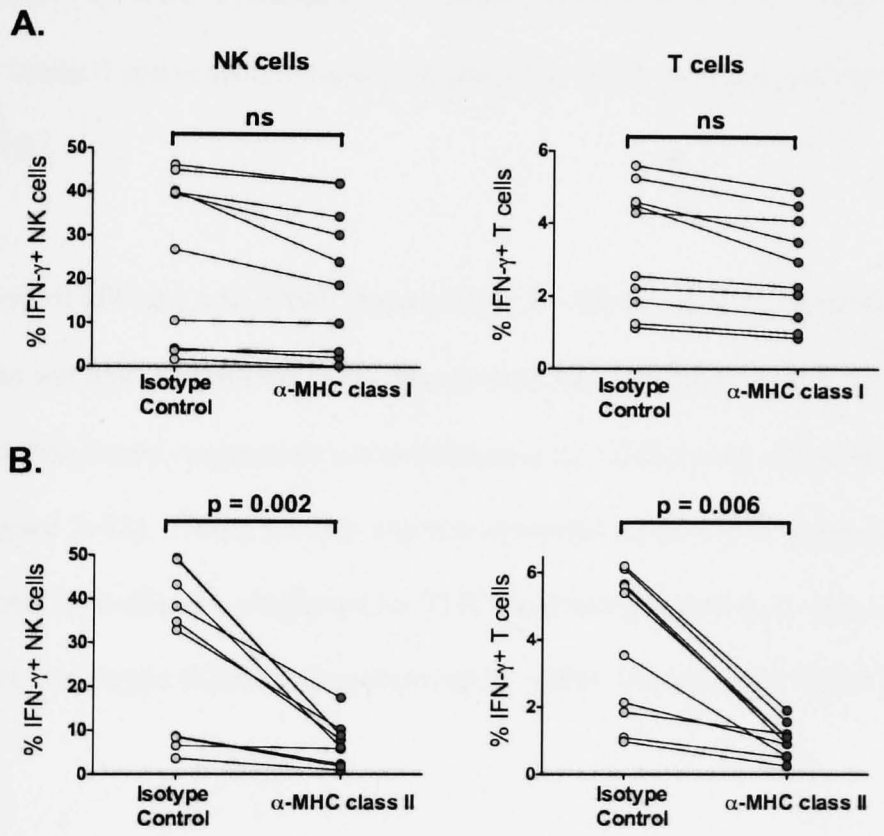


Figure 3.11 NK cell and T cell responses to BCG are also MHC-class II dependent PBMC from 10 donors were cultured with BCG for 24 hours in the presence of blocking antibodies to (**A**) MHC class I molecules molecules or (**B**) MHC class II molecules or with isotype control antibodies and the percentages of NK cells (left columns) and T cells (right columns) expressing intracellular IFN-γ determined by flow cytometry. P-values are for 2-tailed paired Wilcoxon test with 95% CI.

Once I had established that 10 BCG : 1 PBMC was the optimal MOI, I tested the NK cell IFN-γ response to BCG in the same 50 malaria naïve donors (**Figure 3.10**). Of the 50 donors, 36 were previously BCG-vaccinated and 14 were not. I did not observe any significant differences in NK cell IFN-γ responses between the BCG-vaccinated (exact range unknown but all donors >20 years) and unvaccinated group.

I next co-incubated PBMC from 10 donors with BCG for 24 hours either in the presence of blocking antibodies for MHC class I and MHC class II molecules or with their respective isotype control antibodies. As for the experiments using *Pf*-RBC, blocking MHC class I presentation had no significant effect on BCG-induced IFN-γ production by NK cells or T cells (**Fig. 3.11A**) but blocking MHC class II presentation nearly ablated the IFN-γ response in both NK cells and T cells (**Fig. 3.11B**).

This observation of NK cell and T cell dependency on MHC class II presentation of antigen is not universal as we also measured IFN-γ production from NK cells and T cells in response to LPS and found that these responses were independent of blocking either MHC class I or II molecules (**Figure 3.12**). These results are not unexpected as it is considered well-established that LPS-induced activation is mediated by TLR 4 pathways leading to the activation of NF_KB signalling in macrophages (Moore, Goodrum et al. 1976; Beutler and Rietschel 2003).

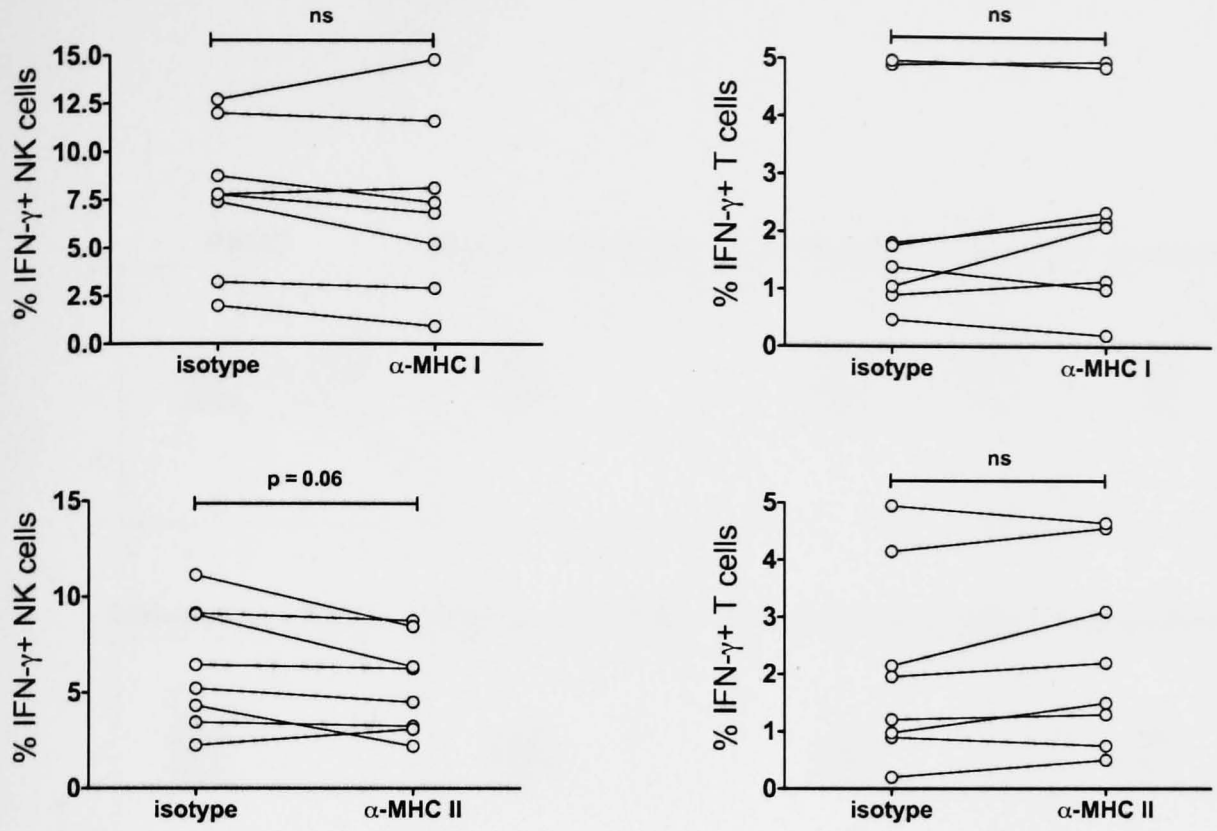


Figure 3.12 NK cell and T cell responses to LPS are independent of MHC class I and class II presentation

PBMC from 8 donors were cultured with LPS for 24 hours in the presence of blocking antibodies to (**A**) MHC class I molecules molecules or (**B**) MHC class II molecules or with isotype control antibodies and the percentages of NK cells (left columns) and T cells (right columns) expressing intracellular IFN-γ determined by flow cytometry. P-values are for 2-tailed paired Wilcoxon test with 95% CI.

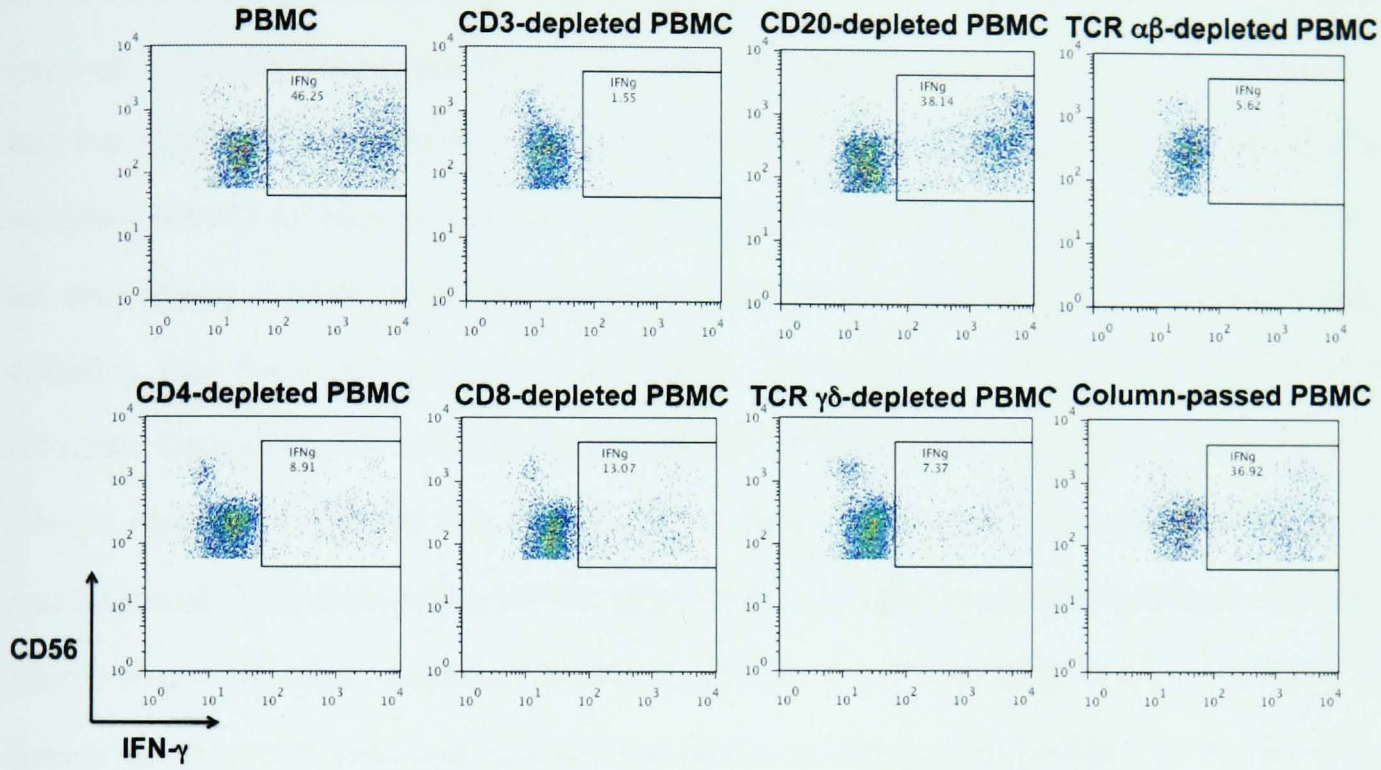


Figure 3.13 Representative flow cytomtery plots demonstrating effects of depleting T cell subsets on IFN-γ production by NK cells in response to *Pf*-RBC
Representative flow cytometry data from one donor showing the percentages of NK cells expressing intracellular IFN-γ after 24 hours co-incubation with *Pf*-RBC.

3.3.3. *NK cell responses to Pf-RBC are dependent upon T cell help*

The observation that NK cell responses to *Pf*-RBC were highly dependent upon both IL-2 and MHC Class II raised the possibility that – as previously described for human NK cell responses to influenza (Fehniger, Cooper et al. 2003; He, Draghi et al. 2004) - NK cells require signals (such as IL-2) from antigen-specific CD4+ T cells in order to respond optimally to *Pf*-RBC. To test this hypothesis, I depleted PBMCs of various lymphocyte populations and tested the remaining PBMCs for their ability to make IFN-γ in 24 hr cultures (**Figure 3.13 and Figure 3.14**). NK cells among CD3-depleted PBMCs made strong responses to rhIL-12+IL-18 (**Fig. 3.14A**) indicating that these two cytokines (at optimal concentrations) are sufficient for NK cell activation. Conversely, NK cells among CD3-depleted PBMCs were completely unable to make IFN-γ in response to *Pf*-RBC (**Fig. 3.14B**) but NK cells among CD20 (B cell)-depleted PBMCs made a robust IFN-γ response to *Pf*-RBC **(Fig. 3.14C**). To determine which subset(s) of T cells provide help to NK cells, I depleted PBMCs of just $CD4^+$ T cells, just $CD8^+$ T cells, all $\alpha\beta TCR^+$ T cells or all $\gamma\delta TCR^+$ T cells and cultured the remaining PBMCs with *Pf*-RBC for 24 hrs (**Fig. 3.14D-G**). All depletions reached levels of >97% efficiency. It was clear that the NK cell response to *Pf*-RBC was only completely ablated when all T cells were removed but that removal of any T cell subset significantly reduced the NK cell IFN-γ response. Finally, to rule out any non-specific effects of magnetic bead treatment, unlabelled PBMC were incubated with anti-PE microbeads and passed through the magnetic column (**Fig. 3.14H**); a robust IFN-γ response was seen.

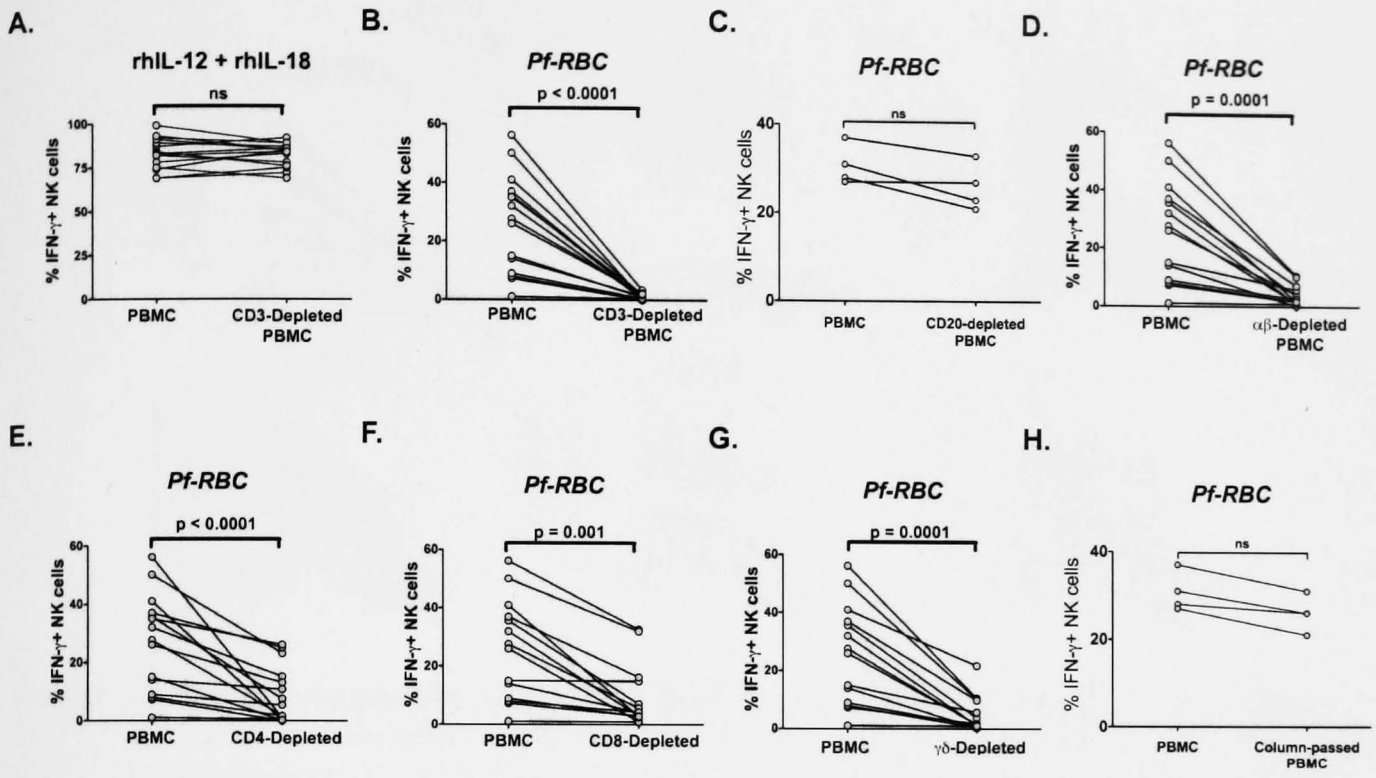


Figure 3.14 NK cell responses to *Pf*-RBC are dependent upon CD4$^+$ T cell help.
Intact PBMC or PBMC depleted of various T cell subsets were incubated for 24 hours with (**A**) 100ng/ml each of rhIL-12 + rhIL-18 or with (**B-F**) *Pf*-RBC, and the percentages of NK cells staining for intracellular IFN-γ were measured by flow cytometry. PBMC were depleted of (**A,B**) all CD3$^+$ cells; (**C**) CD20$^+$ B cells; (**D**) αβ T cells; (**E**) CD4$^+$ T cells; (**F**) CD8$^+$ T cells and (**G**) γδ T cells or (**H**) incubated with anti-PE microbeads (without antibody) and passed through the magnetic column. P-values in are for 2-tailed paired Wilcoxon tests, 95% CI, for 15 donors.

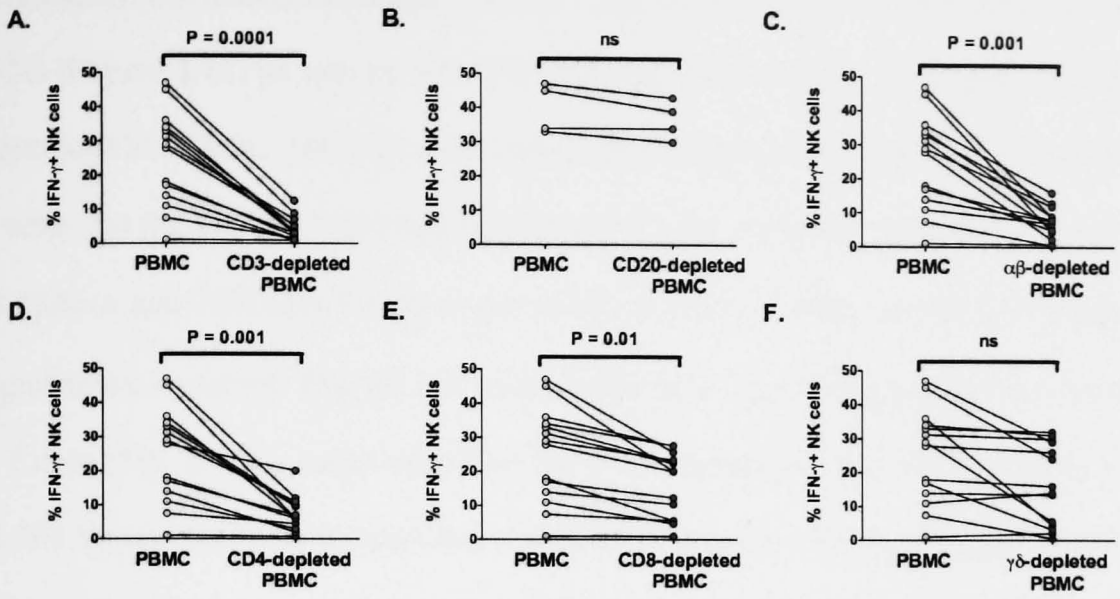


Figure 3.15 NK cell responses to *M. bovis* BCG are dependent upon CD4$^+$ T cell help. Intact PBMC or PBMC depleted of various T cell subsets were incubated for 24 hours with (**A-F**) BCG, and the percentages of NK cells staining for intracellular IFN-γ were measured by flow cytometry. PBMC were depleted of (**A**) all CD3$^+$ cells; (**B**) CD20$^+$ B cells; (**C**) αβ T cells; (**D**) CD4$^+$ T cells; (**E**) CD8$^+$ T cells and (**F**) γδ T cells. P-values in are for 2-tailed paired Wilcoxon tests, 95% CI, for 15 donors.

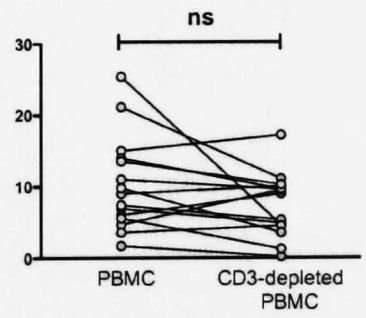


Figure 3.16 NK cell responses to LPS are independent of T cell help. Intact PBMC or PBMC depleted of all CD3$^+$ cells were incubated for 24 hours with LPS, and the percentages of NK cells staining for intracellular IFN-γ were measured by flow cytometry. P-values in are for 2-tailed paired Wilcoxon tests, 95% CI, for 15 donors.

I also measured the effects of subset-specific T cell depletion on NK cells when co-incubating with BCG (**Figure 3.15**) as well as with LPS (**Figure 3.16**). In contrast to what was seen for NK cell responses to *Pf*-RBC, NK cell responses to BCG seem to be completely dependent upon αβ+ T cells, but there was no obvious role for γδ+ T cells. When depleting PBMC of all T cells (**Fig. 3.15A**) or just TCR-αβ+ T cells or just CD4+ or CD8+ T cells, the NK cell response to BCG was significantly impaired. The NK cell response to BCG was, however, independent of γδ-TCR+ T cells (**Fig. 3.15F**). I also examined the T cell dependence of NK cell responses to LPS (**Fig. 3.16**). I found that only in one case was the NK cell response apparently dependent upon T cell help and overall there was no significant effect of T cell depletion on the NK cell IFN-γ response to LPS, suggesting an alternative pathway for NK cell activation by LPS.

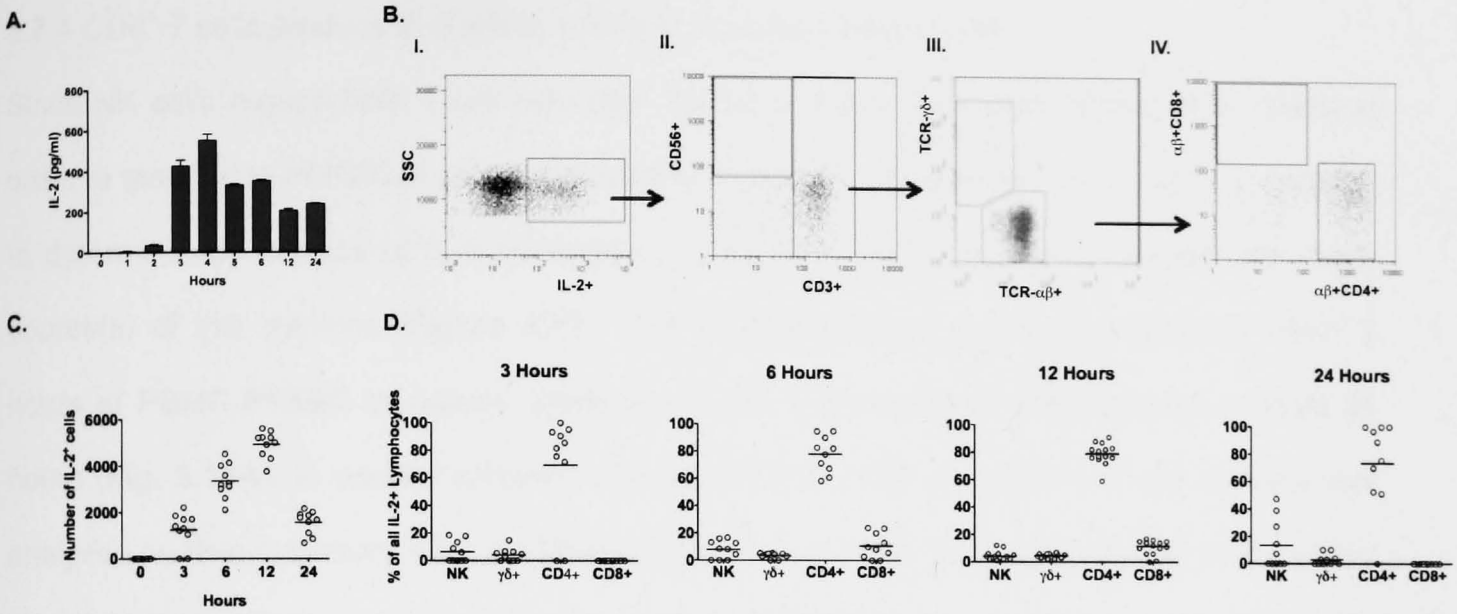


Figure 3.17 Rapid IL-2 production by CD4$^+$ T cells after co-culture with *Pf*-RBC
PBMC were incubated with *Pf*-RBC for up to 24 hours. (**A**) Median concentration of IL-2 in culture supernatants, determined by cytometric bead array, over time. N = 5 donors. (**B**) Gating strategy for identification and phenotypic characterisation of IL-2-producing cells. (**C**) The total number of IL-2$^+$ lymphocytes at each time point. N = 10 donors. (**D**) The proportion of all IL-2$^+$ lymphocytes that are either NK cells or are γδ$^+$ T cells, CD4$^+$ T cells or CD8$^+$ T cells when comparing responses over 24 hours. Data are presented for 10 donors at all time point except at 12 hours for which data from 14 donors are shown. Horizontal lines indicate median values.

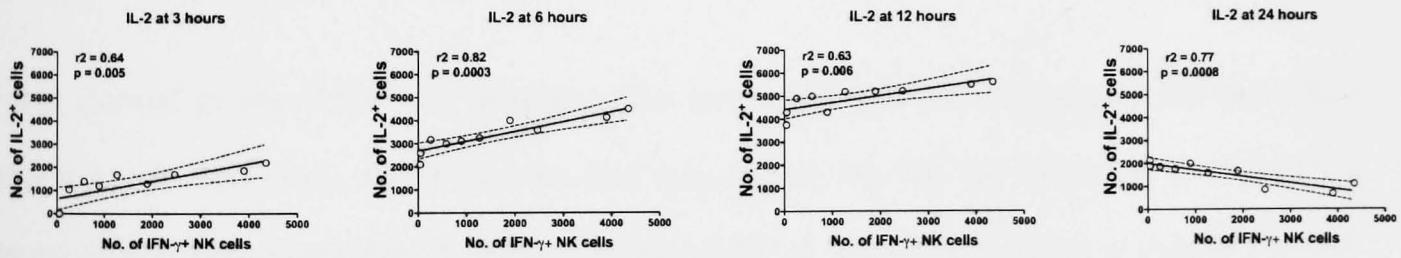


Figure 3.18. Early IL-2 production is highly correlated with NK cell IFN-γ production in response to *Pf*-RBC. PBMC from 10 donors were co-incubated with Pf-RBC for up to 24 hours. 2-tailed Spearman correlation tests were performed to compare IL-2 responses at various time points with the peak NK cell IFN-γ response (at 24 hours). Solid lines represent the best fit line and dotted lines represent 95% confidence intervals.

3.3.4 *CD4$^+$ T cells produce IL-2 within 12 hrs of co-culture with Pf-RBC*

Since NK cells require both T cell help (this thesis) and IL-2 (Newman, Korbel et al. 2006) in order to respond to *Pf*-RBC, it seemed likely that T cells are an essential source of IL-2. I sought to determine the kinetics of IL-2 production in response to *Pf*-RBC and to identify the major source(s) of this cytokine (**Figure 3.17**). IL-2 was detected in culture supernatants within 3 hours of PBMC-*Pf*-RBC co-culture, peaked at 4 hrs and remained detectable for at least 24 hours (**Fig. 3.17A**). In parallel cultures, intracellular IL-2 in NK cells and in T cell subsets was analysed by flow cytometry (**Fig. 3.17B-D**). All IL-2$^+$ cells were gated (**Fig. 3.17B**) and counted (**Fig. 3.17C**). IL-2$^+$ cells were first detected at 3hrs after initiation of co-culture and their numbers peaked at 12 hrs; the discrepancy in timing between the accumulation of soluble IL-2 in culture medium (**Fig. 3.17A**) and the peak of IL-2 secreting cells (**Fig. 3.17C**) presumably reflects the balance between secretion and consumption of IL-2 in the culture. At all time points, the vast majority (>80%) of IL-2-producing cells were CD4$^+$ T cells with only minor contributions from CD8$^+$ T cells at 6 and 12 hours and from γδT cells and NK cells at 3hrs (**Fig. 3.17D**). Interestingly, IL-2 responses were much less heterogeneous among the various donors than were IFN-γ responses.

I next wanted to see if the IL-2 response was correlated with the subsequent NK cell IFN-γ response, i.e. how likely was it that the IL-2 was driving the NK cell response to *Pf*-RBC? I examined IL-2 responses from 10 donors (**Figure 3.17**) at various time points in culture side-by-side with the NK cell IFN-γ response at 24 hours using XY scatter plots with 2-tailed spearman correlations (**Figure 3.18**). These data show that IL-2, produced as early as 3 hours post-incubation of PBMC with Pf-RBC, is highly correlated with the activation of NK cells. As the number of IL-2-producing lymphocytes increased, so did that number of IFN-γ+ NK cells.

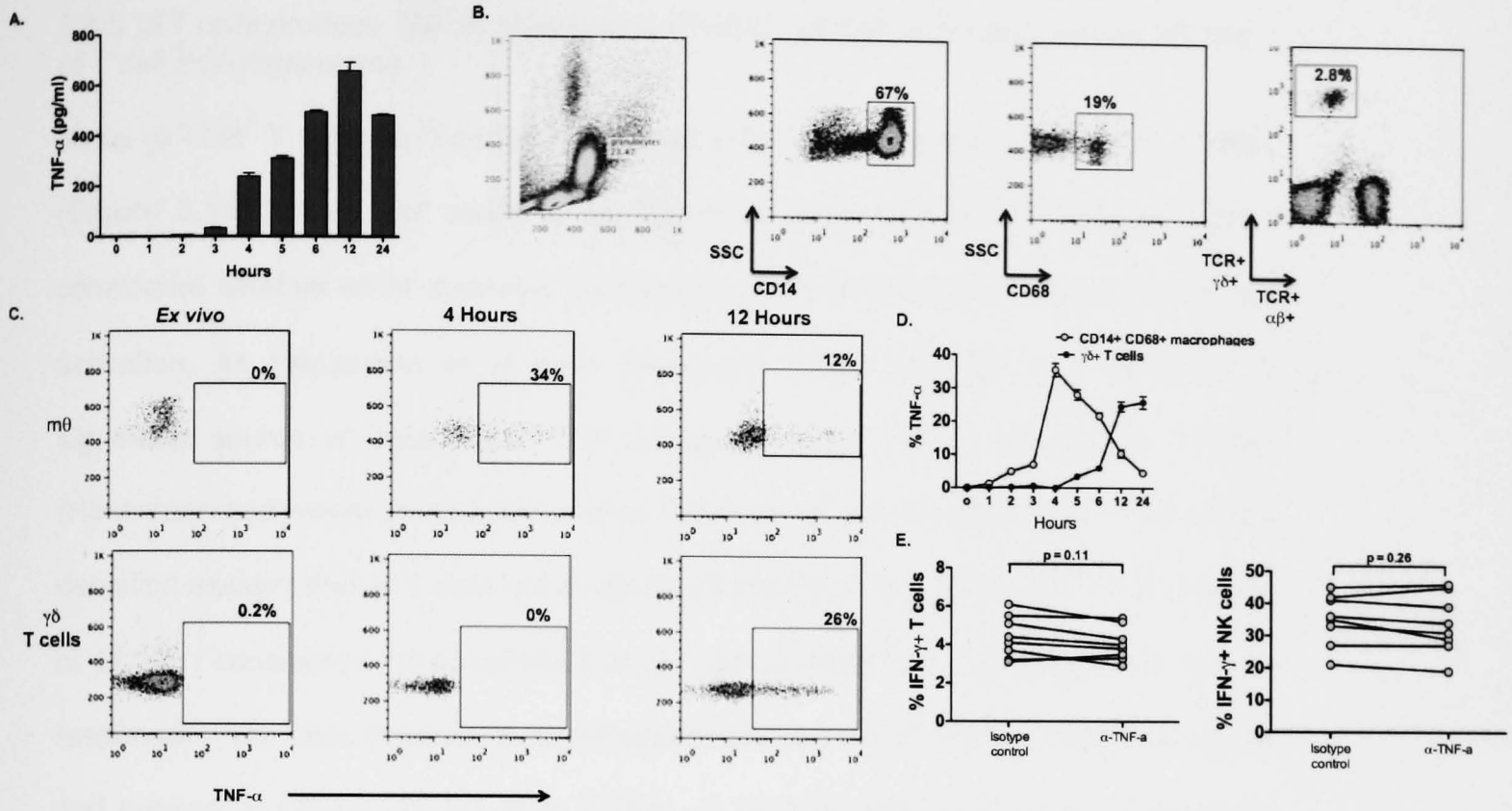


Figure 3.19 Rapid TNF production from macrophages and γδ+ T cells in response to *Pf*-RBC

PBMC were incubated with *Pf*-RBC for up to 24 hours. (**A**) Median concentration of TNF in culture supernatants, determined by cytometric bead array, over time. N = 5 donors. (**B**) Gating strategy for phenotypic characterisation of TNF-producing macrophages ($CD14^+CD68^+$) and $\gamma\delta^+$ T cells (TCR-$\gamma\delta^+$ TCR-$\alpha\beta^-$ when gated on all $CD3^+$ cells) after 12hrs PBMC co-culture with Pf-RBC. (**C**) Representative flow cytometry data from one donor showing the percentages of macrophages (top row) or $\gamma\delta^+$ T cells (bottom row) expressing intracellular TNF after 4 or 12 hours. (**D**) PBMC from 8 donors were incubated for up to 24 hours with *Pf*-RBC and percentages of macrophages (light circles) and $\gamma\delta^+$ T cells (dark circles) producing TNF-α were measured by flow cytometry. Data represent medians (±SE). (**E**) PBMC from 8 donors with previously documented high IFN-γ responses to *Pf*-RBC were incubated for 24 hours with *Pf*-RBC in the presence of neutralising antibody to TNF or with an isotype-matched control antibody and the percentages of T cells (left side) and NK cells (right side) expressing IFN-γ were measured. P-values are for 2-tailed paired Wilcoxon tests, 95% CI.

3.3.5 *γδ T cells produce TNF in response to Pf-RBC but TNF is not required for NK cell or T cell IFN-γ responses*

Since γδ TCR$^+$ T cells seemed to be necessary for an optimal NK response to *Pf*-RBC (**Figure 3.14**) but did not seem to be significant producers of IL-2 (**Figure 3.17**) I considered whether other cytokines produced by γδ T cells might contribute to NK cell activation. As Hensmann *et al* have previously shown that γδT cells represent a significant source of intracellular TNF during the early (18hr) response to *Pf*-RBC (Hensmann and Kwiatkowski 2001) and as Robinson *et al* concluded (from T cell subset depletion assays) that γδ T cells are a significant source of TNF (Robinson, D'Ombrain et al. 2009), I considered the possibility that γδ T cell-derived TNF might potentiate NK cell responses. TNF was detected in supernatants within 4 hrs of PBMC-*Pf*-RBC co-culture and peaked at approx. 12 hrs (**Fig. 3.19A**). In parallel, PBMCs from 8 malaria naïve donors were incubated with *Pf*-RBC for up to 24hrs and then stained for intracellular TNF and for surface markers to identify macrophages (CD14$^+$ CD68$^+$) and γδ T cells (**Fig. 3.19B**). When gating on all macrophages (**Fig. 3.19C**), TNF$^+$ macrophages were detectable within 2 hrs of *Pf*-RBC co-culture, the response peaking (with >35% of all macrophages being TNF$^+$) at 4hrs and declining to barely detectable levels by 24 hrs (**Fig. 3.19D**). In comparison, TNF+ γδ T cells were detected from approx 5-6 hrs after initiation of *Pf*-RBC stimulation and the response was maximal (with ~30% of all γδT cells being TNF$^+$) at 12-24hrs (F**ig. 3.19C-D**). However, I found no evidence that TNF was required for induction of an early IFN-γ response since when I stimulated PBMC from the same donors with *Pf*-RBC in the presence of neutralising antibodies to TNF the NK cell and T cell IFN-γ responses were unaffected (**Fig. 3.19E**).

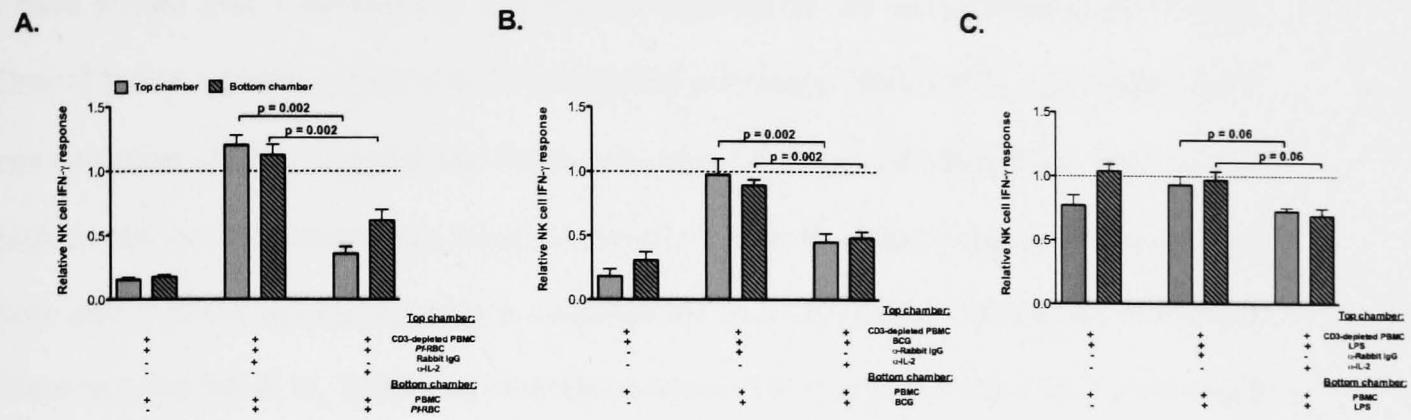


Figure 3.20 T cell help for NK cell activation by *Pf*-RBC does not require NK cell-T cell contact. PBMC were placed in the lower well, and CD3-depleted PBMC were placed in the upper well of a transwell chamber separated by a semi-permeable membrane and cultured in the presence (+) or absence (-) of *Pf*-RBC, anti-IL-2 antibody or isotype-matched control antibody (rabbit IgG). The percentage of NK cells in each chamber expressing IFN-γ was determined after 24 hrs. Data for each donor were normalised to the response given by their own PBMC cultured directly with *Pf*-RBC in the lower chamber (*Pf*-RBC: median 41%, inter-quartile ranges 6%; BCG: median 27%, inter-quartile ranges 5%; and LPS: median 14%, inter-quartile ranges 3%) and are shown as medians and inter-quartile ranges for 5 donors. P-values are for 2-tailed paired Wilcoxon tests, 95% CI.

3.3.6 *T cell help for NK cell activation by Pf-RBC does not require NK cell-T cell contact*

I have shown that T cell-derived signals are required for NK cell activation by *Pf*-RBC. One of these signals is clearly IL-2, emanating principally from CD4+ αβ T cells, but T cell depletion studies suggest that there may also be an, as yet undefined, role for γδ T cells in NK cell activation. We have previously shown that direct contact between NK cells and myeloid accessory cells is required for optimal NK cell responses to *Pf*-RBC (Newman, Korbel et al. 2006)and I therefore considered the possibility that T cells might also provide contact-dependent signals to NK cells (**Figure 3.20**). Intact PBMC and CD3-depleted PBMC were cultured in separate compartments of a transwell plate separated by a semi-permeable membrane; intact PBMC in the lower chamber and CD3-depleted PBMC in the upper chamber. When *Pf*-RBC (**Fig. 3.20A**) or BCG (**Fig. 3.20B**) were added to the CD3-depleted (upper) PBMC compartment but not to the intact PBMC (lower compartment), NK cells in neither compartment produced IFN-γ, suggesting that *Pf*-RBC/BCG need to be in the same compartment as the T cells in order for NK cells to become activated, consistent with a need for antigen presenting cells to take up antigen and present it to T cells in an MHC-Class II-dependent manner. However, if *Pf*-RBC/BCG were added to both compartments, NK cells in both the intact PBMC population and in the CD3-depleted NK cell population were able to respond fully, indicating that soluble T cell-derived signals passing through the semi-permeable membrane were sufficient to activate the NK cells. To determine if this soluble signal is IL-2, I added neutralising anti-IL-2 antibody to the CD3-depleted PBMCs in the upper chamber and added *Pf*-RBC/BCG to both compartments. In this case, the NK cell IFN-γ response was significantly inhibited in both compartments. Having already learned that the NK cell response to LPS was T cell-independent, I also used LPS (**Fig. 3.20C**) to stimulate NK cells as an internal negative control. When comparing conditions where

LPS was added to both the top and bottom chambers with only adding LPS to the top chamber, I did not observe any significant decrease in the NK cell response. Interestingly, blocking IL-2 seemed to reduce the response, although did not reach a level of significance.

3.4 *Discussion*

Whilst there is a general consensus that a rapid, cell-mediated, inflammatory response is required to contain the initial stages of human blood stage malaria infections there has been considerable debate over many years as to the key cellular effectors of this response (Stevenson and Riley 2004). I have therefore conducted a detailed kinetic analysis of all the likely sources of IFN-γ and find that the very early (< 24hrs) IFN-γ response to *Pf*-RBC is dominated - in most PBMC donors - by NK cells, that NK cells and T cells contribute more-or-less equally to the response at 24hrs and that T cells come to dominate the response from 48 hrs onwards. This rapid NK cell response to *Pf*-RBC among PBMCs from malaria naive donors is in full agreement with both earlier studies from our laboratory (Artavanis-Tsakonas and Riley 2002) and others (Baratin, Roetynck et al. 2007). D'Ombrain *et al (D'Ombrain, Hansen et al. 2007)* concluded that NK cells do not contribute significantly to the innate IFN-γ response to *Pf*-RBC however their studies did not include the very early time points (12-18 hrs) at which I observe maximal NK cell responses, suggesting that an NK response may have been present in their donors but was missed, although it is surprising that D'Ombrain *et al* report so few IFN-γ^+ NK cells at 24hrs. Although Hensmann *et al* (Hensmann and Kwiatkowski 2001) stained for CD3, CD56 and IFN-γ after 18hrs co-culture with Pf-RBC they studied only 4 donors and did not explicitly report the NK cell IFN-γ response; from the data presented in their paper it is possible that between 10 and 40% of IFN-γ^+ cells in their donors could have been NK cells, which is well within the range that we have observed in our much larger donor panel.

More problematic is our observation that the vast majority of IFN-γ-producing T cells (at all time points from 6 hrs to 6 days of *Pf*-RBC co-culture) are TCR $\alpha\beta^+$ and $CD4^+$; this

finding is in direct contradiction to the findings of both Hensmann *et al* (Hensmann and Kwiatkowski 2001) and D'Ombrain *et al* (D'Ombrain, Hansen et al. 2007) which suggested that $\gamma\delta^+$ T cells are the major producers of IFN-γ in response to *Pf*-RBC stimulation. The reason for these conflicting results is unclear. It is possible that there are technical explanations. The three studies used different *P. falciparum* clones, which might differ in their production of the phosphorylated nonpeptidic antigens which are believed to be the ligands for $V\gamma9^+$ T cells (Behr, Poupot et al. 1996) although our observation of very robust TNF response to *Pf*-RBC by $\gamma\delta$T cells tends to argue against this explanation. We have previously observed that $\gamma\delta$T cells require quite considerable amounts of IL-2 in order to proliferate in response to *Pf*-RBC (Waterfall, Black et al. 1998); I do not routinely add exogenous IL-2 or other co-stimulatory agents to my cultures which may explain the lack of $\gamma\delta$T cell IFN-γ responses in my experiments, but again the robust $\gamma\delta$ T cell TNF response suggests that this is not the explanation. Lastly, I have taken great care to avoid misclassification of lymphocyte populations by (i) using simultaneous 7-colour analysis of CD3, CD56, $\alpha\beta$TCR, $\gamma\delta$TCR, CD4 and CD8 and IFN-γ expression, (ii) by inclusion of anti-CD3 in the intracellular mix to identify activated T cells which may have down-regulated surface expression of CD3 and (iii) by using a biotinylated anti-$\gamma\delta$TCR antibody which does not suffer from the tendency that we have observed of some supposedly $\gamma\delta$TCR-specific antibodies to stain both $CD3^-$ and $\alpha\beta$ TCR^+ cells. I note with some concern that the majority of the IFN-γ^+ $\gamma\delta TCR^+$ cells in the study by D'Ombrain *et al* (D'Ombrain, Hansen et al. 2007) also express NK cell markers, raising interesting questions as to the true identity of these cells. Alternatively, the differences seen in the various studies may reflect genuine heterogeneity in $\gamma\delta$T cell responses between individuals or populations, reflecting the known inter- and intra-

population diversity in numbers and TCR repertoires of $\gamma\delta^+$ T cells (Carding and Egan 2002).

The strong $\alpha\beta TCR^+$ $CD4^+$ T cell IFN-γ response that I observed after 2-6 days of *Pf*-RBC activation is highly consistent with previous reports in naïve humans (Currier, Sattabongkot et al. 1992; Fell, Currier et al. 1994; Dick, Waterfall et al. 1996), with evidence for cross-reactive priming of malaria-reactive T cells by a diverse array of commonly encountered micro-organisms (Currier, Sattabongkot et al. 1992) and with data from murine infections indicating that $\alpha\beta^+$ T cells can play a crucial role in the IFN-γ response to primary infections (Lertmemongkolchai, Cai et al. 2001; Berg, Crossley et al. 2005; Le-Barillec, Magalhaes et al. 2005). It is difficult to predict whether or not such cross-reactive responses would be beneficial should these individuals become infected with malaria; naïve adults show very variable responses to primary malaria infections with some making a very rapid – and partially protective - pro-inflammatory responses whilst others make little or no inflammatory response and develop rapidly escalating parasitaemia (Walther, Tongren et al. 2005; Walther, Woodruff et al. 2006).

A somewhat unexpected finding of this study was the absolute dependency of NK cells on $CD4^+$ T cell-derived IL-2 for their response to *Pf*-RBC. With hindsight, this should not have been a surprise. We have previously shown that NK cell responses to *Pf*-RBC depend upon cytokine and contact-mediated signals from myeloid cells (Newman, Korbel et al. 2006) and we did notice in those experiments that NK responses were diminished by anti-IL-2 and that adding purified adherent accessory cells to isolated NK cells did not fully restore the NK response seen in intact PBMCs (Newman, Korbel et al. 2006). Moreover, T cell and IL-2 dependency has previously been reported for NK cell

activation by influenza virus (He, Draghi et al. 2004) and, in mice, NK cell cytotoxicity against some tumour cells is $\alpha\beta TCR^+$ T cell dependent (Arina, Murillo et al. 2007; Shanker, Verdeil et al. 2007) but the possibility that this is IL-2-mediated remains to be formally tested. However, although it has long been taken for granted that antigen-specific T cells mediate some of their anti-microbial effects by activation of innate cells such as macrophages, the possibility that antigen-specific T cells might interact with other cells of the innate response, in particular with NK cells, has largely been ignored.

My current hypothesis is that, on exposure to cognate antigen presented by MHC Class II, TCR $\alpha\beta^+$ T cells secrete IL-2, which – in combination with essential signals from myeloid accessory cells – activates NK cells. The preponderance of $CD4^+$ T cells among the IL-2^+ cell population is consistent with the very marked effect on the NK cell response of MHC-Class II blockade. The transwell experiments indicate that the IL-2 is secreted from the T cells and diffuses in solution towards the NK cells; there is no apparent requirement for contact between the T cell and the NK cell nor for the focal secretion of IL-2 into an immune synapse. However, it is likely that T cells also provide other NK cell potentiating signals. For example, although I found no evidence that *Pf*-RBC-activated $\gamma\delta$ T cells produced significant amounts of IL-2, depletion of $\gamma\delta$ T cells from PBMCs substantially reduced the NK cell IFN-γ response. I do not believe that this is simply due to a reduction in the proportion of T cells in the cultures since $\gamma\delta$ T cells represent a very small proportion of the total T cell pool and an equally small proportion of the IL-2+ T cells. I have not yet identified the $\gamma\delta$ T cell contribution to the NK response but it does not appear to be TNF.

My data suggest that full reactivation of IFN-γ-producing T cells also requires IL-2-mediated signals since the peak of the T cell IL-2 response preceded the peak of the T cell IFN-γ response and neutralisation of IL-2 markedly reduced IFN-γ responses of T cells as well as NK cells. However, NK cell IFN-γ responses peaked within 6 hrs of the peak IL-2 response whereas T cell IFN-γ responses did not peak until ~36 hrs after the peak IL-2 response suggesting that T cells may require higher concentrations of IL-2, or more sustained IL-2 signalling, to become fully activated than do NK cells.

My observations imply that enhancing T cell responses to malaria (i.e. by vaccination) should also enhance the NK cell response. Given its speed (which precedes the bulk of the T cell IFN-γ response by hours or days) the NK IFN-γ response may represent an important determinant of vaccine efficacy and the ability of vaccine-induced T cells to support NK cell effector function might be an important biomarker of an effective T cell response to the vaccine. Finally, my demonstration that NK cell responses to *Pf*-RBC are so highly dependent on T cell IL-2 may necessitate reconsideration of data based simply on T cell-depletion of PBMC; the possibility that the IFN-γ–producing cells might be NK cells - and that the main role of T cells might be to secrete IL-2 - needs to be considered and IFN-γ ELISA, CBA or ELISPOT data need to be confirmed by intracellular cytokine staining and flow cytometry.

The following two chapters describe the activation of NK cells and T cells during recall responses to vaccine antigens.

Chapter 4: Markers of T cell and NK cell recall responses to vaccine antigens in a Phase IIb trial of the malaria vaccine RTS,S/ASO1E in Tanzanian children

4.1 *Introduction*

Malaria remains a major cause of human disease and deaths in the developing world (WHO 2008). Whilst recent reports of declining malaria morbidity in several African countries suggest that concerted control efforts may be having an impact on malaria transmission (WHO 2008), it is generally agreed that a safe and effective malaria vaccine would make a substantial contribution to sustainable control and elimination programmes. The most advanced malaria vaccine currently in human trials, RTS,S/AS, has been consistently found to confer partial protection against malaria infection and against clinical malaria episodes in multicentre phase II trials (Ballou 2009). RTS,S is a virus-like particle produced in *Saccharomyces cerevisiae* comprising the C terminus (amino acids 207-395) of the *P. falciparum* circumsporozoite (CS) protein fused to the Hepatitis B virus surface antigen (HBs) together with excess HBs (**Figure 4.1**).

It has recently been reported that RTS,S administered with the GSK proprietary adjuvant AS01 (liposomes containing MPL [derived from lipid A] and QS21 [plant extract from Soap bark tree]) to Tanzanian and Kenyan children aged 5-17 months has an efficacy against clinical malaria caused by *Plasmodium falciparum* of 53 % (95% confidence interval [CI], 28-69%) in children followed up for a mean of 8 months (Bejon, Lusingu et al. 2008), of 39% (95% CI 20-54%) in children followed for 12 months and of 46% (95% CI 24-61%) in a subset of children followed for a mean of 15 months (Olotu, Lusingu et al. 2010).

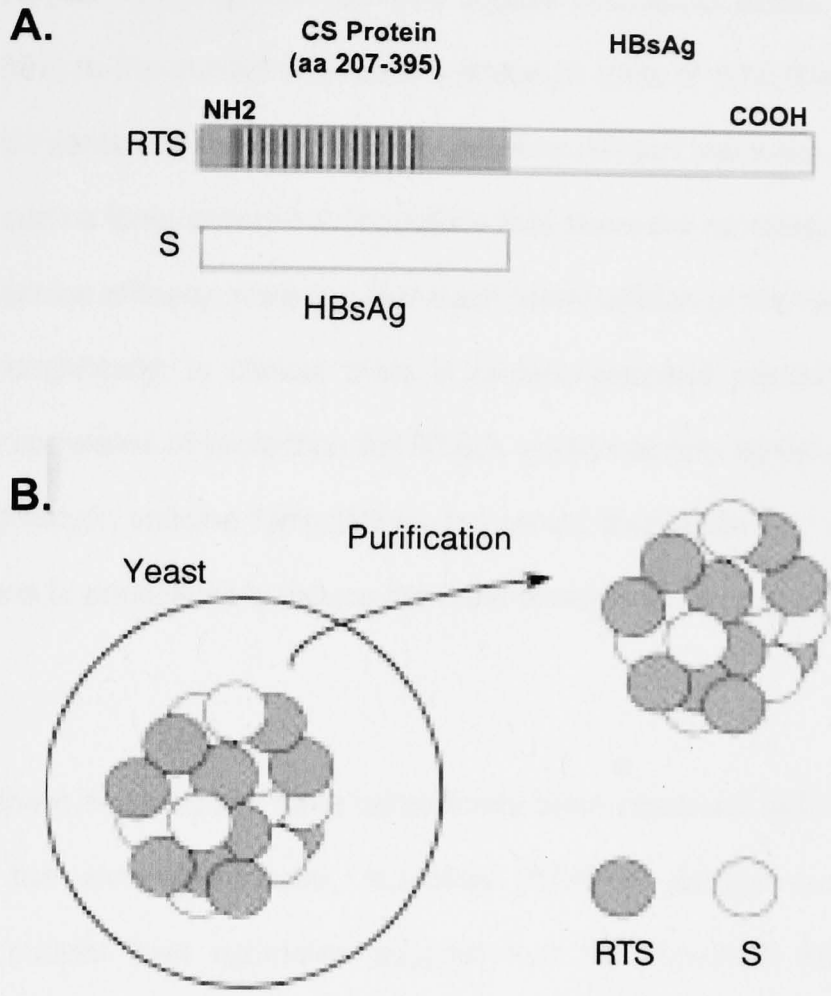


Figure 4.1 Schematic of RTS,S malaria vaccine (adapted from Soares and Rodrigues 1998). (**A**) Schematic representation of the RTS protein, which contains a repeat region of the circumsporozoite protein chemically linked to the HBsAg protein and the S protein, which is free HBaAg protein. These proteins are then co-transfected into (**B**) a yeast hybrid system, producing the RTS,S malaria vaccine.

Several thousand individuals have been enrolled into clinical trials of RTS,S and it has taken almost 15 years to progress from the original successful phase Ib trials (Stoute, Slaoui et al. 1997) to the current multicentre phase III trials of RTS,S/AS01 which aims to enroll 16,000 infants and children in 7 sub-Saharan African countries. One reason that this has been such a long, drawn-out process is that there are no reliable immunological correlates of vaccine efficacy, meaning that each reformulation of the vaccine has had to be evaluated empirically, in clinical trials in malaria-exposed populations. Identifying immunological correlates of protection for RTS,S would not only speed up evaluation of future pre-erythrocytic vaccine formulations but would also allow us to engineer those new formulations to preferentially induce the most protective immune effector responses.

To date, significant associations have consistently been observed between titres of IgG antibodies to the immunodominant, repetitive (NANP)n epitope and resistance to infection and current best estimates suggest that the threshold IgG concentration required for protection is something in excess of 20µg/ml (Moorthy, Diggs et al. 2009). Serum antibodies have been less consistently associated with protection against clinical episodes of malaria but it has recently been calculated that anti-(NANP)n antibody titres of approx 40 ELISA Units/ml (EU/ml) are associated with a highly significant step-change in risk of clinical malaria (Olotu, Lusingu et al. 2010). The contribution of cellular immune responses to RTS,S induced immunity has been less extensively studied. Preclinical data are strongly suggestive of protective roles for IFN-γ-producing $CD8^+$ T cells (Moorthy and Ballou 2009). Among malaria naïve vaccinees, activation of both $CD4^+$ and $CD8^+$ T cells has been observed (by IFN-γ ELISPOT and by flow cytometric analysis of IFN-γ, TNF, IL-2 and CD40L expression) after *in vitro* restimulation of peripheral blood mononuclear cells (PBMC) with peptides representing known T cell

epitopes from the CS protein (Lalvani, Moris et al. 1999; Sun, Schwenk et al. 2003; Kester, Cummings et al. 2009) and there has been a consistent (and, in one case, statistically significant) trend for individuals who were subsequently protected against challenge infection to have stronger responses than individuals who were not protected. Importantly, in a recent trial of RTS,S administered with AS02 (an oil-in-water version of the MPL/ QS21 adjuvant) to infants in Mozambique has revealed marginally statistically significant associations between protection from infection, CS-specific IL-2 secretion (detected by cytometric bead array) and $CD8^+$ T cell IFN-γ production (detected by intracellular staining and flow cytometry) in whole blood assays (Barbosa, Naniche et al. 2009).

In this chapter, I will report the results of a detailed analysis of the post-vaccination cellular immune responses of children (aged 5-17 months at enrolment) enrolled in the Phase I/IIb trial of RTS,S/AS01E in Korogwe, Tanzania. Although the number of clinical cases of malaria among the cohort of children studied was too low for any meaningful analysis of correlates of protection, I have identified additional very sensitive markers of CS-specific T cell recall responses (CD69 and CD25 expression) and a previously unrecognised response by natural killer (NK) cells. In addition to facilitating the search for sensitive and specific immunological correlates of vaccine efficacy, these newly identified markers of cellular immune responses to RTS,S reveal a potential new pathway of vaccine-induced pre-erythrocytic immunity, namely rapid activation of NK cells by antigen-specific secretion of IL-2.

4.2 *Materials and methods*

4.2.1 Study subjects and vaccinations

Four hundred and forty seven, 5- to 17-month old Tanzanian children were enrolled in a Phase IIb, randomised, double blind, controlled trial of RTS,S/AS01E (ClinicalTrials.gov registration number NCT00380393); the trial is described in detail elsewhere (Bejon, Lusingu et al. 2008). Children received either 3 doses of RTS,S/AS01E (n = 224) or 3 doses of human diploid cell rabies vaccine (Sanofi-Pasteur) (n = 223) at approximately 4 week intervals (acceptable range 20-60 days). Venous blood samples for assessment of cellular immune responses and antibody titres were collected approximately 4.5 months after the third vaccination from 93 recipients of the RTS,S/AS01 vaccine and 121 recipients of the rabies vaccine. Subjects to be included in this study were selected from health care centres close to the lab in order that blood could be processed within 2 hours of collection. A full blood count was made for each child and Giemsa-stained thick blood films were examined for malaria parasitaemia. The characteristics of the children included in this analysis are shown in **Table 1**. Ethical approval for the trial, including this immunological assessment, was obtained from the Tanzanian Medical Research Coordinating Committee, the London School of Hygiene and Tropical Medicine Ethics Committee and the Western Institutional Review Board, Seattle, USA. Independent Data Safety Monitoring Board and Local Safety Monitors were appointed. The trial was sponsored by GlaxoSmithKline Biologicals and the PATH Malaria Vaccine Initiative.

Characteristic	RTS,S/AS01E	Rabies
	(N=119)	(N=146)
Mean age (months)*	11.9(±0.22)	12(±0.24)
[Hb] (g/dL)*	10.5(±0.074)	10.6(±0.068)
WBC (x 10^6/mL)*	9.45(±0.17)	9.45(±0.19)
Sex		
Male	47	85
Female	72	61
anti-HBsAg Ab titres (Units/ml)		
Pre-vaccination**	169 (60-493)	290 (103-630)
Post-vaccination (Month 3)**	66,519 (11,503-140,007)	215(79-483)
Post-vaccination (Month 12)**	12,472 (5367-28015)	157 (39-427)
# *P. falciparum* positive (blood smear)	2	4
Clinical malaria cases	3	13

Table 4.1. *Baseline characteristics of subjects according to vaccine group*
* Data are presented as mean values ± SE
** Data are presented as median values plus inter-quartile ranges (25%-75%)

4.2.2 PBMC preparation and culture

Venous blood was collected into vacutainers containing sodium heparin (10iu/ml blood, CP Pharmaceuticals) and peripheral blood mononuclear cells (PBMC) were isolated by Histopaque 1077 (Sigma) density gradient centrifugation as described in **Chapter 2**. Cells were resuspended at a concentration of 2 x 10^6 cells/ml in complete cell growth medium (GM; RPMI-1640 with 10% heat-inactivated human AB serum, 100 Units/ml penicillin, and 100µg/ml streptomycin) in U-bottom 96-well tissue culture plates (total volume = 200µl) and incubated, with or without antigenic or mitogenic stimulation, at 37°C in 5% CO_2 for 24 hours. All assays were performed on freshly isolated cells. PBMC were stimulated with a pool of peptides (n = 53, 15 mers, overlapping by 11 amino acids, each peptide at a final concentration of 1 µg/ml, purity > 85%) representing the vaccine sequences of the hepatitis B surface antigen (HBs), or with a similar pool (n = 31 peptides) representing the vaccine sequence of the CS protein (both peptide pools manufactured by Eurogentec), or with rhIL-12+IL-18 (0.1µg/ml) as a positive control for NK cell and T cell activation. Negative control cultures contained growth medium (GM) alone. Due to insufficient numbers of PBMCs from some children, the numbers of samples tested varies among the assays performed.

4.2.3 *Antigens: HBs and CS peptides*

Peptides representing CS and HBs sequences were provided by GSK Biologicals and were frozen in dimethylsulfoxide (DMSO) at a concentration of 1mg/ml.

4.2.3 *Cell surface and intracellular staining for flow cytometry*

Surface and intracellular staining was performed using standard protocols as described previously (**Chapter 2**). The antibodies/reagents used were: anti-CD3 PerCP (BD Biosciences); anti-CD56 APC (Beckman Coulter); anti-CD69 PE (Serotec) and anti-IFN-γ FITC (Serotec); anti-CD4 FITC, and anti-CD25 APC (eBioscience). All antibodies were mouse monoclonals. Flow cytometric data were acquired using a Becton Dickinson FACSCalibur and analyses were performed using FloJo analysis software (TreeStar).

4.2.4 *Cytokine secretion assays*

Secreted IL-2, IFN-γ and IL-10 were measured, according to the manufacturer's instructions, by Luminex (Millipore). All samples were acquired using the Luminex analyzer® and analysis was performed using Luminex xPONENT® software (Millipore).

4.2.5 *Serology*

Antibodies to the CS protein were assessed by ELISA at CEVAC, Ghent, Belgium using as antigen the recombinant antigen R32LR that contains the sequence $[NVDP(NANP)_{15}]_2$. The method has been reported in detail elsewhere (Macete, Sacarlal et al. 2007). Antibody titres are reported in EU/mL.

4.2.5 *Hepatitis B antibody measurement*

In accordance with Tanzanian Ministry of Health policy for implementation of the expanded programme of childhood immunisations, all children were assumed to have received Hepatitis B vaccinations at 6, 10 and 14 weeks of age. HBV vaccination responders were confirmed based on anti-HbsAg antibody titres from samples collected at the baseline timepoint (i.e. at enrolment into the study). Anti-HBsAg antibody titres were measured by ELISA at GSK Biologicals, Belgium.

4.2.6 *Statistical analysis*

Values for each antigen stimulated well were divided by values for their respective negative control well (GM) for each assay. Geometric mean responses by vaccination group were calculated and students t test on log-transformed values was used to determine statistical significance. Stata version 10 was used. For cytokine secretion assays, Luminex xPONENT software was used to export data and then students t test was used to determine statistical significance using GraphPad Prism version 5.

4.3 *Results*

4.3.1 *T cell responses to HBs and CS peptides in RTS,S/ASO1E vaccinated subjects*

PBMC isolated from RTS,S/AS01E-vaccinated and rabies-vaccinated children were cultured for 24 hours without stimulation (GM) or with HBs or CS peptide pools in order to quantify systemic effector memory T cell responses to each of the vaccine antigen components. Cell surface expression of the early activation marker CD69 and intracellular expression of IFN-γ were analysed in all $CD3^+$ T cells and cell surface expression of the high affinity IL-2 receptor α chain (CD25) was analysed in all CD4+ lymphocytes. A representative example of IFN-γ, CD69 and CD25 expression in PBMC cultured in GM, HBs or CS from one child vaccinated with RTS,S is shown in **Fig. 4.2A**. Aggregated data from 93 RTS,S/AS01E-vaccinated and 121 rabies-vaccinated children are shown for the three different parameters in **Fig. 4.2B-D;** data for HBs- and CS-stimulated cells are presented as fold-change (also known as the stimulation index) compared to GM cultures.

IFN-γ responses to HBs were significantly higher ($p<0.0001$) among RTS,S-vaccinated children than among the rabies-vaccinated children (**Fig. 4.2C**) indicating that, even in this recently vaccinated cohort, revaccination with HBs in AS01 significantly enhances the anti-HBs effector memory T cell response. By contrast, IFN-γ responses to CS peptides did not differ significantly between RTS,S-vaccinated and rabies-vaccinated children and, among RTS,S-vaccinated children, anti-CS responses were significantly lower than responses to HBs. Thus, despite being administered as part of a highly immunogenic HBs vaccine, the CS protein did not appear to induce a sustained systemic effector memory T cell response.

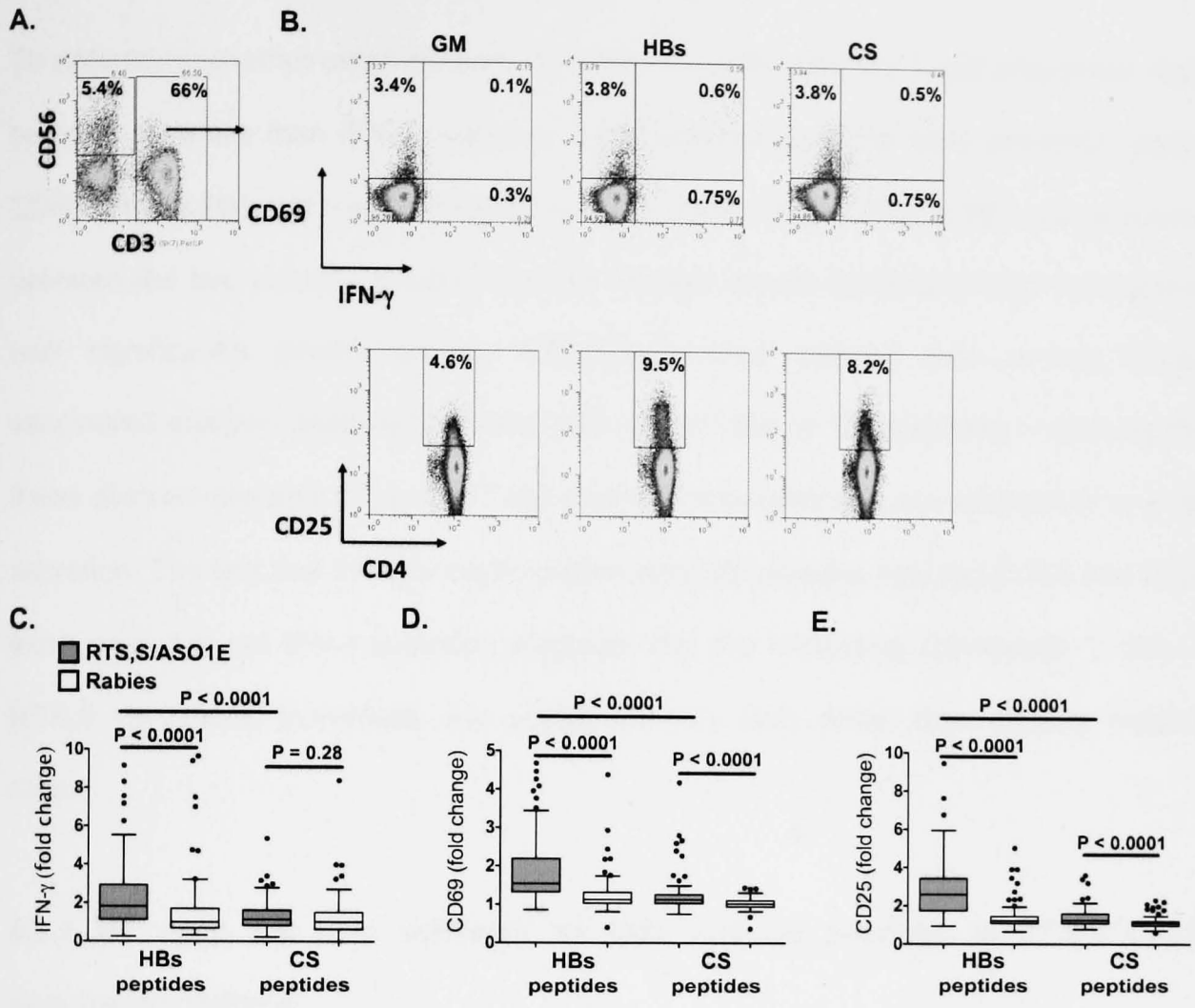


Figure 4.2. After RTS,S/ASO1E vaccination, T cells upregulate expression of CD69 and CD25 and produce IFN-γ in response to vaccine antigens

PBMC were isolated and cultured *in vitro* for 24 hours in growth medium (**GM**) or with peptide pools representative of either Hepatitis B surface antigen (**HBs**) or circumsporozoite (**CS**) proteins and analyzed by flow cytometry for surface expression of CD69 and CD25 and intracellular IFN-γ. T cells were defined as all CD3-expressing lymphocytes (**A**) and then stained for (**B**) expression of CD69 and intracellular IFN-γ (top row) or expression of CD4 and CD25 as a marker of activation (bottom row). Proportion of either (**C**) IFN-γ+ T cells, (**D**) CD69+ T cells and (**E**) CD25+CD4+ T cells represented as fold change increases relative to activation response when cultured in GM. Grey bars represent median responses from RTS,S/ASO1E-vaccinated children and open bars represent median responses from rabies vaccinated children ($N_{RTS,S/ASO1E}$ = 93; N_{rabies} = 121). P values are derived from unpaired Mann Whitney test. The horizontal line within each box represents the median; the top and bottom of each box represent the 25th and 75th percentiles, respectively, and the "I" bars represent the upper and lower limits within 1.5 times the interquartile range. Circles denote outliers.

To determine whether other markers of antigen-specific memory T cell responses might be more sensitive than IFN-γ secretion, T cell expression of the early activation marker CD69 (**Fig. 4.2D**) and the high affinity IL-2R α chain (CD25) (**Fig. 4.2E**) was compared between the two vaccine groups. The fold change in both CD69 and CD25 expression was significantly greater among RTS,S-vaccinated children than among rabies-vaccinated children after restimulation with either HBs or CS peptides, indicating that these markers are able to identify T cell memory responses that are not evident by IFN-γ secretion. The fact that 24 hour restimulation with CS peptides induced CD69 and CD25 expression but not IFN-γ secretion suggests that the circulating CS-specific T cells in RTS,S vaccinated individuals are central memory cells rather than effector memory cells.

4.3.2 *NK cells are also activated by HBs and CS peptides in RTS,S/ASO1E vaccinated children*

Whilst analysing T cell IFN-γ responses (above) we noticed a population of $CD3^-$ but IFN-γ^+ cells (**Fig. 4.3A**), which we suspected might be natural killer (NK cells). To explore this further, we compared IFN-γ and CD69 expression in $CD3^-$ $CD56^+$ NK cells between the two groups of vaccinated children. Representative flow cytometry plots for NK cell responses in one RTS,S vaccinee are shown in **Fig. 4.3B**; aggregate data for 93 RTS,S-vaccinated and 121 rabies-vaccinated children are shown in **Fig. 4.3C** (IFN-γ) and **Fig. 4.3D** (CD69).

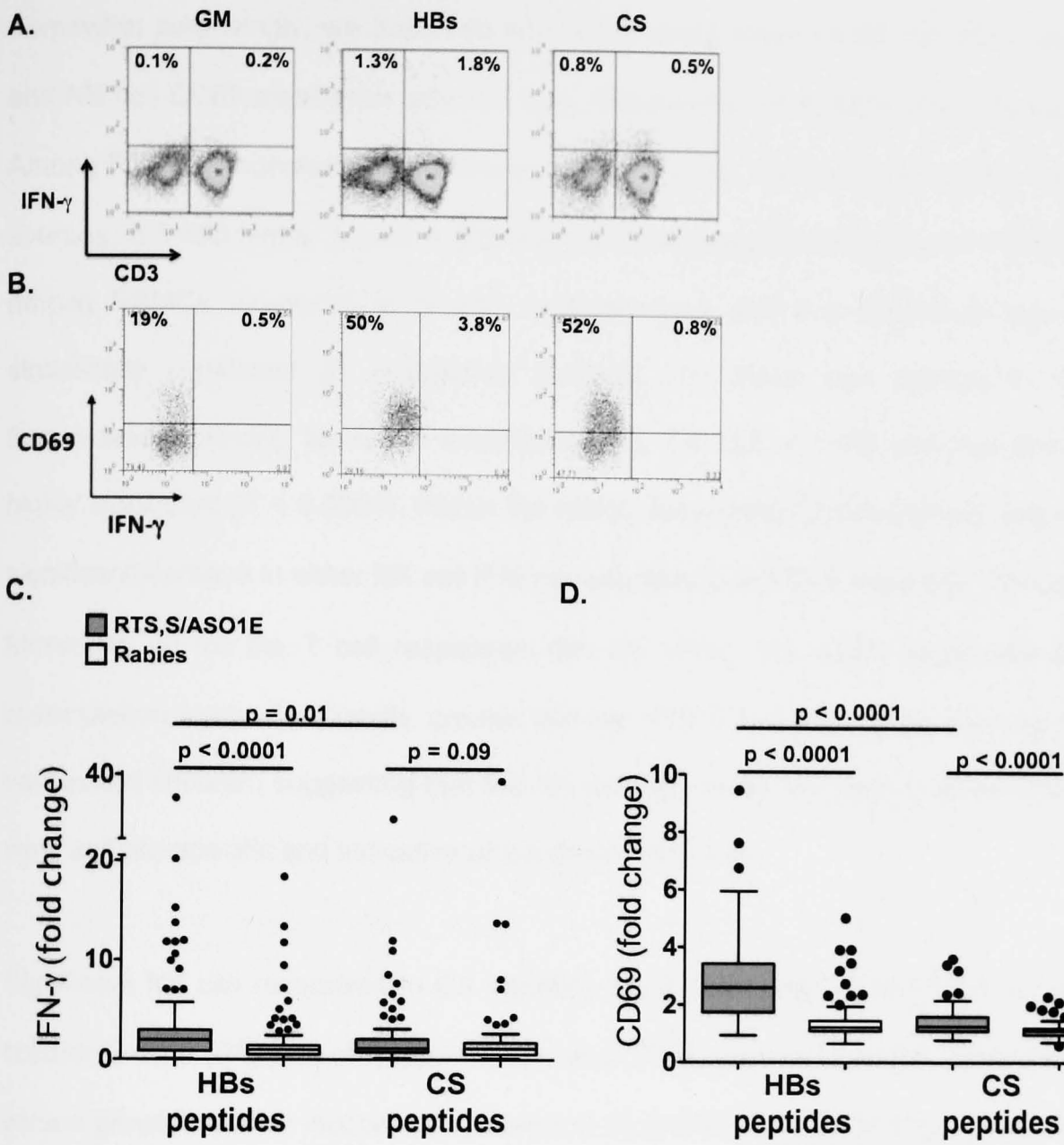


Figure 4.3 NK cells upregulate expression of CD69 and produce IFN-γ in response to RTS,S-vaccine antigens

PBMC were isolated and cultured *in vitro* for 24 hours in growth medium (**GM**) or with peptide pools representative of either Hepatitis B surface antigen (**HBs**) or circumsporozoite (**CS**) proteins and analyzed by flow cytometry for surface expression of CD3 and IFN-γ (**A**) or CD69 and intracellular IFN-γ when gating on all NK cells (**B**). Proportion of either (**C**) IFN-γ+ NK cells cells, (**D**) CD69+ NK cells represented as fold change increases relative to activation response when cultured in GM. Grey bars represent median responses from RTS,S/ASO1E-vaccinated children and open bars represent median responses from rabies vaccinated children ($N_{RTS,S/ASO1E}$ = 93; N_{rabies} = 121). P values are derived from unpaired Mann Whitney test. The horizontal line within each box represents the median; the top and bottom of each box represent the 25th and 75th percentiles, respectively, and the "I" bars represent the upper and lower limits within 1.5 times the interquartile range. Circles denote outliers.

Somewhat surprisingly, we observed significant upregulation of NK cell IFN-γ secretion and NK cell CD69 expression after 24 hour restimulation of PBMCs with HBs peptides. Among RTS,S-vaccinated children the percentage of NK cells expressing IFN-γ was, on average, 3.5 fold higher (mean = 3.5, SE = 0.5) among HBs-restimulated PBMCs than among PBMCs incubated in growth medium alone and this difference was highly statistically significant ($P < 0.0001$); similarly, the mean fold change in median fluorescence intensity for CD69 expression was 2.8 (SE = 0.16) and this was again highly significant ($P < 0.0001$). Within the rabies vaccinated (control) group, the was no significant increase in either NK cell IFN-γ or expression of CD69 relative to GM controls. Moreover, as for the T cell responses, NK cell IFN-γ and CD69 responses to HBs restimulation were significantly greater among RTS,S vaccinees than among rabies-vaccinated children, suggesting that the NK cell responses are also, in some undefined way, antigen-specific and indicative of a memory response.

Significant NK cell responses to CS peptides were also observed in RTS,S-vaccinated children (mean [SE] fold changes in IFN-γ and CD69 expression in CS stimulated cells versus growth medium incubated cells were 2.24 [±0.38] $p = 0.0008$ and 1.4 [[±0.06] $p < 0.0001$, respectively). Similar trends, however, were observed in rabies-vaccinated children (mean [SE] fold changes in IFN-γ and CD69 expression in CS stimulated cells versus growth medium incubated cells were 1.4 [±0.07] $p < 0.0001$ and 1.02 [±0.03] $p < 0.0001$, respectively) and, as for T cell responses, CD69 but not IFN-γ NK cell responses were significantly higher among RTS,S-vaccinated than among rabies-vaccinated children ($p = 0.0001$ and $p = 0.09$, respectively).

4.3.3 *NK cells and T cells contribute equally to RTS,S-vaccine antigen recall responses*

Since there are far greater numbers of T cells than NK cells circulating in the peripheral blood (~10 T cells : 1 NK cell) when I gated on all IFN-γ producing lymphocytes, I observed that NK cells and T cells contributed equally to the IFN-γ response. I performed a similar analysis to compare the IFN-γ responses among the RTS,S- and rabies-vaccinated children in response to HBs and CS peptides and found nearly identical trends (**Figure 4.4**). Interestingly, when gating on all IFN-γ+ lymphocytes, we noticed a significant population that was neither T cells nor NK cells (CD3-CD56-). I assume that these are most probably T cells that have down regulated TCR expression as a result of antigen engagement (CD3 was stained extracellularly only). If we are to assume that this double-negative population really are CD3+ T cells then these data demonstrate that NK cells and T cells are contributing equally to the IFN-γ-producing pool of lymphocytes.

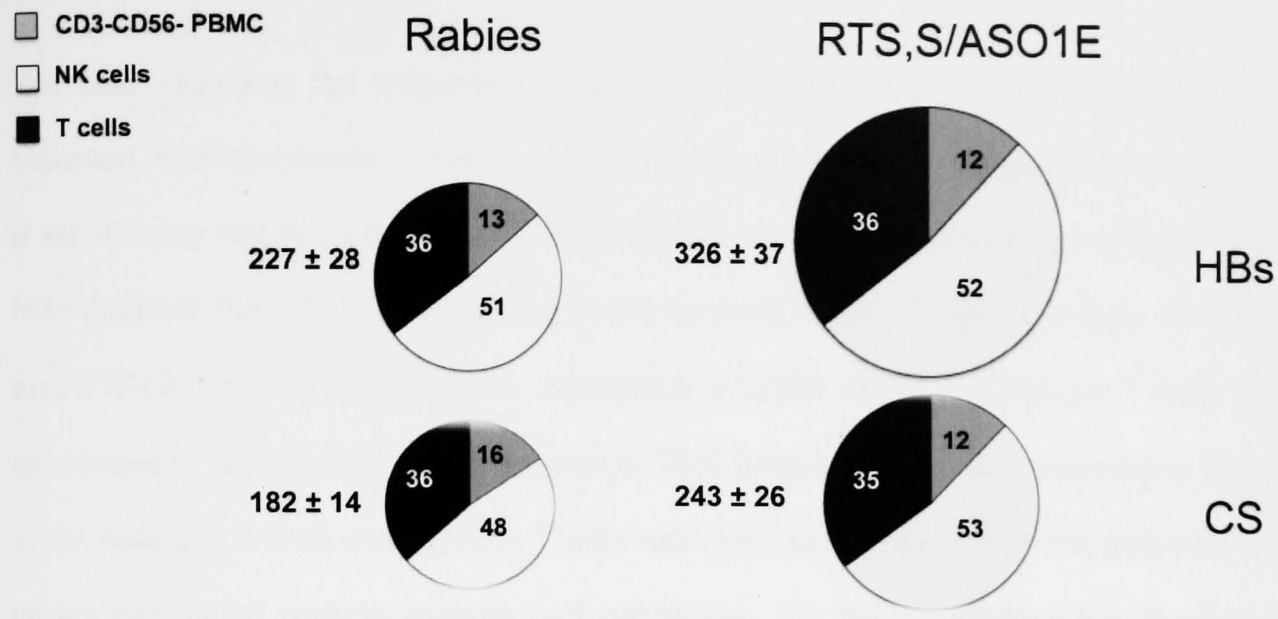


Figure 4.4 NK cells and T cells contribute equally to the IFN-γ recall response to RTS,S vaccine antigens

PBMC were isolated and cultured *in vitro* for 24 hours with peptide pools representative of either Hepatitis B surface antigen (**HBs**) or circumsporozoite (**CS**) proteins and analyzed by flow cytometry for intracellular expression of IFN-γ. All IFN-γ+ lymphocytes were gated on and surface expression of CD3 and CD56 were measured to determine the proportions made up of T cells (black), NK cells (white) or CD3-CD56- lymphocytes (grey). Numbers outside of pie charts represent the mean of IFN-γ+ cells ± SE. $N_{RTS,S/ASO1E}$ = 93; N_{rabies} = 121.

We also assessed the proportion of individuals within each vaccination group that mounted recall responses to HBs and CS peptides. As shown in **Table 4.2**, we observed a significantly higher proportion of RTS,S/ASO1E vaccinees that mounted responses to HBs peptides than did the rabies vaccinated controls based on the expression of CD69 and IFN-γ in NK cells as well as for expression of CD25, CD69 and IFN-γ in T cells. For responses to CS peptides, the proportion of RTS,S/ASO1E vaccinees expressing CD69 in NK cells and T cells and CD25 in T cells was significantly higher than the proportion of rabies vaccinated controls making such responses. We did not observe any significant difference in the proportion of individuals within each group that mounted NK and T cell recall IFN-γ responses to the CS peptides. Responders were defined as having at least twice as many NK cells or T cells expressing CD25, CD69 or IFN-γ after restimulation with HBs or CS than after culture in GM alone.

Antigen:	Group:		CD69:		IFN-γ:		CD25:
			T cells	NK cells	T cells	NK cells	T cells
HBS	RTS,S/ASO1E n = 89	n(%)	32(35.9%)	59(66.2%)	33(37%)	43(48.3%)	59(59%)
	Rabies n = 105	n(%)	4(3.8%)	9(8.5%)	21(20%)	17(16.1%)	9(8.5%)
		P	< 0.0001	< 0.0001	0.008	< 0.0001	< 0.0001
CS	RTS,S/ASO1E n = 85	n(%)	6(7%)	11(12%)	13(15.2%)	21(24.7%)	11(12.9%)
	Rabies n = 101	n(%)	0(0%)	3(2.9%)	17(16.8%)	21(20.7%)	3(2.9%)
		P	0.007	0.01	0.776	0.525	0.01

Table 4.2 *The proportion of vaccinated children whose PBMCs made an NK or T cell response to vaccine.* All responders are defined as expressing CD25, CD69 or producing IFN-γ at levels 2-fold greater than respective GM values. P values are derived from unpaired Mann Whitney test.

4.3.4 *Detection of soluble cytokines in culture supernatants from PBMC in response to RTS,S vaccine antigens*

We collected supernatants from the PBMC cultures described above and measured secreted levels of various cytokines by luminex assay. We measured the secretion of IFN-γ, IL-2 and IL-10 by PBMCs in response to HBs and CS. As shown in **Fig. 4.5A**, we observed significantly greater amounts of secreted IFN-γ in culture supernatants of RTS,S vaccinees in response to HBs as compared to rabies vaccine controls. Consistent with our flow cytometric data, no significant difference in the levels of CS-specific IFNγ secretion was observed between RTS,S- and rabies vaccinees (**Fig. 4.5A**). Furthermore, IFN-γ levels in response to CS peptides for both RTS,S- and rabies vaccinees were not significantly higher than in cultures with just GM.

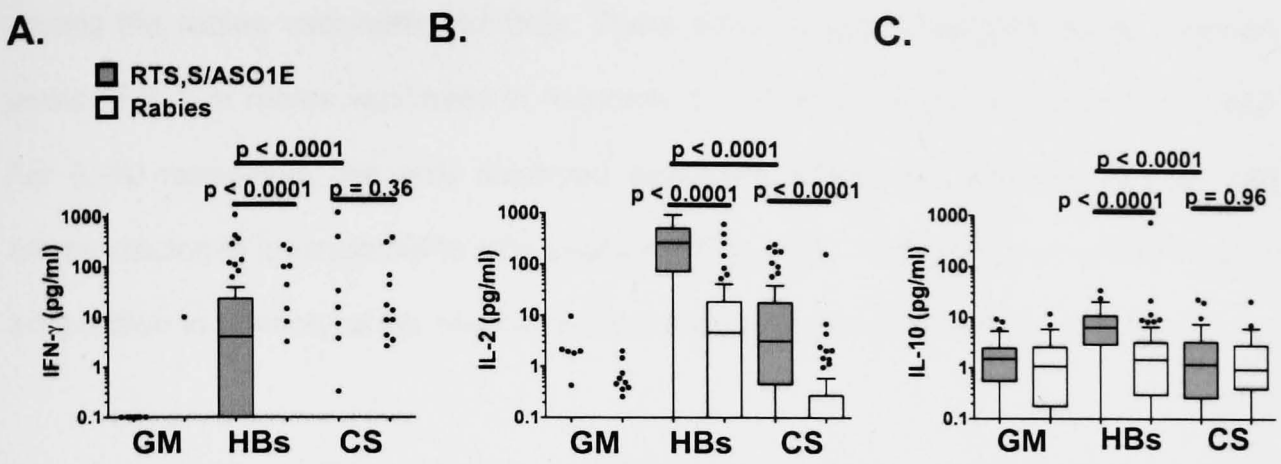


Figure 4.5 Cytokines secreted in response to RTS,S vaccine antigens
PBMC were isolated and cultured *in vitro* for 24 hours in GM or with peptide pools representative of either HBs or CS proteins. Culture supernatants were collected and analyzed by luminex for concentrations of (**A**) IFN-γ, (**B**) IL-2 and (**C**) IL-10. Grey bars represent median responses from RTS,S/ASO1E-vaccinated children and open bars represent median responses from rabies-vaccinated children. P values are derived from unpaired Mann Whitney test. **IFN-γ:** (**HBs**): $N_{RTS,S/ASO1E}$ = 43; N_{rabies} = 60; (**CS**): $N_{RTS,S/ASO1E}$ = 44; N_{rabies} = 61. **IL-2:** (**HBs**): $N_{RTS,S/ASO1E}$ = 47; N_{rabies} = 60; (**CS**): $N_{RTS,S/ASO1E}$ = 48; N_{rabies} = 61. **IL-10:** (**HBs**): $N_{RTS,S/ASO1E}$ = 43; N_{rabies} = 60; (**CS**): $N_{RTS,S/ASO1E}$ = 44; N_{rabies} = 61. The horizontal line within each box represents the median; the top and bottom of each box represent the 25th and 75th percentiles, respectively, and the "I" bars represent the upper and lower limits within 1.5 times the interquartile range. Circles denote outliers.

Strikingly, we observed very robust production of IL-2 in response to both HBs and CS in RTS,S-vaccinees (**Fig. 4.5B**). These responses were significantly greater, both relative to their respective GM values ($p < 0.0001$) and significantly higher than in the rabies vaccinated controls. Interestingly, we also observed that rabies vaccinees still mounted an IL-2 response to HBs peptides that was significantly greater than in cultures with GM ($p=0.01$). This significant difference can be accounted for by previous priming of HBV-specific CD4+ T cells after HBV vaccination. Similarly, in response to CS peptides, RTS,S/AS-vaccinated children mounted strong IL-2 responses which were significantly greater than their respective GM values ($p < 0.0001$) as well as significantly higher than

among the rabies vaccinated controls. There were no significant differences between levels of IL-2 in rabies vaccinees in response to CS peptides and in cultures with GM. For IL-10 responses, we only observed significant differences between RTS,S- and rabies vaccinees in response to HBs peptides (**Fig. 4.5C**). Unfortunately, we were not in the position to phenotype the source of cells responsible for the production of IL-10.

We also compared the proportion of RTS,S- and rabies- vaccinees that produced detectable levels of IFN-γ, IL-2 and IL-10 in response to HBs and CS peptides (**Table 4.3**). In response to HBs peptides, there was a significantly greater proportion of RTS,S vaccinees that mounted detectable IFN-γ, IL-2 and IL-10 as compared to the proportion of rabies vaccinees that mounted positive responses. Interestingly, in response to CS peptides we only observed a significantly greater proportion of RTS,S vaccinees in the production of IL-2 as compared to rabies vaccinees.

Antigen:	Group:		IFN-γ	IL-2	IL-10
HBS	RTS,S/ASO1E	% Positive	53.4	89.3	59.7
		Number Positive	23	42	30
		$N_{RTS,S}$ =	43	47	43
	Rabies	% Positive	11.6	63.3	21.6
		Number Positive	7	38	13
		N_{rabies} =	60	60	60
		P	**< 0.0001**	**0.002**	**< 0.0001**
CS	RTS,S/ASO1E	% Positive	13.6	60.4	11.3
		Number Positive	6	29	5
		$N_{RTS,S}$ =	44	48	44
	Rabies	% Positive	19.6	13.1	3.2
		Number Positive	12	8	2
		N_{rabies} =	61	61	61
		P	0.418	**< 0.0001**	0.101

Table 4.3 *The proportion of vaccinated children whose PBMCs secreted cytokines in response to RTS,S-vaccine antigens.* All responders are defined as expressing CD25, CD69 or producing IFN-γ at levels 2-fold greater than respective GM values. P values are derived from unpaired Mann Whitney test.

4.3.5 *Production of IL-2 is correlated with activation of NK cells in RTS,S vaccinees in response to HBs and CS peptides*

Based on the ability of NK cells to respond to RTS,S vaccine antigens in a recall-specific manner (**Figure 4.3**) and the robust production of IL-2 in response to RTS,S vaccination (**Figure 4.5**), we wanted to determine whether the same individuals that had strong activation of NK cells in response to HBs and CS peptides also mounted robust IL-2 responses. We performed spearman correlation tests on all vaccinees for HBs and CS responses where data was available for both IL-2 production and IFN-γ-producing NK cells as well as for IL-2 production and CD69-expressing NK cells. Data is shown for responses to HBs peptides in **Figure 4.6** and for responses to CS peptides in **Figure 4.7**. While there were no significant correlations observed between IFN-γ+ NK cells and IL-2 production in RTS,S vaccinees, there was a strong correlation between CD69-

expressing NK cells and IL-2 production in response to HBs peptides. Significant correlations were seen for RTS,S vaccinees but not for rabies vaccinees.

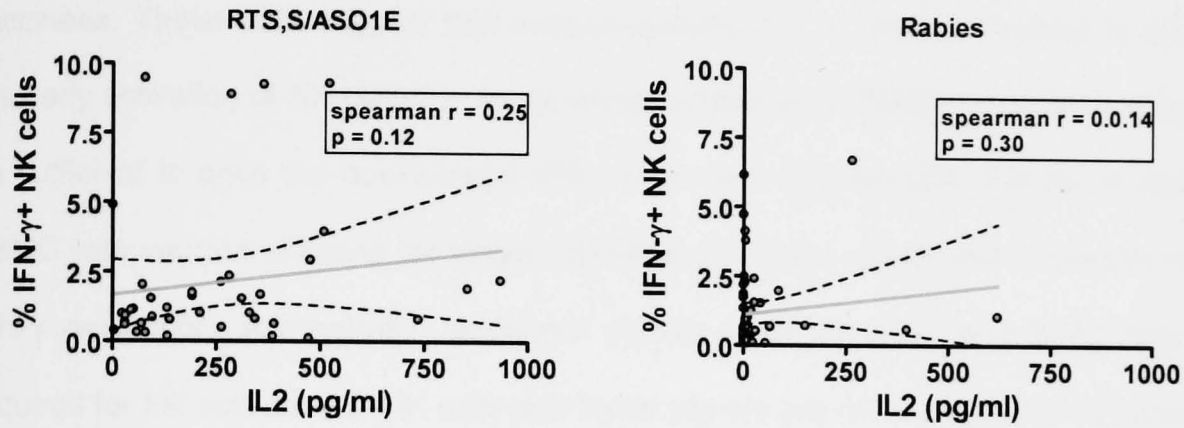


Figure 4.6 Correlations between expression levels of CD69 on NK cells and levels of secreted IL-2 in RTS,S/ASO1E-vaccinated children in response to HBs peptides
PBMC were isolated and cultured *in vitro* for 24 hours in GM or with HBs peptides and analyzed by flow cytometry for surface expression of CD69 and intracellular IFN-γ. Culture supernatants were collected and analyzed by luminex for concentrations of secreted IL-2. Circles represent individual children in the RTS,S/ASO1E-vaccinated group (left side) and rabies-vaccinated group (right side) ($N_{RTS,S/ASO1E}$ = 39; N_{rabies} = 56). P values are derived from non-parametric spearman correlation test and dashed lines represent 95% confidence intervals.

Data are shown for responses to CS peptides in **Figure 4.7**. Similarly to responses to HBs peptides, we observed a strong correlation between the percentage of NK cells expressing CD69 and IL-2 production and this response was only observed in RTS,S vaccinees. These data suggest that antigen-specific IL-2 is directly involved in driving the early activation of NK cells by upregulating expression of CD69; however, it may not be sufficient to drive the downstream IFN-γ response. It is possible that by incubating PBMC with vaccine antigens for longer (30-48 hours) that I would have observed more IFN-γ production. Alternatively, additional signals such as IL-12 and IL-18 may be required for full activation of NK cells and these signals are not expected to be provided by HBs or CS peptides.

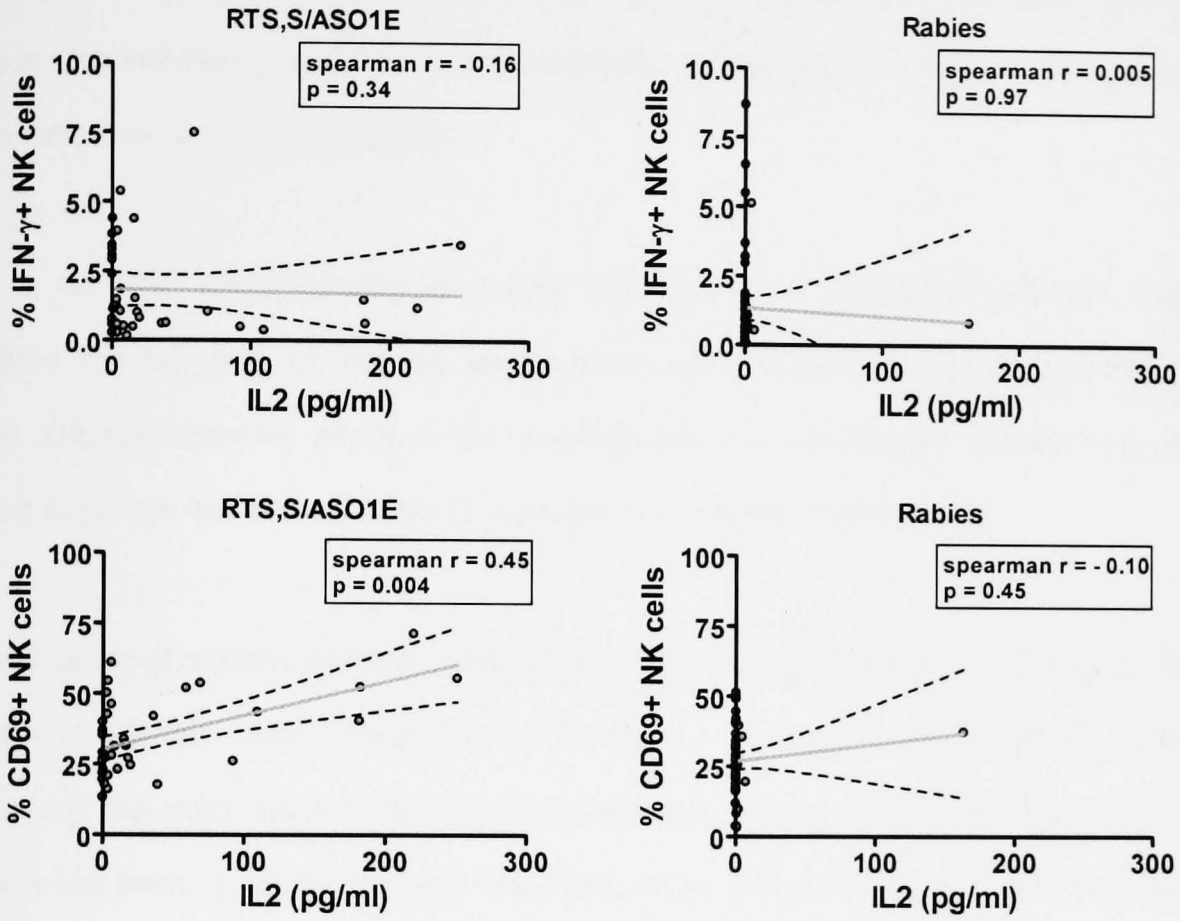


Figure 4.7 **Correlations between expression levels of CD69 on NK cells and levels of secreted IL-2 in RTS,S/ASO1E-vaccinated children in response to CS peptides** PBMC were isolated and cultured *in vitro* for 24 hours in GM or with CS peptides and analyzed by flow cytometry for surface expression of CD69 and intracellular IFN-γ. Culture supernatants were collected and analyzed by luminex for concentrations of secreted IL-2. Circles represent individual children in the RTS,S/ASO1E-vaccinated group (left side) and rabies-vaccinated group (right side) ($N_{RTS,S/ASO1E}$ = 39; N_{rabies} = 56). P values are derived from non-parametric spearman correlation test and dashed lines represent 95% confidence intervals.

4.4 *Discussion*

Vaccination has had a very large impact on a wide range of infectious diseases. Attenuated or inactivated pathogens, pathogen-derived toxins and recombinant proteins have been used as vaccines. Nevertheless, our current understanding of how any particular vaccine works on a mechanistic level is extremely limited.

There is no clear understanding of natural immunity to *P. falciparum* infection, which has hampered the progress of vaccine development with respect to choosing a model target antigen. Despite intensive efforts in the development of a vaccine for malaria, the only truly effective approach has been the use of irradiated sporozoites (Clyde 1975).

The prevalence of malaria infection peaks during the first 5 years of life. A significant proportion of deaths and severe clinical malaria occur in infants < 18 months (Saute, Aponte et al. 2003). Thus, one of the major tasks of the Clinical Development Plan (CDP) of RTS,S malaria vaccine for Africa has been to collaborate with the efforts of the WHO EPI to administer the vaccine as early as possible in infants in order to prevent malaria-induced mortality during the first few years of life.

In this study, I investigated the cell mediated immune responses to the RTS,S/ASO1E (paediatric formulation)-adjuvanted sub-unit vaccine in infants 5-17 months of age. I characterised expression of CD69 and production of IFN-γ in NK cells and T cells and expression of CD25 in T cells. My data demonstrate that robust recall NK cell and T cell responses are mounted during *in vitro* re-stimulation with the RTS,S vaccine antigen, HBs peptides and while CS-induced IFN-γ responses were not as strong, expression of CD25 in T cells and CD69 in NK cells and in T cells were significantly higher in RTS,S vaccinated children

than in rabies vaccinated controls. Furthermore, nearly half of the IFN-γ production is derived from NK cells. I also measured secreted levels of IFN-γ, IL-2 and IL-10 in culture supernatants. I observed high levels of secreted IFN-γ in culture supernatants of RTS,S/ASO1E vaccinated PBMC only in response to HBs peptides. Furthermore, I observed strong vaccine antigen-specific IL-2 production to both HBs as well as CS peptides, which was significantly more robust in the RTS,S/ASO1E vaccinees than in the rabies vaccinated controls. Where IL-2 levels in response to HBs peptides were significantly higher than respective GM negative controls among RTS,S and rabies vaccinees, IL-2 responses to CS peptides were significantly higher relative to GM in RTS,S recipients but not in rabies vaccinees.

That NK cells did not mount as robust a response to CS peptides as to HBs peptides seems to be correlated with significantly lower levels of IL-2 secreted in response to CS as compared to HBs as IL-2 secretion in response to HBs and CS peptides was highly correlated with the early activation of NK cells (expression of CD69).

My data presented in **Chapter 3** suggest that optimal indirect activation of NK cells requires whole pathogen, presumably triggering APC-derived signals such as IL-12, IL-18 and IFN-α due to recognition of pathogens via TLRs. It is difficult to accurately interpret these data when using overlapping peptides representing the CS protein as there are many different epitopes represented and of varying immunodominace relative to the array of MHC haplotypes in the RTS,S-vaccinated children. In this context, using live or killed sporozoites may have served as a more immunogenic "recall" antigen. The reason why NK cells responses to HBs peptides were significantly more robust than responses to CS peptides probably reflects the boosting effects of HBs from the RTS,S vaccine. Every child received a full course of HBV vaccine, but only the RTS,S recipients were re-exposed to HBs, as is clearly demonstrated in **Table 4.1** which

compares anti-HBsAg antibody titres, following vaccination, in RTS,S/ASO1E and rabies vaccinees. Furthermore, based on the levels of secreted IL-2, both in response to HBs peptides in RTS,S recipients as well as by comparing these responses between RTS,S and rabies vaccinees, there is possibly a lower threshold for activation of NK cells in the presence of high dose IL-2.

Recently completed trials have demonstrated the immunogenicity (superior CSP-specific IgG titres as well as induction of higher frequencies of CSP-specific CD4+ T cells producing ≥ 2 activation markers [e.g. IFN-γ, TNF-α and IL-2]), efficacy and safety of the RTS,S/ASO1B liposomal adjuvant system formulation. This formulation appears to be both more immunogenic and more efficacious than the ASO2A [oil-in-water emulsion] formulation (Stewart, McGrath et al. 2006; Kester, Cummings et al. 2009). Similar to efficacy studies for RTS,S/ASO1B (adult formulation), efficacy rates for RTS,S/ASO1E (paediatric formulation), vary from 49% (unadjusted) to 56% (adjusted) ($p < 0.0001$), suggesting that the vaccine offers partial protection against re-infection and the development of severe disease (Bejon, Lusingu et al. 2008). The significant but modest protection offered by RTS,S vaccination suggests that insights regarding the mechanisms by which this vaccine works may allow the vaccine to be modified to be more protective.

Single cell assays, which allow the enumeration as well as phenotype of responding cells, have been quite widely applied in field studies but they have been mostly focused on characterisation of antigen-specific effector T cell responses, overlooking the possibility that other cells may contribute to effector responses. A couple of recent studies have demonstrated this point. One study employing functional genomics, systems biology and the use of polychromatic flow cytometry to assess the global responses to yellow fever vaccination only characterised effector functions of CD4+ T cells by FACS and measured secretion of IFN-γ in PBMC cultures. The

authors make the assumption that the IFN-γ is derived from T cells without any further dissection of the response (Gaucher, Therrien et al. 2008). Similarly, another study analysed responses to yellow-fever vaccination but only examined correlates of CD8+ T cells and antibody responses (Querec, Akondy et al. 2009).

My data presented in **chapter 3** demonstrated that human NK cell responses to *P. falciparum*-infected RBC are markedly potentiated by CD4+ T cells, such that NK cells - rather than T cells - form the vast majority of IFN-γ-secreting cells in the hours immediately following exposure to the infected RBC (**Chapter 3**). I described and defined the kinetics and cellular sources of the very early IFN-γ response and believe that given its speed (which precedes the bulk of the T cell IFN-γ response by hours or days) the NK cell IFN-γ response may represent an important determinant of vaccine efficacy. Thus, the ability of vaccine-induced T cells to support NK cell effector function might be an important biomarker of an effective T cell response to the vaccine.

It has been known for several years that NK cells may contribute to production of IFN-γ during the initial stages of a recall response (Desombere, Clement et al. 2005) and that IL-2 can enhance NK cell activation (Malek 2008). Furthermore, NK cell responses to influenza virus have been shown to be dependent upon IL-2 from T cells (He, Draghi et al. 2004) and elevated frequencies of IFN-γ-producing NK cells have been documented following influenza vaccination (Long, Michaelsson et al. 2008). The connection between the vaccine-induced activation of memory CD4+ T cells producing IL-2 leading to the recruitment of NK cells as effectors of the adaptive immune response and vaccination potentiating NK cell recall responses has been largely overlooked. From a practical vaccine development and testing perspective, my results indicate that NK cells are major contributors to the post-vaccination IFN-γ responses and that

the contribution of these cells as well as IL-2-producing T cells needs to be considered when evaluating vaccine efficacy in clinical trials or in vaccine potency tests.

In the following chapter, using rabies virus vaccination as a model, I will present data which conclusively demonstrate that NK cells may be recruited as effector cells by IL-2 secreting vaccine antigen-specific CD4+ T cells to amplify the adaptive effector response, following vaccination.

Chapter 5: NK cells as effectors of acquired immune responses: effector CD4+ T cell-dependent activation of NK cells following Rabies virus vaccination

---

Adapted from (Horowitz, Behrens *et al.*, 2010)

5.1 *Introduction*

My investigations of the pathways of NK cell activation by *Plasmodium falciparum* (malaria)-infected red blood cells revealed an essential role for IL-2 produced by CD4+ T cells. These observations are bolstered by reports of T cell and IL-2-dependent activation of human NK cells (Fehniger, Cooper et al. 2003; He, Draghi et al. 2004). Furthermore, my characterization of the RTS,S malaria vaccine-specific NK cell recall responses to vaccine antigens demonstrated that NK cell activation was highly correlated with IL-2 production. These observations are also in line with a previous report demonstrating an increase in the frequency of IFN-γ-producing NK cells among re-stimulated PBMCs after influenza vaccination (Long, Michaelsson et al. 2008). This led me to speculate that IL-2 from antigen-specific T cells might allow NK cells to contribute to the effector arm of adaptive immune responses and thus that NK cells may make a significant contribution to the cellular effector response to vaccination.

In this chapter, I tested the hypothesis that NK cell responses are specifically enhanced, after vaccination, by IL-2 emanating from antigen-specific T cells. Using rabies virus vaccination as my model system (as inapparent exposure to rabies virus is likely to be extremely rare and my volunteers are thus expected to be fully naïve prior to vaccination) I demonstrate that NK cells are the major contributors to the immediate vaccine-specific cytokine and cytotoxic recall response, that this response is dependent upon IL-2 from antigen-specific memory T cells and is associated with extensive NK cell proliferation.

5.1.1 *The burden of rabies virus*

More than 55,000 people die of rabies each year and more than 95% of all rabies virus-related fatalities occur in developing countries within Africa and Asia, where most fatalities are directly attributed to bites from rabies-infected dogs (**Figure 5.1**) (WHO 2008). Of these, 30% to 60% are children under the age of 15. It is widely recognized that the number of fatalities, which are reported is a gross underestimation of the true incidence of rabies infections. There is minimal political commitment to controlling rabies infections, as there are no accurate data on the true public health impact of this disease. This can be attributed to poor surveillance as well as patients not presenting to health treatment centers. Even fewer reported cases ever get laboratory confirmation and clinical cases are often not officially documented with central authorities (WHO 1998; Cleaveland, Fevre et al. 2002).

Perhaps the most accurate estimates to date on risk of clinical rabies developing in a person bitten by a dog suspected of infection with rabies have come from a study done in Tanzania based (WHO 1998; Cleaveland, Fevre et al. 2002; Cleaveland, Kaare et al. 2003). This group showed that dog bites are reported more frequently than human cases of rabies and could potentially provide a source of data from which to infer the incidence of human fatalities. Their data suggest that the death toll may be 100-fold greater than official reports declare.

Education is a major factor in disease control. It has been shown that wound cleansing and immunizations immediately following 'unnatural' encounters with animals, nearly eliminates the chance of contracting the disease. Once bite victims become symptomatic, mortality is nearly 100%. Global vaccination strategies in dogs

are currently viewed as the most cost-effective strategy for rabies prevention in humans. There is a wide range of commercially available rabies vaccines marketed towards animals, both small and large. **Table 1** provides a comprehensive listing of these vaccines, their manufacturers as well as their recommended dosage and booster regimens as deemed by the Centers for Disease Control. While effective and economical control measures are available, rabies virus infection remains a neglected disease throughout the developing world (WHO 1998).

While the use of post-exposure treatment is effective if started early enough, its use is extremely cost-prohibitive in the developing world. A rabies immunization following exposure to animal suspected of rabies infection is US$40 in Africa and US$49 in Asia and 3 doses are recommended.

Product Name	Produced By	For Use In	Dosage	Age at Primary Vaccination a	Booster Recommended	Route of Inoculation
A) MONOVALENT (Inactivated)						
RABVAC 1	Fort Dodge Animal Health	**Dogs**	1 ml	3 months b	Annually	IM c or SC d
		Cats	1 ml	3 months	Annually	IM or SC
RABVAC 3	Fort Dodge Animal Health	**Dogs**	1 ml	3 months	1 year later and triennially	IM or SC
		Cats	1 ml	3 months	1 year later and triennially	IM or SC
		Horses	2 ml	3 months	Annually	IM
RABVAC 3 TF	Fort Dodge Animal Health	**Dogs**	1 ml	3 months	1 year later and triennially	IM or SC
		Cats	1 ml	3 months	1 year later and triennially	IM or SC
		Horses	2 ml	3 months	Annually	IM
DEFENSOR 1	Pfizer, Incorporated	**Dogs**	1 ml	3 months	Annually	IM or SC
		Cats	1 ml	3 months	Annually	SC
DEFENSOR 3	Pfizer Incorporated	**Dogs**	1 ml	3 months	1 year later and triennially	IM or SC
		Cats	1 ml	3 months	1 year later and triennially	SC
		Sheep	2 ml	3 months	Annually	IM
		Cattle	2 ml	3 months	Annually	IM
RABDOMUN	Pfizer, Incorporated	**Dogs**	1 ml	3 months	1 year later & triennially	IM or SC
		Cats	1 ml	3 months	1 year later & triennially	SC
		Sheep	2 ml	3 months	Anually	IM
		Cattle	2 ml	3 months	Annually	IM
Rabdomun 1	Pfizer, Incorporated	**Dogs**	1 ml	3 months	Annually	IM or SC
		Cats	1 ml	3 months	Annually	SC
Continuum Rabies	Intervet, Incorporated	**Dogs**	1 ml	3 months	1 year later & triennially	SC
		Cats	1 ml	3 months	1 year later & quadrennially	SC
Prorab-1	Intervet, Incorporated	**Dogs**	1 ml	3 months	Annually	IM or SC
		Cats	1 ml	3 months	Annually	IM or SC
		Sheep	2 ml	3 months	Annually	IM
Imrab-1	Merial, Incorporated	**Dogs**	1 ml	3 months	Annually	SC
		Cats	1 ml	3 months	Annually	SC
Imrab-1 TF	Merial, Incorporated	**Dogs**	1 ml	3 months	Annually	SC
		Cats	1 ml	3 months	Annually	SC
Imrab 3	Merial, Incorporated	**Dogs**	1 ml	3 months	1 year later & triennially	IM or SC
		Cats	1 ml	3 months	1 year later & triennially	IM or SC
		Sheep	2 ml	3 months	1 year later & triennially	IM or SC
		Cattle	2 ml	3 months	Anually	IM or SC
		Horses	2 ml	3 months	Anually	IM or SC
		Ferrets	1 ml	3 months	Annually	SC
Imrab 3 TF	Merial, Incorporated	**Dogs**	1 ml	3 months	1 year later & triennially	IM or SC
		Cats	1 ml	3 months	1 year later & triennially	IM or SC
		Ferrets	1 ml	3 months	1 year later & triennially	SC
Imrab Large Animal	Merial, Incorporated	**Cattle**	2 ml	3 months	Annually	IM or SC
		Horses	2 ml	3 months	Annually	IM or SC
		Sheep	2 ml	3 months	1 year later & triennially	IM or SC

Product Name	Produced By	For Use In	Dosage	Age at Primary Vaccination a	Booster Recommended	Route of Inoculation
B) MONOVALENT (Rabies glycoprotein, live canary pox vector)						
PUREVAX Feline Rabies	Merial, Incorporated	**Cats**	1 ml	8 weeks	Annually	SC
C) COMBINATION (Inactivated rabies)						
CONTINUUM DAP-R	Intervet, Incorporated	**Dogs**	1 ml	3 months	1 year later and triennially	SC
CONTINUUM Feline HCP-R	Intervet, Incorporated	**Cats**	1 ml	3 months	1 year later & quadrennially	SC
Equine POTOMAVAC + IMRAB	Merial, Incorporated	**Horses**	1 ml	3 months	Anually	IM
D) COMBINATION (Rabies glycoprotein, live canary pox vector)						
PUREVAX Feline 3/ Rabies	Merial, Incorporated	**Cats**	1 ml	8 weeks	Annually	SC
PUREVAX Feline 4/ Rabies	Merial, Incorporated	**Cats**	1 ml	8 weeks	Annually	SC
E) ORAL (Rabies glycoprotein, live vaccinia vector) - RESTRICTED TO USE IN STATE AND FEDERAL RABIES CONTROL PROGRAMS						
RABORAL V-RG	Merial, Incorporated	**Coyotes**	N/A	N/A	As determined by local authorities	Oral
		Raccoons	N/A	N/A	As determined by local authorities	Oral

a: Minimum age (or older) and revaccinated one year later.

b: One month = 28 days.
c: Intramuscularly.

d: Subcutaneously

e: Non-rabies fractions have a 3 year duration (see label)

Table 5.1 *Rabies vaccines licensed and marketed in the United States, 2008* (CDC 2008)

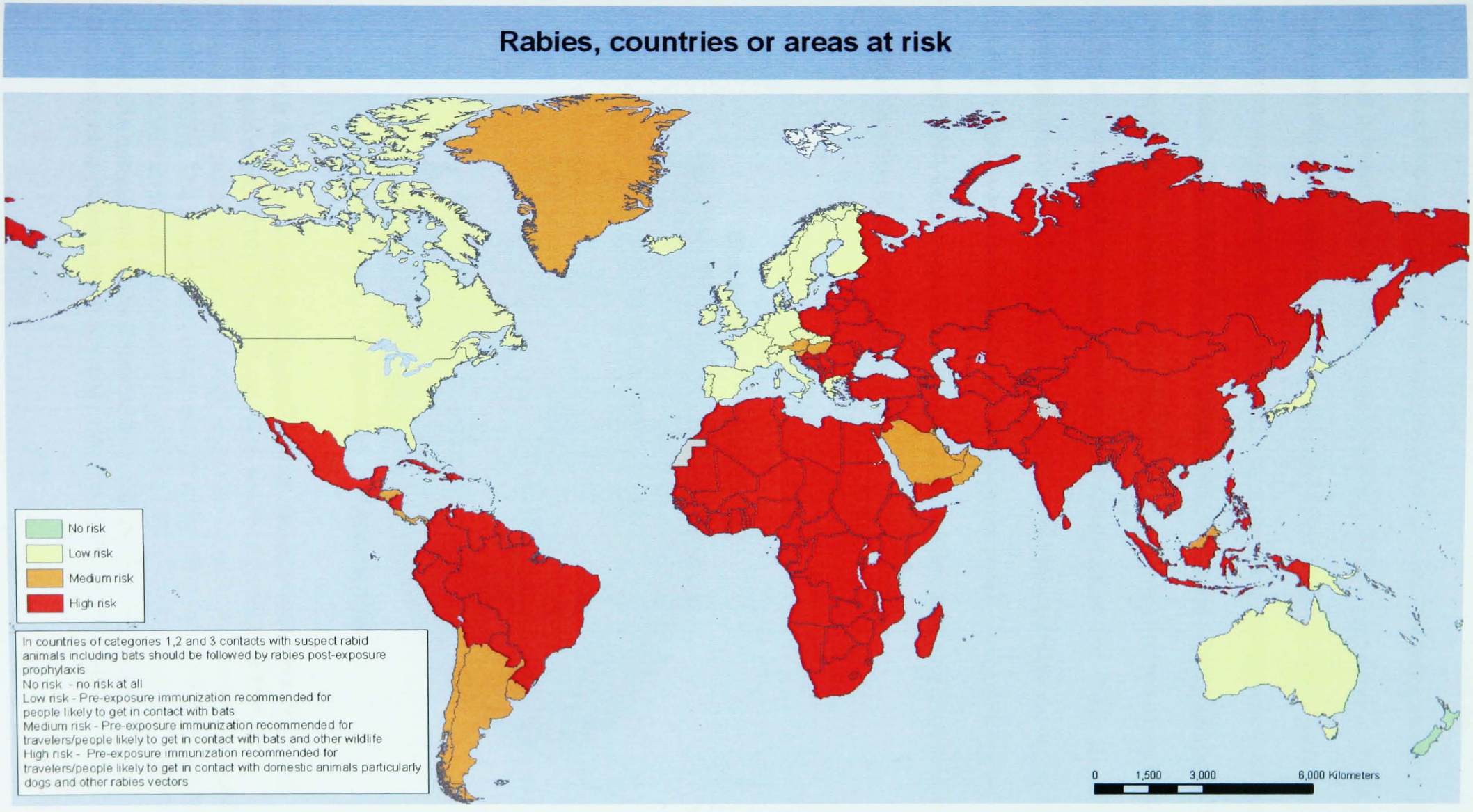


Figure 5.1 Countries at risk of spread of rabies virus (WHO 1998).

5.1.2 Rabies virus and pathogenesis

Rabies virus belongs to the genus lyssavirus and is in the *Rhabdoviridae* family and this genus is made up of 10 other viruses as well (Hanlon, Kuzmin et al. 2005; Nadin-Davis and Fehlner-Gardiner 2008). Genotype 1 is the classical rabies virus and is considered responsible for the vast majority of human and animal infections and deaths (Nadin-Davis and Fehlner-Gardiner 2008). Bat rabies virus (Genotype 2) is also responsible for a significant number of infections. The other 9 viruses all have caused or are capable of causing rabies virus in the human host. While they are less commonly encountered, all are highly infectious and require treatment with great urgency. Rabies virus is a single-stranded, negative sense, unsegmented and enveloped RNA virus which encodes 5 proteins. A schematic of a fully assembled rabies virus virion is shown in **Figure 5.2**.

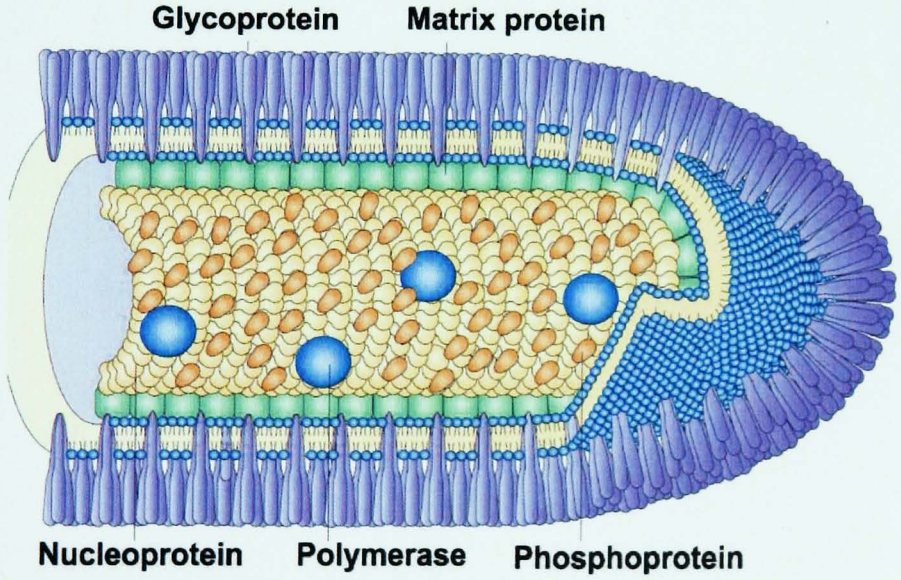


Figure 5.2 A schematic of a fully assembled rabies virus virion (Schnell, McGettigan et al. 2010). The RNA genome is tightly enclosed within the nucleocapsid protein (yellow) - referred to as ribonucleoprotein or nucleocapsid protein (NCP). The polymerase (blue) and phophoprotein (orange) make up the internal capsid structure and associate with the RNP. The matrix protein (green) joins together the capsid and the viral membrane. The glycoprotein (purple) is a transmembrane protein which stretches into the matrix protein layer.

The rabies virus life cycle can be broken into 3 phases and a schematic is shown in **Figure 5.3 (Schnell, McGettigan et al. 2010)**:

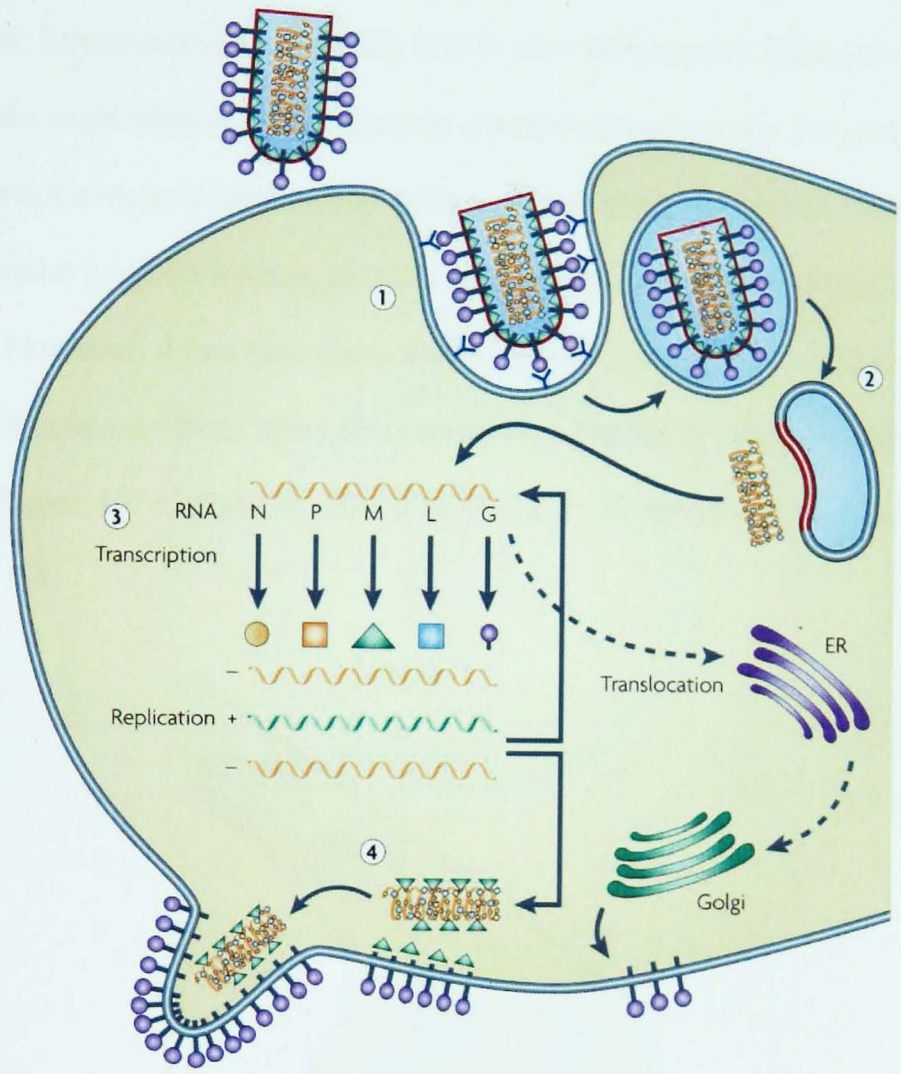


Figure 5.3 A schematic of the rabies virus life cycle (Schnell, McGettigan et al. 2010).

1) virus recognizes host receptor(s) and binds to host cell initiating endocytosis.
2) Endocytosis is quickly followed by fusion between the endosomal and viral membrane proteins which releases the viral genome into the cytoplasm, a process known as 'uncoating'. The classical wild-type rabies virus enters the motor neuron at the neuromuscular junction, and rabies virus particles are shuttled – in a retrograde direction – through the axon of the infected neuron when they finally reach the neuron's cell body.
3) The production of the virion components begins, which includes transcription, replication and protein synthesis.
4) Viral protein assembly begins, followed by transportation to the sites of viral budding, which releases mature rabies virus particles, each of which are capable of infecting a new cell.

This process is highly regulated, however, it is not fully understood. It has been suggested that the rabies glycoprotein is the viral protein responsible for ligation with host-cell receptors. Previous studies have demonstrated that deletions in the glycoprotein gene prevent mature virus particles spreading from an infected cell *in vivo* (Etessami, Conzelmann et al. 2000) and *in vitro* (Mebatsion, Konig et al. 1996). It has also been postulated that the nicotinic acetylcholine receptor (nAchR), located at the post-synaptic muscle membrane, allows for the accumulation of rabies virus at the neuromuscular junction leading to more efficient infection of the motor neurons (Lafon 2005). However, it has also been shown that rabies virus can replicate in striated muscle cells and that rabies virus uses the nAchRs to infect muscle cells (Murphy and Bauer 1974; Watson, Tignor et al. 1981). A schematic of this process is shown in **Fig. 5.4**.

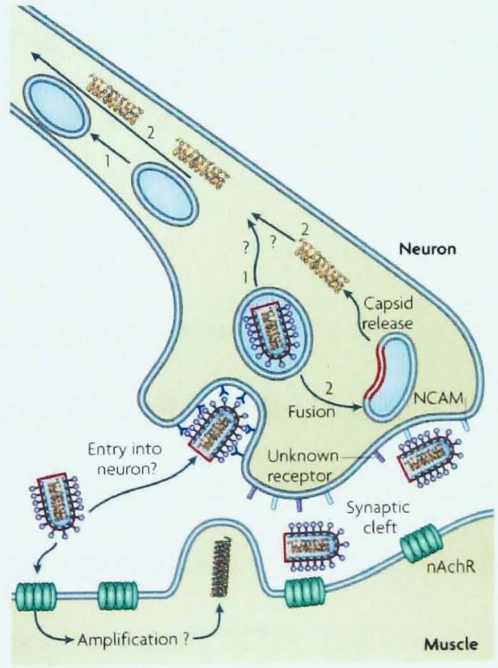


Figure 5.4 A schematic of rabies virus infecting a motor neuron (Schnell, McGettigan et al. 2010). Rabies virus is enriched for at the neuromuscular junction by nAchR, which are located on the postsynaptic muscle membrane. The rabies virus enters the cell via binding to the neural cell adhesion molecule (NCAM) and possibly through binding to unknown receptor(s).

Early diagnosis of rabies virus has proven very difficult. The current guidelines state that rabies should be treated for in the event of unexplained viral encephalitis with a known history of an animal bite. Non-vaccinated individuals require more time to develop neutralizing antibodies, and they usually do not develop until later in the course of disease after symptoms have begun. A conclusive diagnosis of rabies infection includes: 1) isolated virus from saliva; and 2) positive immunofluorescent skin biopsies or immunofluorescent neutralizing antibodies isolated from cerebrospinal fluid or from serum (WHO 2008).

Virus may directly enter the peripheral nervous system and migrate to the brain or may replicate for a period in the muscle tissue. The latter creates a period of sequestration in close proximity to the entry site, and this period is referred to as the incubation period. Once completion of incubation period occurs, the virus can then spread into other organs. The duration of the incubation period has been noted between 5 days to more than 2 years.

The clinical manifestations of the disease can be seen in 2 ways: 1) the furious (classical) form - accounts for 80% of infections; and 2) the numb (paralytic) form – 20% of infections. Furious infection is described as hydrophobia and defined as terror and excitation-induced spasm of inspiratory muscles, larynx and pharynx leading to an overwhelming need to drink (Warrell 1976). Hallucinations are commonly associated with the furious form of rabies virus infections. These symptoms often include extreme aggression leading to outburst attacks on other animals or humans. The numb form of infection is associated with weakness in the form of mild paralysis and is associated with a greater frequency of misdiagnoses at the onset of disease (Schnell, McGettigan et al. 2010). In both forms of infection, once symptoms begin,

there is an acute neurologic period which usually lasts up to 7 days and this then leads to coma (5-14 days) and finally death (Warrell 1976).

5.1.3 *Immunoprophylaxis*

Fortunately, post-exposure prophylaxis (PEP) is available for humans after having encounters with animals suspected of infection. Current guidelines in the United States for use of PEP in previously non-immunized people state to give 1 dose of rabies immunoglobulin (RIG) along with 5 doses of rabies vaccine (Manning, Rupprecht et al. 2008). There are clear disadvantages to this approach in developing countries as the cost of this is not sustainable and patient compliance and adherence to medication treatment regimes are often a concern. There are currently alternative approaches being investigated on behalf of the WHO which include the use of DNA vaccines (Lodmell, Ray et al. 1998; Lodmell, Ray et al. 1998; Lodmell and Ewalt 2000; Lodmell, Ray et al. 2000; Lodmell and Ewalt 2001; Lodmell, Parnell et al. 2001; Lodmell, Parnell et al. 2002) and viral vectors (Vos, Neubert et al. 2001; Lees, Briggs et al. 2002; Ito, Sugiyama et al. 2005; Morimoto, Shoji et al. 2005; Cenna, Tan et al. 2008). Studies have also begun to assess the safety, efficacy and cost-benefit for implementing pre-exposure vaccinations in children in areas of high endemnicity (Dodet, Adjogoua et al. 2008; Shanbag, Shah et al. 2008; Lang, Feroldi et al. 2009).

As for many other vaccines, the mechanisms of vaccine-induced immunity to rabies virus infection remain unknown. Vaccination induces virus neutralising antibodies and it is widely assumed that this is how the vaccine protects. There is a great need to identify effective correlates of protection. However, there is very little known about the cell-mediated immune response to rabies vaccination or whether it contributes to protective immunity.

Rabies vaccination, however, serves as an excellent model in that screening for previous exposure to rabies virus can be performed with a relatively high degree of certainty. Furthermore, rabies virus can infect multiple species of animal other than *homo sapiens*, such as mice and dogs, which affords us the opportunity to study this virus in its native environment as compared to working with cultures and cell lines. Finally, the same vaccine, which elicits protection in humans, is also used in other animals.

5.2 Materials and Methods

5.2.1 *Vaccination of study participants*

Thirty healthy adult volunteers (median age = 28 yrs; i.q.r. = 26-31 yrs) received three doses (Days 0, 7 and 21) of 2.5 IU heat-inactivated rabies virus (Flury LEP strain) (RABIPUR, Novartis Vaccines) by intramuscular injection. All subjects gave fully informed, written consent and the study was approved by the London School of Hygiene and Tropical Medicine Ethics Committee.

5.2.2 *Blood sampling, PBMC preparation and cell culture*

PBMC collected immediately before the first and third vaccinations (i.e. Day 0, Day 21) were cryopreserved in liquid nitrogen so that pre- and post-vaccination samples could be analyzed side-by-side at the end of the study. PBMC (2×10^6 cells/ml) were cultured in 96 well U-bottom plates for up to 7 days (with or without 25 ng/ml PMA (phorbol 12-myristate 13-acetate) plus 1µg/ml Ionomycin (both Sigma) (P/I), rhIL-12, rh-IL-18 or rhIL-2 (0.1µg/ml) or 200 arbitrary units/ml inactivated rabies virus 07/162 (5^{th} International Standard; National Institute for Biological Standards and control, UK). Neutralizing anti-IL-2 (MQ1-17H12; BD Biosciences), or anti-IL-12 and anti-IL-18 (R & D Biosystems) were each used at final concentrations of 10µg/ml. Recombinant rabies virus nucleocapsid protein (rNCP) (M. Juozapaitis, Institute of Biotechnology, Vilnius, Lithuania) and was used at a final concentration of 10µg/ml. Brefeldin A (3 µg/ml) and monensin (1.33 µg/ml) were added to all cultures for the last 5 hours of the culture.

$CD3^+$ cells were positively selected using FlowComp human CD3 Dynabeads and a Dynal Magnetic Particle Concentrator-2 (both Invitrogen), according to the manufacturer's instructions and purity confirmed with anti-human CD3 antibody (clone UCHT-1; BD Biosciences). Purity of both $CD3^+$ and $CD3^-$ cell populations was

> 97%. T cell subsets were depleted on LD separation columns (Miltenyi Biotec) using monoclonal antibodies to CD4 (BD Biosciences) or CD8 (Caltag) or anti-CD45RO-coated microbeads (Miltenyi Biotec).

PBMC labeled with 10μM carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Invitrogen) were cultured at 37℃ with 5% CO_2 for up to 7 days with or without heat-killed rabies virus 07/162 and analyzed by flow cytometry.

5.2.3 *Cell surface and intracellular staining for flow cytometry*

Surface and intracellular staining was performed as previously described (Korbel, Newman et al. 2005). Antibodies used were: CD56 PE (N901), CD56 APC (N901) (both Beckman Coulter), CD56 PE-Cy7 (B159), CD45RO PE-Cy7 (UCHL1), CD3-PerCP (SK7), CD4 APC-Cy7 (RPA-T4), CD4 PE (RPA-T4), CD8 PE (SK1), LAMP-1 biotin (H4A3), Perforin PE (δG9), IL-2 APC (MQ1-17H12), Streptavidin-PerCP (all BD Biosciences), anti-CD69 PE (CH/4) and CD3 PE-Texas Red (S4.1) (both Caltag/Invitrogen), CD8 Pacific Blue (LT8; eBiosciences) andIFN-γ FITC (D9D10; Ab Serotec).

5.2.4 *Statistical analysis*

Data analyses were performed using prism5 (GraphPad) or Stata10 (StataCorp). To estimate the proportion of the precursor lymphocyte population proliferating through each division, the number of CFSE-diluted cells in each division was divided by $2^{(\text{number of divisions} + 0.5)}$ (Hawkins, Hommel et al. 2007).

5.3 *Results*

5.3.1 *T cells and NK cells upregulate CD69 and produce IFN-γ in response to rabies antigen, after rabies vaccination*

PBMC isolated from vaccinees immediately before their 1st and 3rd vaccinations (Day 0 and Day 21) were cultured for 21 hours without stimulation (GM), with P/I (positive control) or with heat-inactivated rabies virus (07/162) as the specific recall antigen. Cell surface expression of CD69 and intracellular expression of IFN-γ were analyzed in $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T cells and in $CD56^+$ $CD3^-$ NK cells (**Fig. 5.5a**). A representative example of IFN-γ and CD69 expression before (left panels) or after (right panels) two doses of rabies vaccine is shown in **Fig. 5.5b.** and data from 5 individual vaccinees are shown in **Figs. 5.5c and 5.5d**. In the pre-vaccination samples, P/I induced upregulation of both IFN-γ and CD69 in $CD4^+$ and $CD8^+$ T cells and in NK cells but none of these cells responded to rabies virus 07/162. By contrast, in the post vaccination (Day 21) samples, rabies virus 07/162 induces highly significant upregulation of IFN-γ and CD69 in $CD4^+$ and $CD8^+$ T cells *and* in NK cells. Moreover, among NK cells the IFN-γ responses are as high as those induced by the polyclonal stimulator P/I (with at least 30% of all NK cells staining positively for IFN-γ) and the MFI for IFN-γ staining is significantly higher among NK cells than among T cells (**Figs. 5.5b and 5.5d**). Both $CD56^{bright}$ and $CD56^{dim}$ NK cells produce IFN-γ in response to rabies virus 07/162 and although a higher percentage of $CD56^{bright}$ NK cells than $CD56^{dim}$ cells make IFN-γ (~50% vs. ~20%) (**Figure 5.6**), since $CD56^{bright}$ cells make up only a small proportion of the NK cell population, the majority (>75%) of the IFN-γ+ NK cells are in fact $CD56^{dim}$. Furthermore, there was no significant difference in the amount of IFN-γ being produced by $CD56^{bright}$ and $CD56^{dim}$ NK cells as judged by MFI.

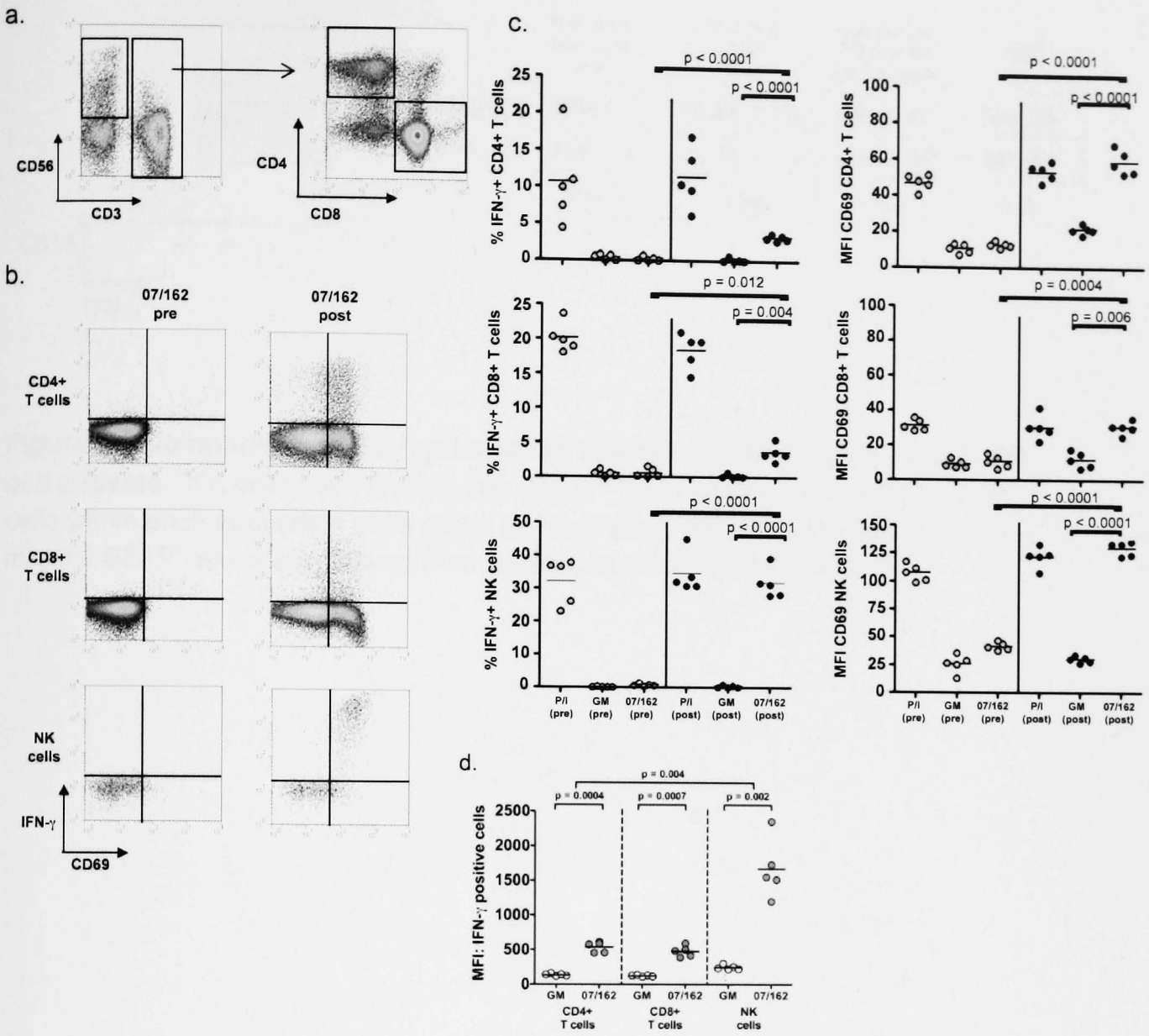
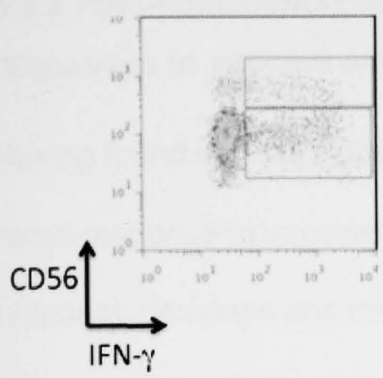


Figure 5.5 After rabies vaccination, T cells and NK cells upregulate CD69 and produce IFN-γ in response to rabies virus

PBMC were isolated before (pre) and after (post) 2 doses of rabies vaccine, cultured *in vitro* for 21 hours in growth medium alone (GM) or with PMA and Ionomycin (P/I) or heat-inactivated rabies virus (07/162) and analyzed by flow cytometry for surface expression of CD69 and intracellular IFN-γ. (**a**) Lymphocytes (identified by characteristic FSC and SSC) were identified as NK cells ($CD56^+$ $CD3^-$), $CD4^+$ $CD3^+$ T cells or $CD8^+CD3^+$ T cells. (**b**) Representative flow cytometry plots for PBMC obtained before or after rabies vaccination from one donor, showing IFN-γ and CD69 expression in $CD4^+$ T cells, $CD8^+$ T cells and NK cells after restimulation in vitro with rabies virus 07/162. (**c**) Percentages of IFN-γ^+ cells (left plots) and MFI of CD69 expression (right plots) among $CD4^+$ T cells, $CD8^+$ T cells and NK cells isolated from 5 subjects, before (open circles) and after (filled circles) vaccination, and restimulated *in vitro*. (d) MFI of IFN-γ^+ $CD4^+$ T cells, $CD8^+$ T cells and NK cells among post-vaccination PBMCs from 5 subjects cultured with (07/162; filled circles) or without (GM; open circles) inactivated rabies virus. P values are derived from 2-tailed paired Wilcoxon test.



	% of subset positive for IFN-γ	% of total positive for IFN-γ	Total number of IFN-γ+ NK cells/10^5 PBMC	MFI
CD56 Bright	**52% ± 7**	**4.5% ± 1.2**	**490 ± 28**	**344.23**
CD56 Dim	**21% ± 4**	**17% ± 2.3**	**1630 ± 87**	**341.97**
P =	**0.063**	**0.063**	**0.063**	**1.0**

Figure 5.6 Comparison of IFN-γ production between CD56^{bright} and CD56^{dim} NK cell subsets. NK cells were divided into CD56^{bright} and CD56^{dim} and all IFN-γ+ NK cells within each population were gated on separately. Numbers are presented are mean ± SE. P values are derived from 2-tailed paired Wilcoxon test. N = 5.

5.3.2 *Post-vaccination, NK cells make both early and sustained IFN-γ responses to vaccine antigens*

Having found that vaccination primes NK cells to make a robust and immediate IFN-γ recall response to vaccine antigen, I sought to determine how quickly this NK response develops and for how long it is sustained, by sampling cultured PBMCs at intervals over a 7 day period (**Figure 5.7**). Restimulation of post-vaccination PBMC with rabies virus 07/162 leads to an initial, modest peak in IFN-γ production from both $CD4^+$ and $CD8^+$ T cells at 18-24 hours but there is a second much more robust response that begins on day 5 and which is still increasing on Day 7 (**Fig 5.7*a***). By contrast, a very robust NK cell recall response is clearly underway within 12 hrs and peaks at 18 hrs; however there was also a second wave of NK cell IFN-γ production - coinciding with the secondary wave of the T cell response – such that ~30% of NK cells were still making IFN-γ 7 days into the recall response. This biphasic NK cell and T cell response may reflect an initial responding population giving rise to a second generation of responsive cells or providing essential stimuli for further cell activation. In either case, it is apparent that NK cells are not only the earliest contributors of IFN-γ to the post-vaccination recall response but also contribute to this response for a protracted period of time.

Although the proportion of NK cells producing IFN-γ in the restimulated post-vaccination samples was much higher than the proportion of T cells producing IFN-γ, circulating T cells outnumber NK cells by approx 10:1. I therefore calculated the contribution of NK cells to the total pool of IFN-γ-producing cells (**Fig. 5.7b**). Twelve and 18 hrs after *in vitro* re-stimulation with rabies virus 07/162, more than 70% of all IFN-γ^+ cells were NK cells. Although this dropped to ~40% by 24 hours, NK cells continued to represent 30-50% of all IFN-γ^+ cells until at least 7 days after re-

stimulation. Thus, not only are NK cells readily activated post-vaccination, but they contribute very significantly to the total effector cell pool during the first 7days of the recall response. When taken together with the very high MFI for IFN-γ staining among NK cells (**Figure 5.5d**) these data suggest that the overwhelming majority of the early IFN-γ emanates from NK cells.

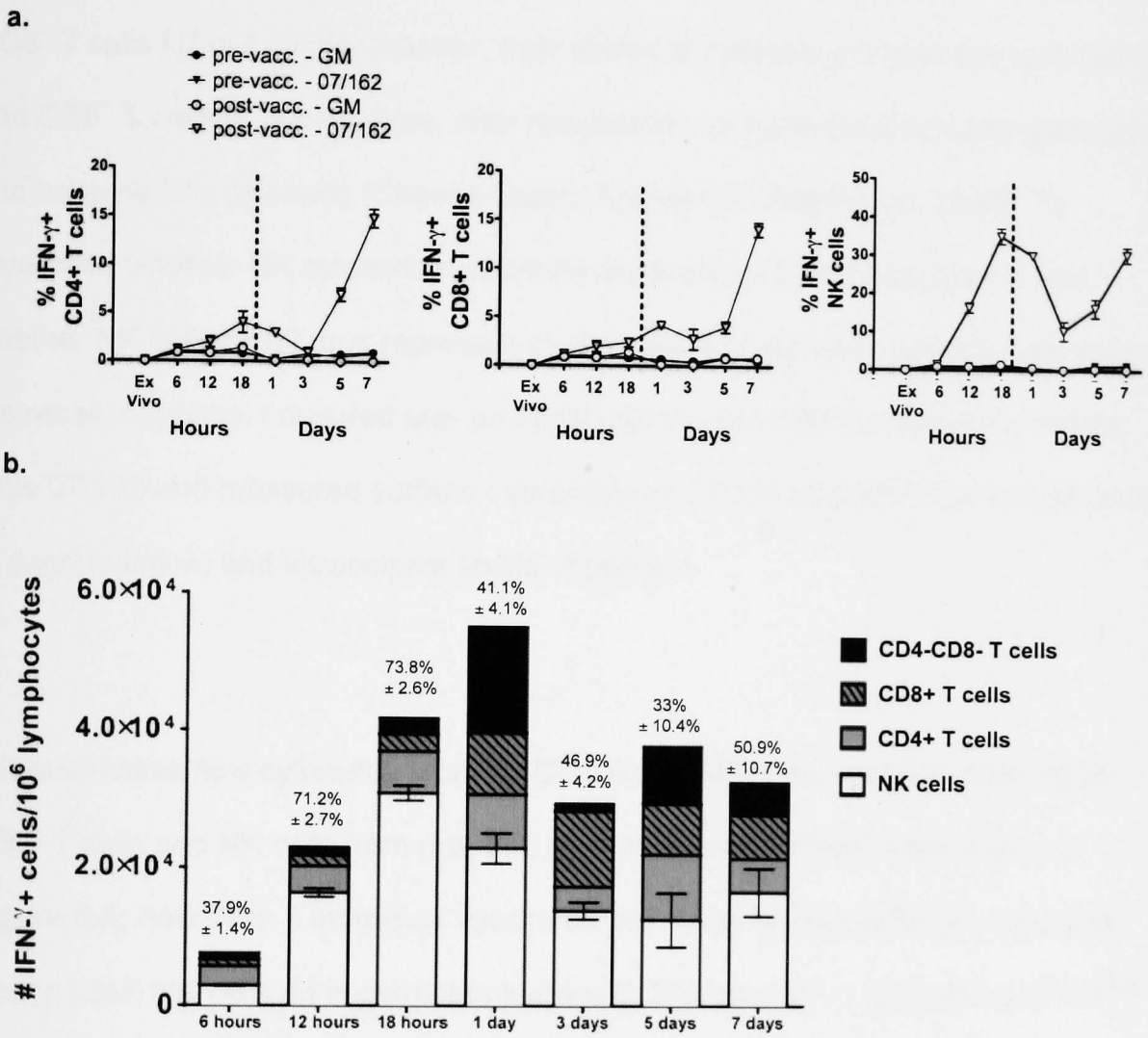


Figure 5.7 Post-vaccination, NK cells make both early and sustained IFN-γ responses to vaccine antigens. PBMC from 5 subjects were isolated before (pre) and after (post) 2 doses of rabies vaccine, analyzed immediately (ex vivo) or cultured *in vitro* for up to 7 days in growth medium alone (GM) or with heat-inactivated rabies virus (07/162) and analyzed by flow cytometry for intracellular IFN-γ. (a) The percentage of IFN-γ^+ $CD4^+$ T cells, $CD8^+$ T cells and NK cells detected in PBMC cultures at each time point. The vertical dashed line separates data from a short-term restimulation (up to 18 hours) and a long-term restimulation experiment. (**b**) The absolute number of IFN-γ^+ lymphocytes was calculated at each time point and stratified according to whether these were NK cells or $CD4^+$, $CD8^+$ or $CD4^-CD8^-$ T cells. Numbers above each column indicate the proportion of all the IFN-γ+ cells that are NK cells (mean ± SE).

5.3.3 *NK cells degranulate and release perforin in response to rabies antigen after rabies vaccination*

One of the hallmarks of NK cells is their ability to rapidly detect and kill (within minutes) infected, transformed, or non-self cells by releasing pre-formed cytolytic molecules, such as perforin and granzymes, stored in secretory granules. Whilst $CD8^+$ T cells kill in a similar manner, their stores of cytolytic granules are very limited and $CD8^+$ T cells thus need time, after reactivation, to synthesize cytolytic granules and become fully cytotoxic (Chavez-Galan, Arenas-Del Angel et al. 2009). To determine whether NK cytotoxic responses are enhanced post-vaccination and whether NK cells might thus represent the first wave of actively cytotoxic cells during the recall response, I cultured pre- and post-vaccination PBMCs with P/I or rabies virus 07/162 and measured surface expression of CD107a/LAMP-1 (as an indicator of degranulation) and intracellular levels of perforin.

Representative flow cytometry plots for CD107a/LAMP-1 and perforin staining of $CD8^+$ T cells and NK cells from pre- and post-vaccination PBMCs are shown in **Figure 5.8;** data from 5 individual vaccinees are shown in **Fig. 5.9a.** As expected, resting (GM) NK cells do not express surface CD107a/LAMP-1 but resting $CD56^{dim}$ (but not $CD56^{bright}$) NK cells do contain pre-formed perforin; 12 hrs polyclonal (P/I) stimulation results in increased surface expression of LAMP-1 and complete loss of intracellular perforin, consistent with NK cell degranulation. On the other hand, resting $CD8^+$ T cells do not contain perforin but do synthesize perforin and upregulate LAMP-1 in response to P/I stimulation. Importantly, stimulation with rabies virus 07/162 also causes marked degranulation (upregulation of LAMP-1 and loss of perforin) of $CD56^{dim}$ NK cells in the post-vaccination but not in the pre-vaccination

samples. Degranulation in 07/162-restimulated post-vaccination $CD8^+$ T cells is less marked but there is clear induction of perforin synthesis.

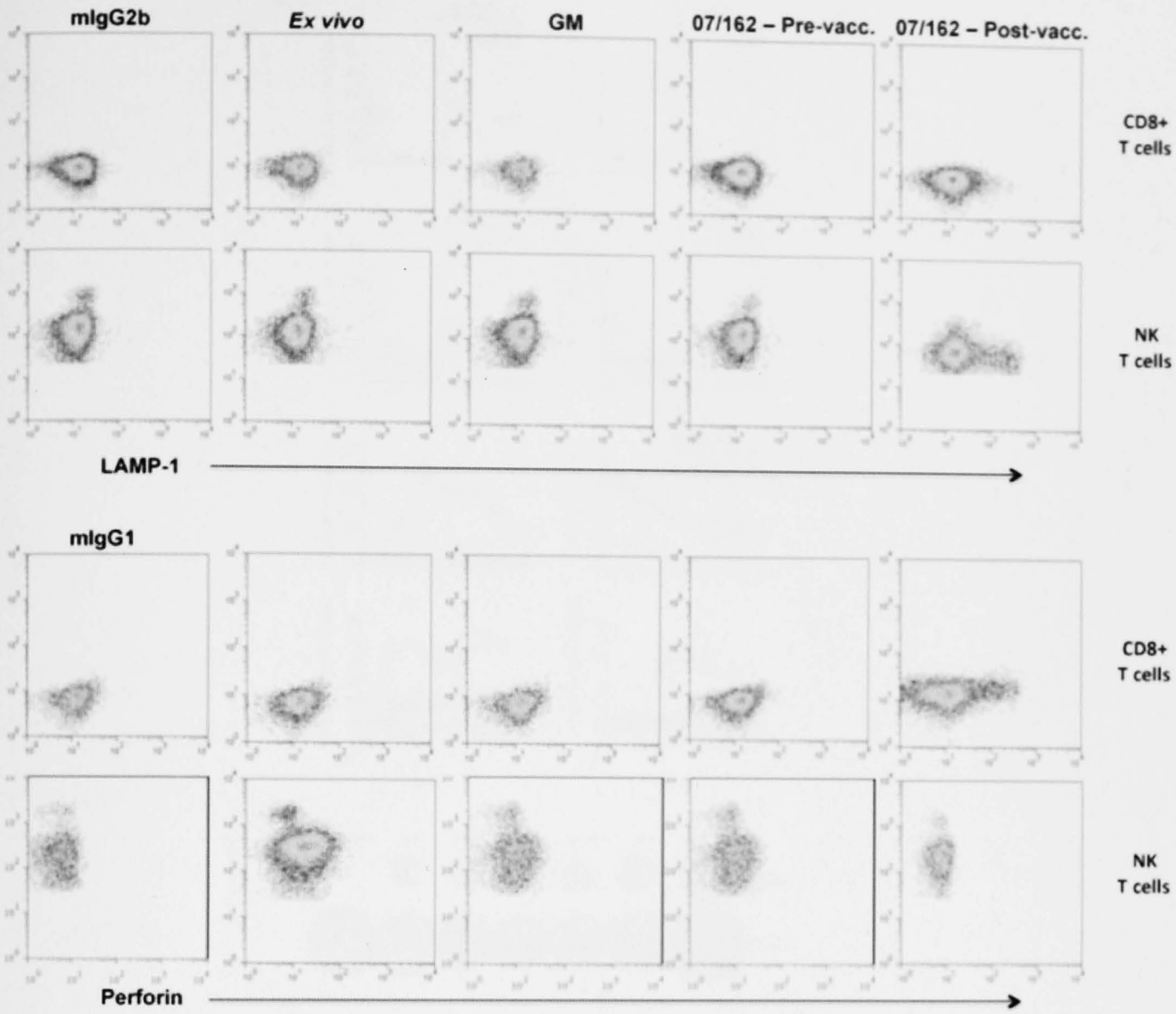


Figure 5.8 Representative staining of LAMP-1 and perforin expression in CD8+ T cells and NK cells. Representative flow cytometry plots showing CD8+ T cell expression of LAMP-1 and perforin (upper panels) and LAMP-1 (middle panels) and perforin (lower panels) expression in NK cells isolated from one donor data before and after rabies vaccination (*ex vivo*) and after culture *in vitro* in growth medium (GM) or with rabies virus 07/162 for 12 hours. Isotype matched control antibodies (mIgG2b or mIgG1; left panels) were used to establish background levels of staining.

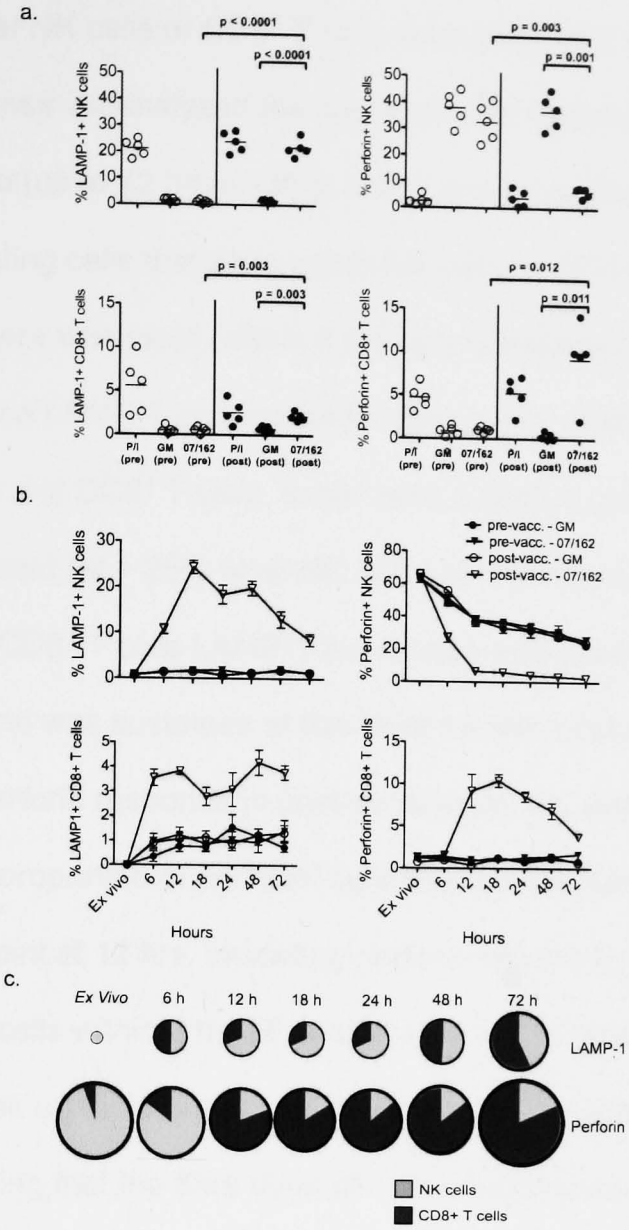


Figure 5.9 Post-vaccination, NK cells degranulate and release perforin in response to rabies virus. PBMC were isolated from 5 subjects before (pre) and after (post) 2 doses of rabies vaccine, analyzed immediately (ex vivo) or cultured *in vitro* for up to 72 hrs in growth medium alone (GM) or with PMA and Ionomycin (P/I) or heat-inactivated rabies virus (07/162) and analyzed by flow cytometry for surface expression of LAMP-1 and intracellular perforin. (**a**) Percentages of LAMP-1$^+$ (left plots) and perforin$^+$ (right plots) NK cells (upper plots) and CD8$^+$ T cells (lower plots) isolated before (open circles) and after (filled circles) vaccination, and restimulated *in vitro* for 12 hrs. Data represent mean ± SE. P values are derived from 2-tailed paired Wilcoxon test. (**b**) The percentages of LAMP-1$^+$ (left plots) and perforin$^+$ (right plots) NK cells (upper plots) and CD8$^+$ T cells (lower plots) detected in PBMC cultures at each time point. (**c**) Pie charts showing the proportion of all LAMP-1 (top row) expressing lymphocytes and all perforin (bottom row) expressing lymphocytes that are either NK cells (light grey) or CD8+ T cells (black) among post-vaccinated PBMC stimulated in vitro for up to 72 hours with rabies virus 07/162. The size of the circles is proportional to the absolute numbers of LAMP-1$^+$ or perforin$^+$ cells per 10^6 PBMC. Data represent mean values from 5 subjects.

To determine whether NK cells or $CD8^+$ T cells were the major contributors to the early cytotoxic response we analyzed the degranulation response of the two cell populations over time (up to 72 hours) (**Fig. 5.9b**) and calculated the absolute number of degranulating cells that were either NK cells or CD8+ T cells at each time point (**Fig. 5.9c**). There was rapid (within 6 hrs) and sustained (at least 72 hrs) upregulation of surface LAMP-1 expression in rabies virus re-stimulated, post-vaccination, NK cells and $CD8^+$ T cells. In NK cells, LAMP-1 upregulation was evident by 6 hrs, peaked (at > 25% of all NK cells) at 12 hrs and then gradually declined whereas in $CD8^+$ T cells LAMP-1 expression was maximal by 6 hrs (at ~4% of all $CD8^+$ T cells) and was sustained at this level for the duration of the experiment. The kinetics of the perforin response in post-vaccination NK cells mirrored the LAMP-1 response, with the proportion of $perforin^+$ cells falling significantly within 6 hrs and reaching its lowest point at 12 hrs, indicating perforin release by degranulation in post-vaccination NK cells within 6 hrs of re-exposure to the vaccine antigen. However, perforin took up to 12 hrs to accumulate in re-stimulated post-vaccination $CD8^+$ T cells suggesting that the 6hrs burst of T cell degranulation may not lead to an optimal cytotoxic response.

The degranulation response was antigen-specific since LAMP-1 expression remained at baseline levels in all unstimulated cells and in rabies virus re-stimulated pre-vaccination NK and $CD8^+$ T cells. Perforin levels remained at baseline in unstimulated and pre-vaccination $CD8^+$ T cells. There was a gradual loss of perforin over time in pre-vaccination NK cells and in unstimulated post-vaccination NK cells, presumably reflecting non-specific leakage of perforin.

The relative contributions of NK cells and $CD8^+$ T cells to the pool of cytotoxic effectors are shown in **Fig. 5.9c**. NK cells represent the majority of both $LAMP\text{-}1^+$

and perforin$^+$ cells 6 hrs after antigen stimulation. The marked decrease at 12 hours in the proportion of perforin$^+$ cells that are NK cells, together with the increased proportion of the LAMP-1$^+$ cells that are NK cells, provides further evidence of a wave of NK cell degranulation between 6 and 12hrs. On the other hand, although CD8$^+$ T cells represent the majority of all perforin$^+$ cells from 12hrs onwards, they do not represent the majority of degranulating, LAMP-1$^+$ cells, until 48-72 hrs into the recall response.

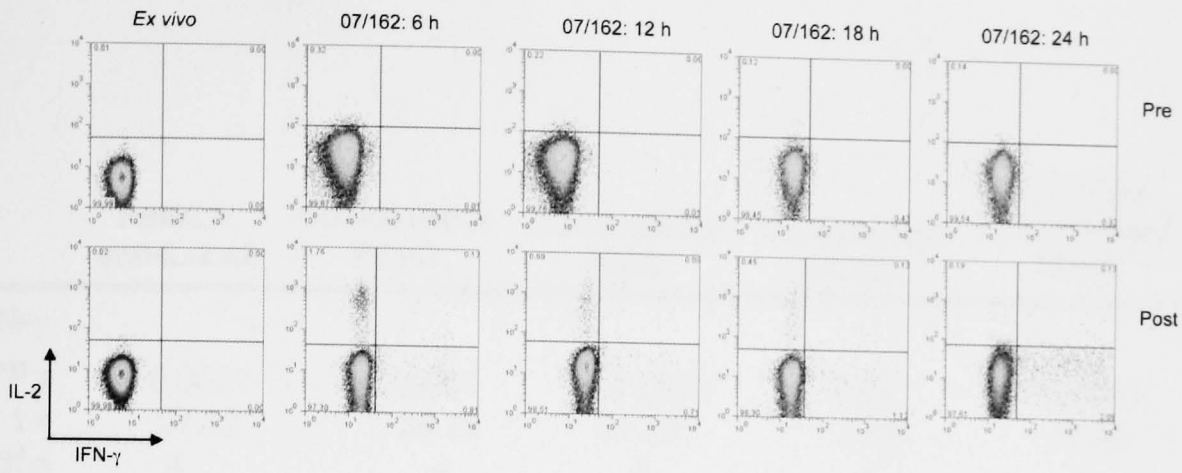
5.3.4 *Antigen-specific IL-2 production from CD45RO$^+$ CD4$^+$ T cells is required for NK cell recall responses*

Our previous data (Newman, Korbel et al. 2006) and that of others (He, Draghi et al. 2004) indicating a role for IL-2 and CD4$^+$ T cells in NK cell activation, led us to speculate that IL-2 from antigen-specific T cells drives the recall response of NK cells after vaccination. I therefore analyzed the kinetics of IL-2 and IFN-γ production by CD4$^+$ T cells among pre- and post-vaccination PBMC re-stimulated *in vitro* with rabies virus 07/162 for up to 24 hrs. Representative flow cytometry plots for one vaccinated subject are shown in **Fig. 5.10a** and post-vaccination data from 4 subjects are summarized in **Fig. 5.10b**.

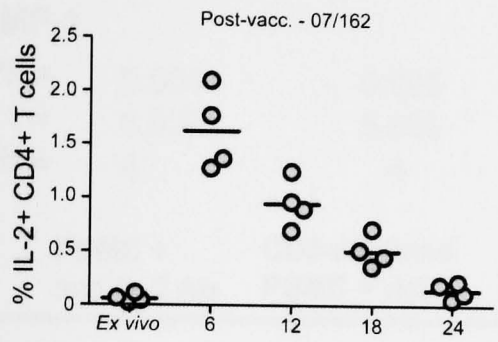
Pre-vaccination, CD4$^+$ T cells produce neither IL-2 nor IFN-γ in response to rabies virus 01/162. Post-vaccination, a clear population of IL-2$^+$ CD4$^+$ T cells can be distinguished, peaking as early as 6 hrs after re-exposure to rabies virus and declining to negligible values within 24 hrs. Very few of the IL-2$^+$ cells produced IFN-γ (**Fig. 5.10a**) suggesting that at this early stage they are Th0 rather than Th1 effector cells. In line with my hypothesis, the peak of T cell IL-2 production (6 hrs) preceded the onset of NK cell IFN-γ production (12 hrs; **Fig 5.7a**).

To determine whether this $CD4^+$ T cell IL-2 response contributed to the post-vaccination NK cell response, post-vaccination PBMCs from 5 subjects were cultured overnight with rabies virus 07/162 in the presence or absence of rh-IL-2 or a neutralizing antibody to IL-2 and analyzed for intracellular IFN-γ, intracellular perforin or cell surface expression of LAMP-1 (**Fig. 5.10c;** statistical analysis is shown in **Table 5.3.1**). In parallel experiments, PBMCs were depleted of all $CD3^+$ T cells, just $CD4^+$ T cells, just $CD8^+$ T cells or just $CD45RO^+CD3^+$ T cells. In the intact PBMC cultures, as before, there were potent NK cell IFN-γ and degranulation (decreased intracellular perforin and increased surface expression of LAMP-1) responses to the recall antigen. Anti-IL-2 antibody completely ablated both the IFN-γ response and degranulation. Moreover, NK cells among PBMC depleted of $CD3^+$, $CD4^+$, or $CD45RO^+CD3^+$ T cells did not mount any significant IFN-γ or degranulation responses; depletion of $CD8^+$ T cells, on the other hand, had no significant detrimental effect on the NK cell recall response. In support of my hypothesis, however, NK cells among $CD4^+$ T cell-depleted and $CD45RO^+$ T cell-depleted PBMCs were able to make robust IFN-γ and degranulation responses when cultures were supplemented with rhIL-2.

a.



b.



c.

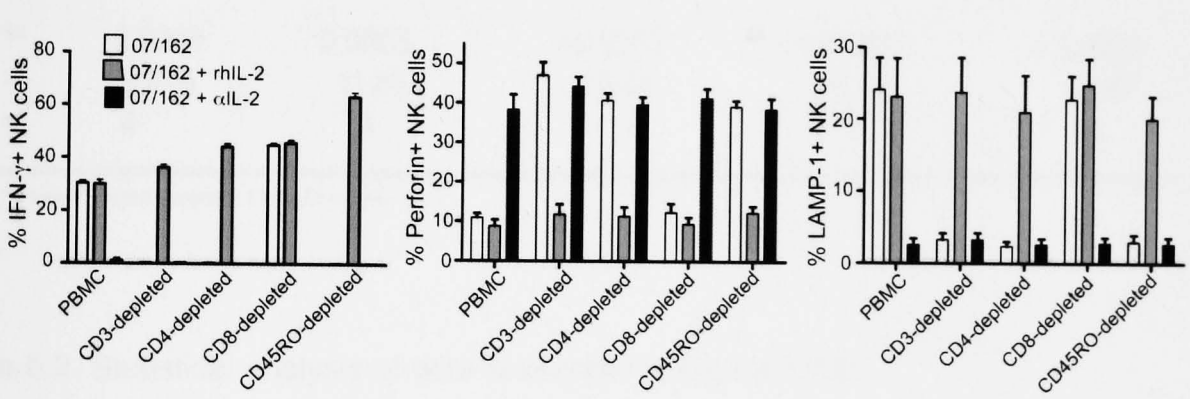


Figure 5.10: **Antigen-specific IL-2 production from CD45RO$^+$ CD4$^+$ T cells is required for NK cell recall responses.** Pre- and post-vaccination PBMC from 4 subjects were cultured for periods of up to 24 hrs without stimulation (GM) or with rabies virus (07/162) and analyzed by flow cytometry for intracellular IL-2 and IFN-γ. (**a**) Representative flow cytometry plots showing IL-2 and IFN-γ expression in pre- (upper plots) and post- (lower plots) vaccination CD4$^+$ T cells from one donor in response to 07/162 stimulation over 24 hours. (**b**) Percentages of CD4+ T cells expressing intracellular IL-2 in response to 07/162 restimulation over time; post-vaccination samples from 4 subjects. (**c**) Post-vaccination PBMCs from 4 subjects were cultured for 12 hrs (perforin and LAMP-1 expression) or 24hrs (IFN-γ expression) with rabies virus 07/162 in the presence or absence of rh-IL-2 or a neutralizing antibody to IL-2 and analyzed for intracellular IFN-γ (left panel), intracellular perforin (middle panel) or cell surface expression of LAMP-1 (right panel). Parallel experiments were carried out using PBMCs that had been depleted of all CD3$^+$ T cells, just CD4$^+$ T cells, just CD8$^+$ T cells or just CD45RO$^+$CD3$^+$ T cells. Data represent mean ± SE. Statistical analysis is presented in **Table 5.3.1**.

	PBMC + anti-IL-2 Ab	CD3-depleted PBMC	CD4-depleted PBMC	CD8-depleted PBMC	CD45RO+ CD3-depleted PBMC
IFN-γ					
***P =**	< 0.0001	< 0.0001	< 0.0001	0.53	< 0.0001
t =	27.42	38.33	38.59	0.68	37.42
df =	4	4	4	4	4
LAMP-1					
***P =**	0.004	0.005	0.005	0.006	0.004
t =	6.037	5.699	5.684	5.81	5.831
df =	4	4	4	4	4
	PBMC + anti-IL-2 Ab	**CD3-depleted PBMC + rhIL-2**	**CD4-depleted PBMC + rhIL-2**	**CD8-depleted PBMC + rhIL-2**	**CD45RO+ CD3-depleted PBMC + rhIL-2**
Perforin					
***P =**	0.0008	0.0003	< 0.0001	< 0.0001	< 0.0001
t =	9.075	11.83	19.44	16.23	20.67
df =	4	4	4	4	4

* 2 tailed paired students t test; CI = 95%

Table 5.2. Statistical analysis of data presented in Figure 5.10c

5.3.5 *Extensive NK cell proliferation during post-vaccination recall response*

To determine whether IL-2 produced by T cells during the post-vaccination recall response induced NK cell proliferation – which might potentiate the NK cell effector response and/or replenish the NK cell pool after activation-induced cell death of the first wave of effector response – I labeled pre- and post-vaccination PBMC from 4 donors with CFSE, cultured them for 7 days without stimulation (GM) or with rabies virus 07/162 and then analyzed CFSE expression separately in $CD4^+$ T cells, $CD8^+$ T cells and NK cells (**Fig. 5.11a**) and in both $CD45RO^+$ and $CD45RO^-$ populations of T cells (**Fig. 5.11b**). Cells cultured without antigen did not proliferate. Extensive proliferation was observed in post-vaccination T cells and NK cells after 7 days restimulation with the recall antigen, rabies virus 07/162. The CFSE dilution patterns (**Fig. 5.11a**) suggested that a higher proportion of NK cells than T cells were proliferating and that NK cells were going through more rounds of proliferation than the T cells. Indeed, we were unable to see any undivided NK cells and we could discern at least 7 distinct CFSE peaks among the NK cells whilst a substantial proportion of T cells remained undivided and only ~4 peaks of divided cells could be seen; this was confirmed by detailed analysis of samples from 4 vaccinees (**Figs. 5.11c and 5.11d**). The average number of divisions undergone by NK cells (mean/SE: 2.55/0.01) was significantly higher than for $CD8^+$ T cells (mean/SE: 1.12/0.18; $p < 0.001$), $CD4^+$ T cells (mean/SE: 0.96/0.07; $p < 0.001$); $p = 0.31$) or $CD45RO^+CD4^+$ T cells (1.20/0.37; $p = 0.005$). Analysis of the proportion of the initial cell population that had gone through one or more divisions confirmed that, after 7 days all NK cells had divided at least once but only ~30% of all $CD45RO^+$ $CD4^+$ T cells and less than 10% of $CD8^+$ T cells or $CD45RO^-$ CD4 T cells had done so (**Fig. 5.11d**) however the time to first cell division did not differ between NK cells and the $CD4^+$ and $CD8^+$ T cells.

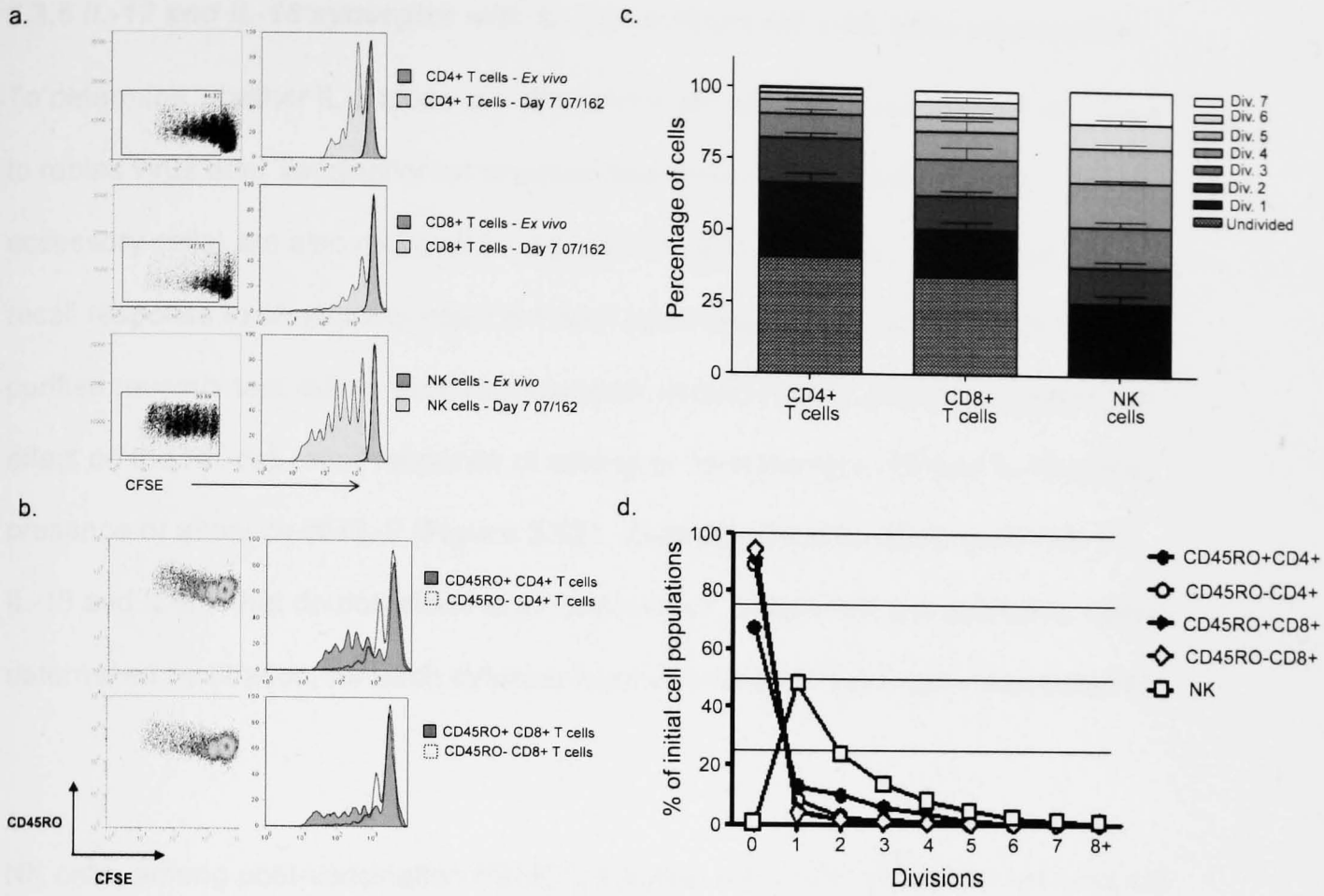


Figure 5.11 Extensive NK cell proliferation during post-vaccination recall response

Pre- and post-vaccination PBMC from 4 donors were labeled with CFSE, cultured for 7 days with rabies virus (07/162) and analyzed by flow cytometry. (**a**) Representative flow cytometry plots of CFSE dilution (left plots) in post-vaccination $CD4^+$ T cells (upper plots), $CD8^+$ T cells (middle plots) and NK cells (lower plots) after 7 days incubation with 07/162. Histogram overlays (right plots) compare CFSE dilution in post-vaccination cells either ex vivo (dark fill) or after 7 days restimulation with 07/162 (light fill). (**b**) Representative flow cytometry plots of CFSE dilution and CD45RO expression (left plots) after 7 days restimulation with rabies virus (07/162) in post-vaccination $CD4^+$ (upper plots) and $CD8^+$ (lower plots) T cells. Histogram overlays (right plots) compare CFSE dilution in $CD45RO^+$ (dark fill) or $CD45RO^-$ (no fill) $CD4^+$ or $CD8^+$ T cells. (**c**) The percentage of $CD4^+$ T cells, $CD8^+$ T cells or NK cells in the post-vaccination, 7 day-restimulated PBMC population that had not divided, or had divided 1 or more times. Data represent mean + SE for 4 donors. (**d**) Estimated proportions of the precursor populations of NK cells and $CD45RO^+$ or $CD45RO^-$ T cells that did not divide, or that divided 1 or more times during the 7 day restimulation assay. Data represent means for 4 donors.

5.3.6 *IL-12 and IL-18 synergize with IL-2 to activate NK cells after vaccination*

To determine whether IL-2 alone is sufficient for NK cells to show a "recall" response to rabies virus after vaccination or whether other signals (e.g. from myeloid accessory cells) are also required for optimal NK cell responses, I compared the recall response to structurally intact but heat-killed rabies virus (07/162) with that to purified recombinant rabies virus nucleocapsid protein (rNCP) and I investigated the effect on the NK cell recall response of adding or neutralizing IL-12 and IL-18 in the presence or absence of rIL-2 (**Figure 5.12**). Suboptimal concentrations of rhIL-12, IL-18 and IL-2 – that do not, alone or in combination, induce NK cell activation - were determined by titration; for each cytokine a concentration of 0.01 ng/ml was selected.

NK cells, among post-vaccination PBMC incubated with rNCP alone, did not produce IFN-γ or upregulate CD69 ($t = 2.1$, $df = 4$, $p = 0.6$), but when cultured with rNCP plus 0.01ng/ml rhIL-12, rhIL-18 and rhIL-2 they responded strongly ($t = 8.94$, $df = 4$, $p = 0.0009$) (**Figs. 5.12a and 5.12b**). No NK cell response was seen in pre-vaccination PBMCs with any of the combinations of stimuli tested (**Fig. 5.12b**). Neutralizing antibodies to IL-12 and IL-18 completely ablated the rabies virus 07/162-induced NK cell IFN-γ response in post-vaccination PBMCs ($t = 8.5$, $df = 4$, $p = 0.001$) (**Fig. 5.12c**), in a very similar manner to IL-2 neutralization. I conclude that signals from two accessory cell populations (myeloid cells and T cells) are required for the recall response of NK cells and that inactivated virus but not rNCP (which presumably lacks the TLR-activating ligands present in whole virus) is able to induce a recall response in NK cells.

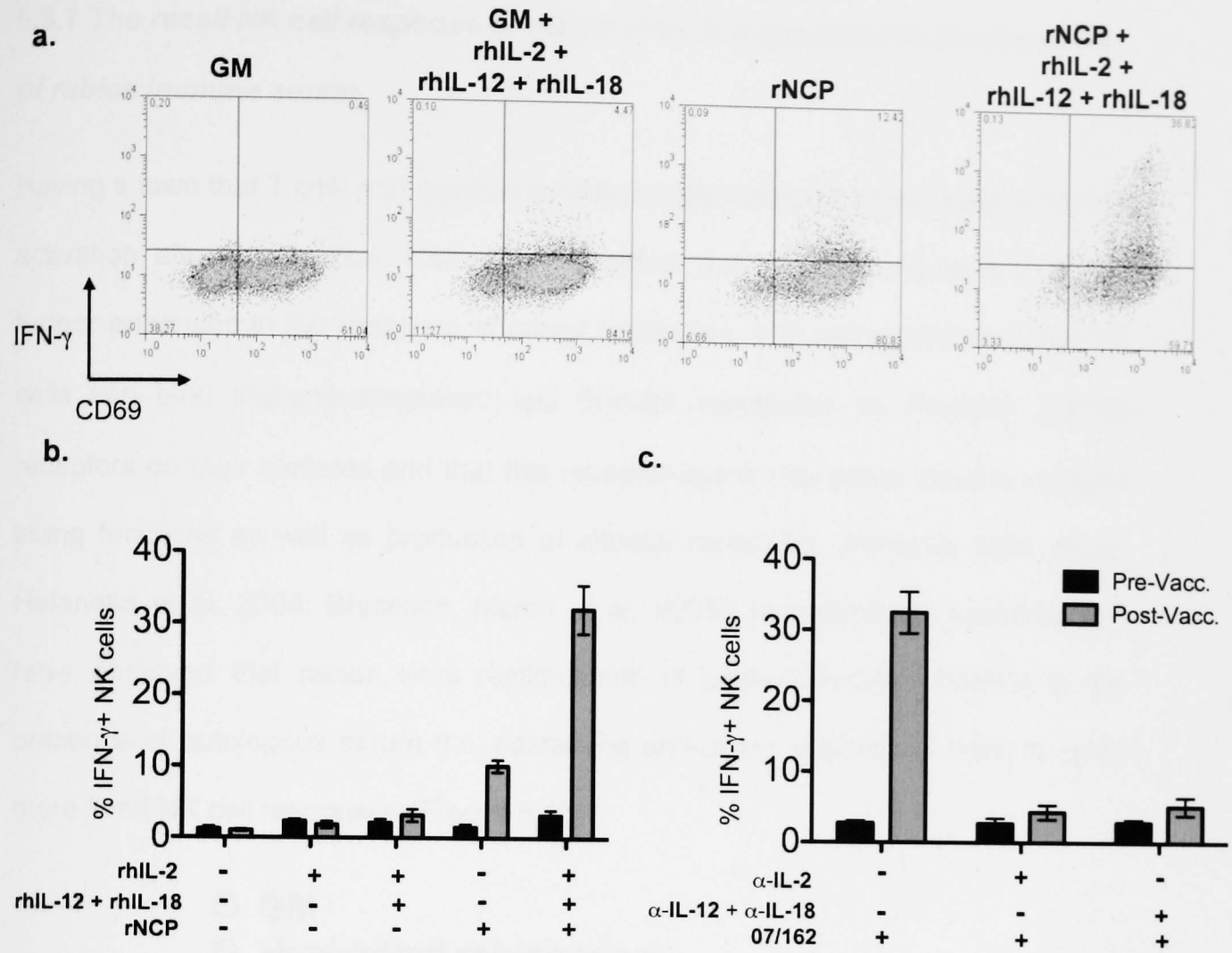


Figure 5.12: **IL-12 and IL-18 synergize with IL-2 to activate NK cells after vaccination**

Pre- and post-vaccination PBMCs from 5 donors were restimulated *in vitro* with inactivated whole rabies virus (07/162) or with purified recombinant viral nucleocapsid protein (rNCP), in the presence or absence of recombinant human IL-2 or IL-12 plus IL-18, or in the presence or absence of neutralizing antibodies to IL-2 or IL-12 plus IL-18, for 24 hours and analyzed by flow cytometry for CD69 and IFN-γ expression. (**a**) Representative flow cytometry plots showing IFN-γ production and CD69 expression in post-vaccination NK cells cultured with or without rNCP in the presence or absence of rhIL-2, rhIL-12 and rhIL-18. (**b**) Percentage of NK cells producing IFN-γ^+ after restimulation with rNCP in the presence or absence of recombinant cytokines. Data represent mean ± SE of 5 subjects. P values are derived from 2-tailed paired Student's t test. (**c**) Percentage of NK cells producing IFN-γ^+ after restimulation with rabies virus 07/162 in the presence or absence of neutralizing antibodies to IL-2, IL-12, IL-18. Data represent mean ± SE.

5.3.7 *The recall NK cell response to rabies virus is augmented in the presence of rabies immune serum*

Having shown that T cell- and myeloid accessory cell-mediated stimuli lead to NK cell activation after vaccination, I wondered whether this NK cell response could be further enhanced in the presence of rabies antibodies. It is well established that NK cells can bind immune-complexed IgG through recognition by FcγRIIIA (CD16) receptors on their surfaces and that this receptor-ligand interaction induces cytolytic killing functions as well as production of effector molecules (Perussia 1998; Niwa, Hatanaka et al. 2004; Bryceson, March et al. 2005). In preliminary experiments I have observed that rabies virus restimulation of post-vaccination PBMCs in the presence of autologous serum (i.e. containing anti-rabies antibodies) leads to even more florid NK cell responses (**Figure 5.13**).

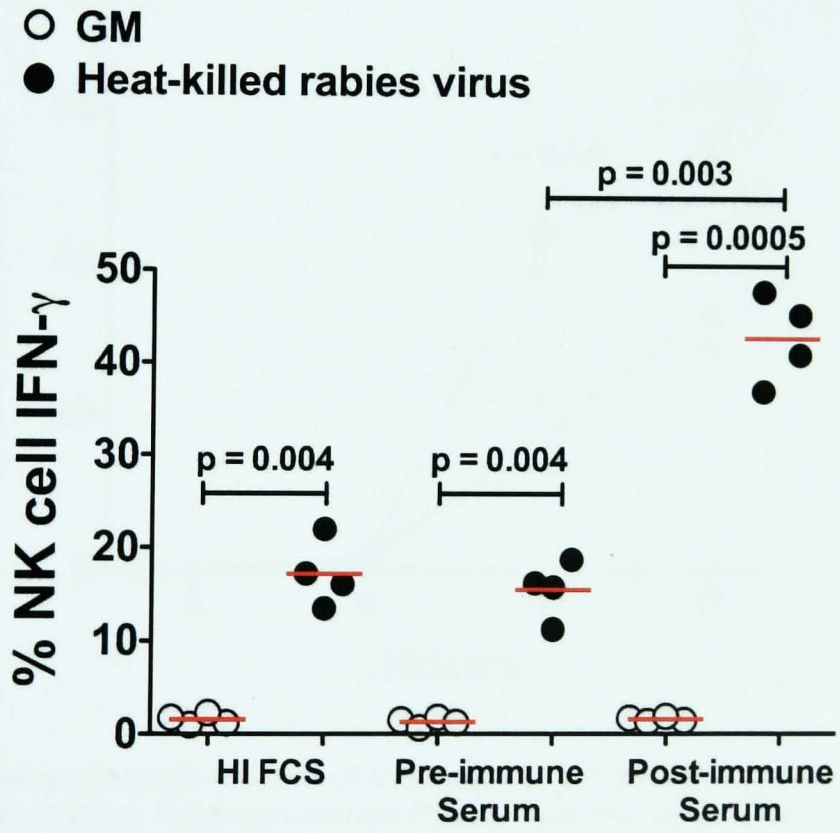


Figure 5.13 Immune serum enhances the recall NK cell response to rabies virus. Post-vaccination PBMC from 4 donors were restimulated *in vitro* with whole rabies virus (07/162), in the presence of heat-inactivated fetal calf serum (HI FCS), pre-immune or post-immune autologous serum. Data represent mean of 4 subjects. P values are derived from 2 tailed paired Wilcoxon test.

While rabies virus clearly induces activation of post-vaccination NK cell IFN-γ when cultured with pre-immune autologous serum or with HI FCS, this response is markedly enhanced when cells are cultured with the post-immune serum. It is not possible from these data to infer whether this enhanced state of NK cell activation is due to a synergistic effect of the IL-2/IL-12 + CD16 pathways or whether there are 2 independent pathways, where the enhanced NK cell response is a cumulative effect. I therefore compared the kinetics of the NK cell IFN-γ response in autologous rabies immune serum versus HI FCS (**Figure 5.14**).

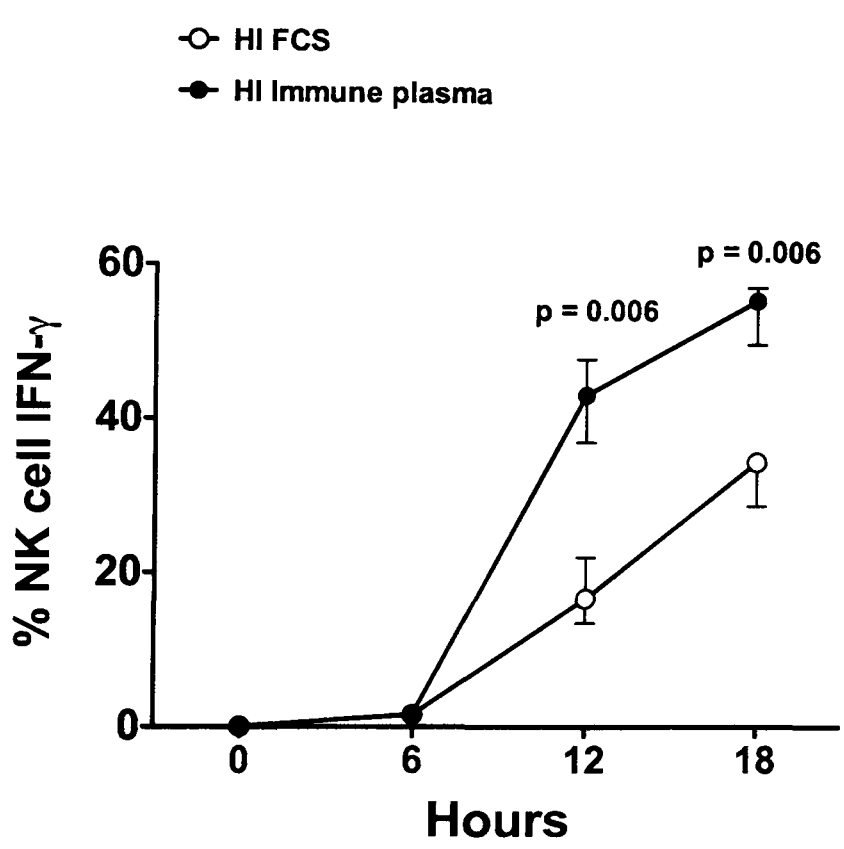


Figure 5.14 Measuring the effects of immune serum on NK cells in response to rabies virus over time. Post-vaccination PBMC from the same 4 donors as in Figure 5.13 were cultured with rabies virus in the presence of HI FCS or autologous post-vaccination serum for up to 18 hours. Data represent mean of 4 subjects. P values are derived from 2 tailed paired students t test.

As early as 6 hours post-incubation, there is a very noticeable difference between NK cell responses in post-immune serum and HI FCS. While the slope of the NK cell response when cultured with HI FCS seems to be quite linear, there is a much steeper slope for the post-immune serum suggesting that the pathways maybe synergistic rather than simply additive, but further work is required to test this.

5.3.8 *Pre-vaccination NK cells respond to rabies virus 07/162 when cultured with primed (post-vaccination) T cells*

Recent reports suggest that exposure to haptenated proteins, cytomegalovirus or cytokines can enhance the subsequent response of murine NK cells to reactivation by the same stimuli, raising the possibility that NK cells can acquire a "memory" phenotype (O'Leary, Goodarzi et al. 2006; Cooper, Elliott et al. 2009; Sun, Beilke et al. 2009). To determine whether the enhanced anti-viral response of post-vaccination NK cells results from such an "adaptive" response to prior antigen exposure, I compared the 07/162-induced IFN-γ, CD69 and LAMP-1 responses of pre- and post-vaccination PBMCs with the responses of pre-vaccination, CD3 T cell- depleted PBMC to which purified post-vaccination T cells had been added; as a control, CD3-depleted post-vaccination PBMCs were mixed with purified pre-vaccination T cells (**Figure 5.15**). I observed that pre-vaccination NK cells responded vigorously to inactivated rabies virus 07/162 when cultured together with post-vaccination T cells and the magnitude of the NK response was proportional to the number of T cells added to the culture. Conversely CD3-depleted post-vaccination NK cells cultured with naïve T cells were fully responsive to high dose IL-12/18 but were unable to respond to rabies virus. These data suggest that there are no intrinsic differences between pre-vaccination and post-vaccination NK cells and that post-vaccination NK cells are simply responding to the high levels of cytokines emanating from rabies virus-specific CD4+ T cells and myeloid accessory cells.

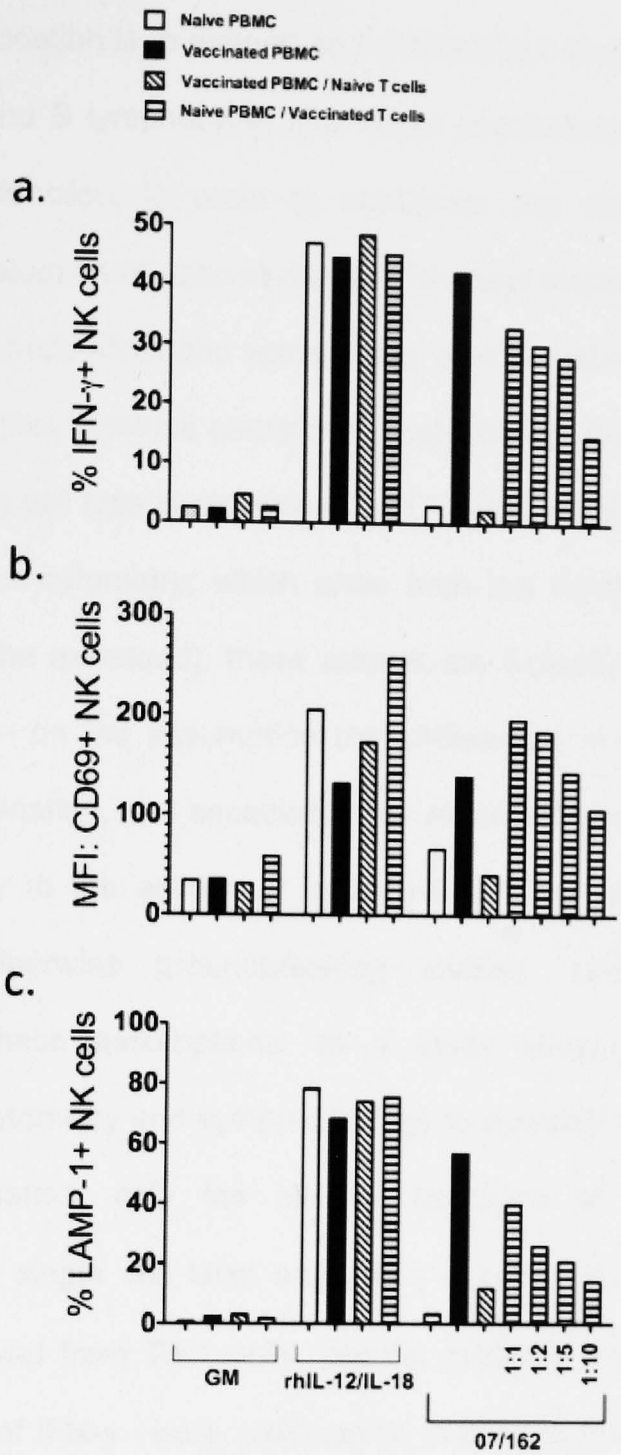


Figure 5.15 Pre-vaccination NK cells respond to rabies virus 07/162 when cultured with post-vaccination T cells. PBMC, collected either before (white bars) or after (black bars) vaccination, were cultured without stimulus (GM) or with rhIL-12 + rhIL-18, or with 07/162 for 24 hours and stained for intracellular IFN-γ (a); CD69 (b); and LAMP-1 (c) . Responses of whole PBMC were compared with those of CD3-depleted post-vaccination PBMC to which naïve (pre-vaccination) T cells had been added (diagonally cross-hatched bars) and with those of CD3-depleted pre-vaccination PBMC to which varying numbers of memory (post-vaccination) T cells had been added (horizontally cross-hatched bars); post-vaccination T cells were added to pre-vaccination PBMCs at ratios of 1 T cell per PBMC, or 1 T cell per 2, 5 or 10 PBMCs.

5.4 *Discussion*

The purpose of vaccination is to expand and differentiate small populations of naïve, antigen-specific T and B lymphocytes into larger populations of memory cells with enhanced effector function, in order to accelerate the clearance of pathogenic microorganisms. Evaluation of vaccine-induced immune responses typically includes measurement of antibody titers and some assay of cell-mediated immunity, such as lymphocyte proliferation, cytokine secretion or cytotoxicity. Although bulk assays (in which the responding cell type is not known) can now be supplemented by single cell assays (such as flow cytometry; which allow both the number and phenotype of responding cells to be assessed), these assays are typically designed – and their outputs interpreted – on the assumption that differences in cell-mediated effector responses post-vaccination, and especially after recall antigen stimulation of cells *in vitro*, are due solely to the actions of antigen-specific effector cells. Two recent examples, from otherwise groundbreaking studies, serve to illustrate the pervasiveness of these assumptions. In a study using functional genomics, polychromatic flow cytometry and systems biology to evaluate the global response to yellow fever vaccination, only the effector functions of $CD4^+$ T cells were characterized at the single cell level and IFN-γ secreted by PBMC cultures was assumed to be derived from Th-1 cells, despite evidence that NK cells – a well-documented source of IFN-γ - were proliferating in the first 7 days after vaccination (Gaucher, Therrien et al. 2008). A similar analysis of yellow fever vaccine-induced responses examined correlates only of $CD8^+$ T cell and antibody responses (Querec, Akondy et al. 2009).

Here I compare, for the first time, pre- and post-vaccination NK cell effector responses (IFN-γ production and exocytosis of cytotoxic granules), demonstrate that these responses are augmented in an antigen-specific manner by vaccination and demonstrate that NK cells contribute significantly to the post-vaccination response,

especially – but not exclusively – during the first hours and days after re-exposure to the vaccine antigen. Moreover, and again for the first time, I have elucidated the activation signals required for this post-vaccination NK cell "recall" response. My findings indicate that IL-2-mediated NK activation should be considered as an additional – and potentially very important – indicator of vaccine efficacy.

In many ways, my findings are not surprising. It has been known for several years that NK cells can represent a significant proportion of IFN-γ^+ cells in the initial stages of a classical recall response (Desombere, Clement et al. 2005), that IL-2 can augment NK cell responses (Malek 2008; Sutlu and Alici 2009), that the NK cell response to influenza virus depends upon IL-2 and T cells (He, Draghi et al. 2004), and that increased numbers of IFN-γ-producing NK cells can be detected after influenza vaccination (Long, Michaelsson et al. 2008). However, the logical conclusion of these observations – that antigen-specific IL-2 secretion from memory T cells may recruit NK cells as effectors of adaptive immunity and, thus, that NK cell responses can be potentiated by vaccination – has not previously been made explicit and has not been formally tested. Indeed, our collective fixation on NK cells as cells that can mediate effector function without prior sensitization (Trinchieri 1989; Moretta, Bottino et al. 2002) has blinded us to the notion that they may perform their effector functions even more effectively after sensitization.

By detailed analysis of the response to rabies vaccination, I have shown that antigen-specific, CD45RO$^+$ CD4$^+$ T cells secrete IL-2 within 6hrs of re-exposure to antigen and that this IL-2 – in combination with IL-12 and IL-18 induced by the interaction of whole virus with other accessory cells – activates NK cells to produce IFN-γ and to degranulate, releasing perforin. A proposed schematic for the NK recall response is shown in **Figure 5.16**.

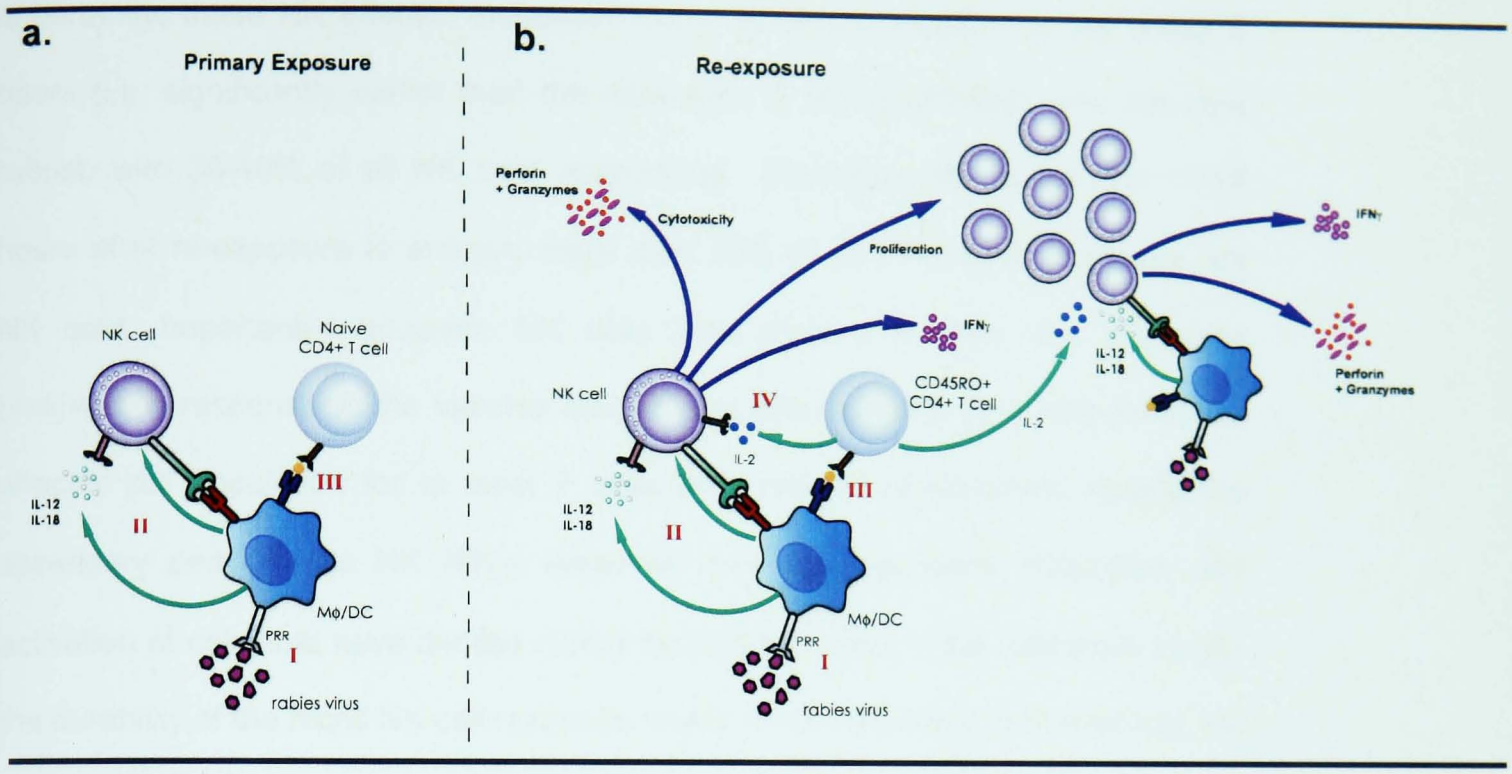


Figure 5.16: Schematic representation of suggested pathway for "recall" NK cell responses (a) On first exposure, pathogens bind to macrophages/DC pattern recognition receptors (PRR) (either on the cell surface or within various intracellular compartments) (I), leading to cytokine secretion and upregulation of co-stimulatory molecules (II). Pathogen uptake and peptide presentation by DCs will prime naïve T cells (III). However, in the absence of a source of IL-2, NK cells will not become activated. (b) On re-exposure to the same pathogen, PRR binding, cytokine secretion and peptide antigen presentation will again take place (I, II, III). In addition, IL-2 from primed effector T cells (IV) provides signals, which allow NK cells to secrete cytokines, become cytotoxic and proliferate. In the presence of ongoing antigen presentation, a second wave of activation may provide for a sustained NK cell response.

Importantly, these NK effector responses occur extremely rapidly, starting within 6 hours (i.e. significantly earlier than the equivalent T cell responses), and are very robust, with 30-40% of all NK cells responding. Consequently, in the first 12-18 hours after re-exposure to antigen, more than 70% of all IFN-γ-producing cells are NK cells. Importantly, however, NK cells also show a marked and prolonged proliferative response to the vaccine antigen and they continue to contribute to the effector cell population for at least 7 days after antigen re-exposure; indeed the secondary peak in the NK IFN-γ response may well represent maturation and activation of cells that have divided during the first few days of the response. Lastly, the durability of the recall NK cell response needs to be thoroughly characterized, but I have repeated this analysis, in 1 donor, more than 4 months after the last vaccination without any noticeable decline in the response (*data not shown*).

My finding that, in the absence of T cells, rIL-2 is sufficient to restore NK recall responses indicates that IL-2 is the only T cell-derived signal that is essential for the NK recall response. In support of this scenario, I have shown that although T cells are required for optimal activation of NK cells by malaria-infected red blood cells there is no requirement for NK cell-T cell contact and that the T cell-dependent signal can be delivered to the NK cells via a semi-permeable membrane (Horowitz, Newman et al. 2010). My data therefore reveal an important new role for vaccine-induced IL-2-secreting memory T cells and may, in part, explain the emerging consensus that polyfunctional T cells, which secrete IL-2 in addition to IFN-γ or TNF-α, are associated with positive outcomes of viral infection (Ciuffreda, Comte et al. 2008; Nebbia, Mattes et al. 2008; Ferre, Hunt et al. 2009) and with particularly effective vaccination regimes (Harari, Dutoit et al. 2006; Aagaard, Hoang et al. 2009).

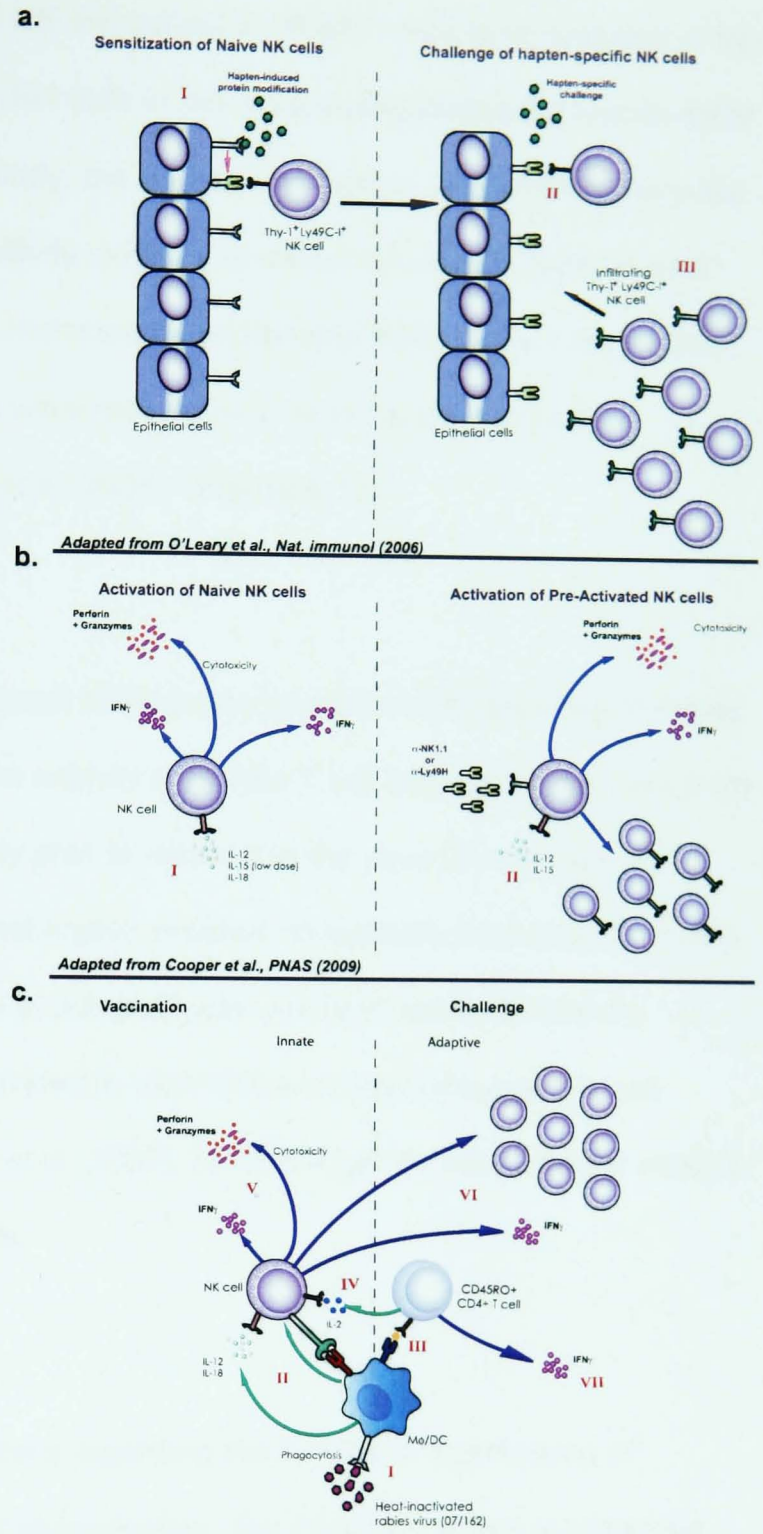
It was noticeable that whichever parameter I assayed (CD69 upregulation, IFN-γ production, degranulation) the NK cell response to rabies vaccination among the vaccinees was remarkably homogeneous. Overall, for different parts of the study, I assayed responses from 30 individuals and in every case there was a robust and persistent NK cell recall response. This is in marked contrast to our previous findings for NK cell responses to malaria-infected red blood cells, *Mycobacterium bovis* BCG and bacterial lipopolysaccharide where NK IFN-γ responses are extremely heterogeneous, but similar (in homogeneity, if not magnitude) to responses we observed to high dose rhIL-12+IL-18 (Korbel, Norman et al. 2009). We have proposed that heterogeneity in NK cell IFN-γ responses to pathogens reflects both differences in the strength of accessory stimuli and variable expression of polymorphic NK cell receptors (which fine-tune the degree of activation) (Korbel, Norman et al. 2009). The results of this vaccination study suggest that if the accessory cell stimulus is sufficiently strong (which may require synergism of signals from myeloid cells and T cells) then the effect of NK cell regulatory receptors may be overcome. If so, genetic diversity in NK cell regulatory receptors may not represent a major hurdle to effective vaccination.

The indirect route of NK cell activation has largely been overlooked until recently but it is now clear that inflammatory cytokines (IL-12, IL-18, IFN-α) and co-stimulatory signals from myeloid accessory cells are essential for optimal NK cell responsiveness to a wide range of viral, bacterial and protozoal infections (Newman and Riley 2007). My finding that intact rabies virus, but not purified recombinant protein, was able to activate NK cells after vaccination in an IL-12- and IL-18-dependent manner, is consistent with a requirement for myeloid accessory cell-derived as well as T cell-derived signals for induction of recall NK responses. Neither the rabies virus encoded ligands for pattern recognition receptors (PRR) nor the PRRs themselves are known.

Although TLR3 is upregulated in brains of RV infected mice (McKimmie, Johnson et al. 2005; Mansfield, Johnson et al. 2008) and humans (Jackson, Rossiter et al. 2006), TLR3 preferentially recognises dsRNA rather than ssRNA and is thus not an obvious candidate for recognition of RV. Human TLR8/murine TLR7 are receptors for some ssRNAs (Diebold, Kaisho et al. 2004; Heil, Hemmi et al. 2004) and may thus be more likely innate receptors for RV. One practical implication of the need for myeloid acessory cell stimuli for induction of recall NK responses is that evaluation of vaccine-induced immune responses by restimulation of PBMCs with purified protein antigens or synthetic peptides may not reveal the full extent of the NK recall response that may occur after exposure to whole pathogens.

Although there are superficial similarities between the "recall" NK response that I have described and the "memory-like" NK cells recently described in mice (O'Leary, Goodarzi et al. 2006; Cooper, Elliott et al. 2009; Sun, Beilke et al. 2009), there are important differences between the cytokine-driven response described here and some of the mouse studies. In mice infected with murine cytomegalovirus (MCMV), NK cells proliferate, persist at higher than normal frequencies for several months and show enhanced cytokine and degranulation responses on reactivation (Sun, Beilke et al. 2009) however NK cell activation in this model is driven by binding of the activating NK Ly49H receptor to the m157 viral protein expressed on MCMV-infected cells (Arase, Mocarski et al. 2002) and may thus occur independently of accessory cell stimuli.

Figure 5.17 Schematic representation of proposed interactions between NK cells and T cells after vaccination.



(**a-I**) Contact hypersensitivity model for sensitization of Thy-1^{+}Ly49C-I^{+} NK cells by hapten-specific challenge. (**a-II**) Upon re-treatment of tissues with same hapten, NK cells bearing the same hapten-specific Thy-1^{+}Ly49C-I^{+} phenotype undergo rapid expansion and infiltrate to the site of challenge. (**b-I**) Activation of naïve NK cells with IL-12, low-dose IL-15 and IL-18 triggers robust NK cell IFN-γ production. (**b-II**) After 7 days post-transfer of pre-activated NK cells into *Rag1*$^{-/-}$ mice, activation of NK cells with IL-12 and IL-15 or by using plate bound antibodies targeting Ly49H or NK1.1 activating receptors, a significantly enhanced NK cell response is observed as well as sustained. (**c-I**) Prior sensitization of naïve NK cells by route of vaccination with heat-killed rabies virus is phagocytosed by antigen presenting cells and processed intracellularly. (**c-II**) Viral ligands binding to TLR3 and TLR9 lead to production of IL-12 and IL-18 as well as direct contact between NK cells and APC. (**c-III**) Peptide antigen is presented by MHC class II molecules on the surface of the plasma membrane and recognised by antigen-specific memory CD4+ T cells causing them (**c-IV**) to secrete IL-2. (c-**V**) Production of IL-2, IL-12 and IL-18 seem to have a synergistic effect leading to effective NK cell IFN-γ production as well as degranulation responses. (c-**VI**) NK cell activation as defined by IFN-γ prodction, degranulation, and robust proliferation is also sustained well after the adaptive T cell response is initiated. (c-**VII**) NK cell derived IFN-γ may promote differentiation of CD4+ T cells from Th0 to Th1 effectors.

Indeed, expansion of the Ly49H$^+$ NK cell subset in MCMV$^+$ mice is reminiscent of the expanded population of NKG2C$^+$ NK cells in individuals seropositive for human MCV (Guma, Angulo et al. 2004). Similarly, the original description of murine memory-like NK cells - in a contact hypersensitivity model – specifically involved Ly49C$^+$/Ly49I$^+$ cells which might conceivably be activated by haptenated MHC Class 1 molecules and, at least inasmuch as T cells were not required, would appear to be IL-2 independent (O'Leary, Goodarzi et al. 2006) (**Figure 5.17**).

Importantly, the "memory" component of the post-vaccination NK response to rabies virus described here appears to lie entirely within the T cell population: NK cells from unvaccinated individuals were fully able to respond to the virus when mixed with autologous memory T cells. It is not known whether nonspecific inflammatory stimuli can maintain human NK cells in a prolonged hyper-reactive state, as recently described for murine NK cells activated *in vitro* with a cocktail of accessory cell-derived cytokines (Cooper, Elliott et al. 2009), but this might be interesting to explore in the context of vaccine adjuvants.

Our study raises interesting questions regarding the functional significance of enhanced NK cell responses after immunisation. For infections where a protective role for NK cells is established, evaluation of NK responses post-vaccination is likely to be a useful indicator of vaccine efficacy but for other infections the implications are less clear and further studies are required. In the case of rabies virus vaccination it is widely accepted that neutralising antibody is essential for protection (Johnson, Cunningham et al. 2010), but experimental infections in mice suggest that cell-mediated immune responses (including signalling through the IFN-γR) are required for efficient viral clearance (Hooper, Morimoto et al. 1998) and that pro-inflammatory

cytokines (IFN-γ and IL-2) enhance vaccine immunogenicity, leading to significantly higher neutralising antibody titres (Claassen, Osterhaus et al. 1998). The potential for NK cell IFN-γ responses to contribute to the efficacy of rabies vaccines thus deserves to be evaluated. The role of the degranulation response is less obvious. In these particular experiments, using killed virus, it is unlikely that NK cells are degranulating in response to infected cells and degranulation may simply be a marker of NK cell activation.

In summary, I have demonstrated that NK cells are major contributors to the effector lymphocyte population during the recall response to rabies vaccination. This should lead us to reconsider the precise roles of antigen-specific memory T cells in vaccine-induced immunity. Assays of $CD4^+$ T cell IL-2 production, NK cell IFN-γ production and NK cytotoxicity need to be included in the arsenal of tools for evaluating correlates of vaccine-induced immunity.

Chapter 6: Final Discussion

6.1 *Study significance*

The conventional view of acquired immunity to infection is that it is mediated by numerous, small populations of effector and memory B and T lymphocytes expressing antigen receptors with high affinity for the particular pathogen. Upon re-exposure to an antigen, these populations undergo rapid clonal expansion. Antigen-specific CD4+ T helper cells augment B cell, CD8+ T cell and macrophage-mediated effector functions. These interactions and their downstream responses, i.e. the production,of antibodies, cytotoxic T lymphocyte (CTL) killing and phagocytosis, form the basis for evaluation of vaccine-induced immunity.

Vaccination is the most sustainable and cost-effective way to reduce the global burden of many infectious diseases. New vaccines are urgently needed for complex infections such as malaria, HIV and TB. Rational design of such vaccines might be informed by comparison with existing effective vaccines but these were, in the main, developed empirically and we have a very incomplete understanding of how they work. Expensive and time-consuming clinical trials remain the only way to evaluate vaccines for most human diseases; correlates of protection – which might allow triage of candidate vaccines – are lacking in most cases. Recently, CD4+ T cell-derived IL-2 has emerged as a potential correlate of protection in several disease and vaccine studies (Harari, Dutoit et al. 2006; Beveridge, Price et al. 2007; Darrah, Patel et al. 2007; Ciuffreda, Comte et al. 2008; Kamath, Rochat et al. 2008; Nebbia, Mattes et al. 2008; Aagaard, Hoang et al. 2009; Barbosa, Naniche et al. 2009; Ferre, Hunt et al. 2009; Lindenstrom, Agger et al. 2009).

In this thesis, I have investigated an additional, little-appreciated but potentially very important, function of CD4+ T cells, namely their ability to activate NK cells in an IL-2-dependent manner. I have thoroughly characterised human NK cell responses to *Plasmodium falciparum* malaria (*Pf*-RBC) (**Chapter 3** and **Chapter 4**), *Mycobacterium bovis* BCG (BCG) (**Chapter 3**) and rabies virus (**Chapter 5**). My data demonstrate that antigen-specific CD4+ T cell-derived IL-2 is an essential component of the cocktail of cytokines required for the activation of NK cells to secrete IFN-γ and release cytotoxic granules, and that vaccination-primed effector/memory CD4+ T cells lead to rapid and potent activation of NK cells upon re-exposure to the pathogen. I hypothesize that IL-2-driven NK cell effector functions may contribute to containment and elimination of certain pathogens in vaccinated individuals. If this holds true, IL-2-driven NK cell activation may represent a novel correlate of vaccine-induced immunity and designing vaccines to optimize this response may enhance their efficacy. Furthermore, NK cell recall responses may provide a new tool for rapid evaluation of vaccine efficacy, screening of new antigens and adjuvants, optimising dosing schedules and determining duration of protection. Where appropriate, vaccines could be designed to induce NK cell responses.

NK cells are classically thought of as innate immune effectors that, by cytokine production or cytotoxicity, help to contain an infection (or limit tumour growth) until an effective adaptive response is mounted. NK cells become activated when the balance of activating and inhibitory signals that they receive is disturbed (Newman and Riley 2007). Direct NK cell activation follows interaction with transformed or infected cells, which lack ligands for inhibitory NK cell surface receptors for self-MHC (the "missing self" phenomenon) and/or express stress-induced ligands for NK cell activating receptors. Indirect NK cell activation occurs following microbial ligation of pattern recognition receptors on myeloid accessory cells and is mediated by

cytokines (IL-12, IL-18, IFN-α) and by contact—dependent stimuli from myeloid cells (e.g. ICAM-1/LFA-1). Whether NK cell regulatory functions, such as secretion of IL-10 (Maroof, Beattie et al. 2008), are induced in a similar manner remains to be determined. Recently, it has become clear that bi-directional interactions between NK cells and T cells are also vital for the activation of NK cells (He, Draghi et al. 2004; Newman, Korbel et al. 2006). IL-2 is produced predominantly from effector memory CD4+ T cells and is secreted very early in the secondary response to antigens.

The quality of the antigen-specific CD4+ T cell response after vaccination is increasingly recognized as being as important as, or perhaps even more important than the size of this response. Specifically, induction of multifunctional CD4+ T cells secreting several cytokines (IFN-γ, TNF-α, IL-2) and/or expressing ligands for co-stimulatory receptors (e.g. CD154/CD40L) correlates with protection in murine vaccination models of, for example, leishmaniasis (Darrah, Patel et al. 2007) and tuberculosis (Forbes, Sander et al. 2008) and in human malaria vaccine trials (Roestenberg, McCall et al. 2009). Retention of secretory IL-2 is assumed to endow IFN-γ/TNF-secreting effector CD4+ T cells with long-term memory potential (Darrah, Hegde et al. 2010), but the possibility that IL-2 is an effector molecule in its ownright is rarely considered (Rochman, Spolski et al. 2009).

6.2 *Key findings and future perspectives*

(i) *Malaria*

My research, presented in Chapter 3 of this thesis, has indicated that T cell-derived IL-2 is an essential component of the cocktail of cytokines that activates NK cells to

secrete IFN-γ and release cytotoxic granules upon exposure to *Pf*-RBC. Of note, a weak but significant association between the proportion of CD4+ T cells making IL-2 and protection against challenge infection in malaria-vaccinated humans has been reported and was accompanied by expansion of the circulating NK cell pool and increased NK cell perforin expression (Berthoud, Fletcher et al. 2009). These observations suggest that T cell-derived IL-2 may contribute to the immediate effector response by inducing NK cell-mediated lysis/apoptosis of infected cells or by inducing NK cells to secrete IFN-γ and thereby enhancing phagocytosis and destruction of malaria-infected red blood cells.

Our group has been characterizing the pathways of NK cell activation by *Pf*-RBC for many years now, and our investigations have revealed essential roles for IL-12 and accessory cell contact as well as necessary roles for IL-18 and IFN-α (Artavanis-Tsakonas, Eleme et al. 2003; Korbel, Newman et al. 2005; Newman, Korbel et al. 2006). These studies also demonstrated that IL-2 was necessary for optimal activation of NK cells in response to *Pf*-RBC (Newman, Korbel et al. 2006) and although the data demonstrated that myeloid accessory cells were also crucial for the NK cell response, our group was never able to recapitulate the NK cell response in mixed PBMC by simple addition of myeloid cells and purified NK cells. In this thesis, I have now demonstrated that NK cell responses (CD69 and CD25 expression, proliferation, secretion of IFN-γ, mobilization of cytolytic granules and release of perforin/granzyme) to *Pf-RBC* and to *Mycobacterium bovis* BCG are crucially dependent on IL-2 secreted (in an MHC class II-dependent manner) by CD4+ T cells, indicating that the "innate" response to malaria infection actually relies upon complex interactions between NK cells, antigen-specific T cells and accessory cells (**Figure 5.16**). In the case of *Pf*-RBC, antigen recognition is thought to be mediated by TLR pattern recognition. Specifically, ligands for myeloid accessory cells include

glycosylphosphatidylinositols (GPI) derived from the glycolipid anchors of parasite surface proteins which signal through TLR2 (Gowda 2007) and parasite DNA trapped within hemozoin (the insoluble breakdown product of haemoglobin) which is carried into the cytosol and binds to TLR-9 (Parroche, Lauw et al. 2007). In malaria naïve donors (such as those used throughout **Chapter 3** of this thesis) CD4+ T cells are believed to recognise *Pf*-derived peptides, which cross-react with those on commonly encountered micro-organisms, vaccines or environmental antigens (Currier, Beck et al. 1995).

These observations with *Pf*-RBC, bolstered by reports of T cell and IL-2-dependent activation of human NK cells (Fehniger, Cooper et al. 2003; He, Draghi et al. 2004) and of an increase in the frequency of IFN-γ-producing NK cells among restimulated PBMCs after influenza vaccination (Long, Michaelsson et al. 2008) led us to speculate that IL-2 from antigen-specific T cells might allow NK cells to contribute to the effector arm of adaptive immune responses and thus that NK cells may make a significant contribution to the cellular effector response to vaccination. Our first evidence to support this hypthesis comes from a phase IIb clinical trial of the RTS,S malaria vaccine in children (**Chapter 4**). RTS,S comprises a chimaeric recombinant protein [malaria circumsporozoite surface protein (CS) fused to hepatitis B surface antigen (HBs)] formulated with excess free HBs and adjuvant (Casares and Brumeanu 2010). PBMC isolated 4.5 months after vaccination from RTS,S vaccinated children and from controls (who received rabies virus vaccine instead of RTS,S) were restimulated for 24 hours with CS or HBs and analyzed by flow cytometry for NK cell activation (CD69 expression and IFN-γ production) and T cell activation (CD69 and CD25 expression, IFN-γ production and secretion of IL-2). HBs-induced IL-2 secretion, NK cell CD69 expression and IFN-γ production were all significantly higher among RTS,S vaccinated children than in controls and similar results were obtained for responses to CS. Importantly, NK cell responses were

significantly correlated with secreted IL-2, and after 24 hour stimulation, >50% of all IFN-γ-secreting cells were NK cells.

Whilst my data presented in this thesis demonstrate very clearly that NK cells can respond to malaria-infected red blood cells as well as malarial antigens, *in vitro*, the next steps will be to determine if NK cells actually make any significant contribution to malaria-induced immunopathology or protection from developing clinical disease, *in vivo*, using experimental malaria infections. In order to do so, more detailed studies in mouse models of malaria infections are needed. Specifically,

- To investigate the role of NK cells in experimental cerebral malaria using the *P. berghei* ANKA model in C57/BL6 mice;

- To investigate protective immune mechanisms and non-cerebral forms of malaria using the *P. yoellii* model in C57/BL6 mice;

- To investigate potential roles for NK cells in prevention of clinical disease using novel in vivo NK cell depletion techniques comparing the use of new depletion antibodies (targeting NKp46) (Walzer and Blery 2007) and "E4BP4"-NK cell deficient mice (Gascoyne, Long et al. 2009) or NKG2D-deficient mice (Guerra, Tan et al. 2008).

(ii) *Vaccination*

Whilst highly supportive of my hypothesis, the cross-sectional RTS,S data do not formally prove that vaccination can induce potent NK cell effector responses in that we did not have access to cells collected prior to vaccination. Thus, as a direct test of the hypothesis, I compared NK cell responses to inactivated rabies virus before and after rabies virus vaccination (**Chapter 5**) (Horowitz, Behrens et al. 2010). Inapparent

exposure to rabies virus is extremely rare and our volunteers were thus expected to be fully naïve prior to vaccination. Prior to vaccination, NK cells were unresponsive to inactivated rabies virus whereas potent NK cell responses were observed 21 days after first dose (14 days after the 2nd dose). The NK cell response was completely dependent upon IL-2 from antigen-specific memory (CD45RO+) CD4+ T cells and did not require differentiation of NK cells to a "memory" phenotype (as described for mouse NK cells (Sun, Beilke et al. 2009)) since pre-vaccination NK cells responded as efficiently as post-vaccination NK cells when co-cultured with post-vaccination/primed CD4+ T cells (Horowitz, Behrens et al. 2010). Importantly, in the first 12-18 hours of the recall response, >70% of all IFN-γ-secreting cells were NK cells. However, extensive NK cell proliferation and IFN-γ secretion continued for at least 7 days, suggesting that NK cells make a major contribution to both immediate and sustained effector responses.

Whilst IL-2 is clearly essential for these "recall" responses, there do seem to be some differences between the different pathogens in terms of the other stimuli that are required and ultimately the magnitude of the downstream NK cell response. For *Pf*-RBC response, IL-12 is also essential and IL-18 and IFN-α are beneficial (Artavanis-Tsakonas and Riley 2002; Artavanis-Tsakonas, Eleme et al. 2003; Newman, Korbel et al. 2006). For rabies virus, whole (inactivated) virus induced NK cell activation but recombinant ribonucleocapsid protein antigen did not (Horowitz, Behrens et al. 2010) and the anti-viral response was blocked by anti-IL-12 and anti-IL18 antibodies. In RTS,S recipients, CS peptide antigens were able to induce partial activation of NK cells (CD69 was significantly upregulated but IFN-γ production was not) but HBs peptides induced very potent NK cell responses. More detailed analyses focusing more specifically on antigen recognition/processing by accessory cells and how

these synergize with T cell-derived IL-2 to trigger NK cell activation will need to be investigated before we can accurately define appropriate vaccine design strategies.

It will be necessary to explore the potential for NK cells to contribute to the effector phase of adaptive (acquired) immune responses to infection following vaccination and to define the pathways by which this occurs. This needs to be tested in the setting of a large and very well defined vaccine trial. Specifically, it will be very interesting to test:

- If NK cell "recall" responses are naturally induced by infection and whether these responses can be recapitulated by vaccination while comparing different types of vaccines (i.e. live attenuated, inactivated, recombinant protein or polysaccharide vaccines) as well as if different vaccine delivery systems with and without the use of adjuvants may augment these responses;

- If the post-vaccination NK cell "recall" responses depend upon, and correlate with, CD4+ T cell IL-2 responses;

- If the durability of the NK cell "recall" responses is linked to the durability of the CD4+ T cell IL-2 responses and if these responses are boosted by infection/vaccination;

- If the quality of the NK cell "recall" responses, induced by vaccination, is age-dependent at the time of immunisation;

- If the NK cell response differs both phenotypically and functionally during primary and secondary "recall" responses, following infection/vaccination;

- And if the NK cell "recall" responses may contribute to protective immunity following vaccination by secretion of IFN-γ (enhancing macrophage activity), cytotoxicity, and/or by direct induction of apoptosis of infected cells.

All of the assays presented in this thesis were performed using heat-inactivated fetal calf serum (HI-FCS) in the culture in lieu of autologous serum. Protective responses to rabies virus infection have largely been associated with the presence of neutralising antibodies (specific isotypes remain unknown). Strikingly, when comparing the use of autologous immune serum with HI-FCS in the cultures, the NK cell response was dramatically enhanced both in the speed as well as in the magnitude of their response to inactivated rabies virus. In many ways, this observation was not completely surprising. Celis *et al.* first demonstrated that antigen-antibody immune complexes (specifically anti-rabies IgG with rabies vaccine as a recall antigen) induced enhanced proliferation in culture (Celis and Wiktor 1985). This study predates the implementation of multi-colour flow cytomtery techniques to identify specific populations of proliferating lymphocytes but the authors – understandably - concluded that this proliferation was due exclusively to T cells as they were using T cell lines. However, the cell lines were generated by culturing PBMC from vaccinated donors with rabies vaccine (inactivated virus) and recombinant IL-2 for 1 week and then further expanding antigen-reactive cells by limiting dilution assays and stimulating with irradiated PBMC as a source of antigen. Based on my observations, NK cells were highly responsive to rabies vaccine as a recall antigen and proliferated in a far more robust manner than either CD4+ or CD8+ T cells; it is quite likely that NK cells were also responding in these assays.

Based on my observations, one hypothesis that needs to be tested in the presence of immune serum could be that immune complex recognition through CD16 lowers the threshold for NK cell activation. In other words, there are two potential pathways by which NK cell responses can be potentiated by vaccination: IL-2/CD4+ T cell-mediated and immune complex/CD16-mediated. This hypothesis of CD16-mediated activation of NK cells could be tested alongside the characterisation of IL-2/CD4+ T cells in a vaccine study setting. Specifically, it will be very interesting to test:

- If post-vaccination NK cell "recall" responses are further augmented by vaccine antigen-specific antibodies targeting CD16 by way of antigen-antibody immune complexes.

- If the durability of the NK cell "recall" responses is linked to the durability of serum antibodies as well as if these responses can also be boosted by infection/vaccination.

6.3 *Concluding remarks*

It is increasingly clear over the last 5 years that the dichotomy drawn between innate and adaptive immune responses to antigen has become skewed. Interactions between conventionally defined innate cells and adaptive "recall" immune responses seem to be very different to the interactions between these two arms of the immune response during primary responses to the same pathogens.

My observations with protozoan (*Pf*-RBC), bacterial (BCG) and viral (rabies) pathogens, together with the observations of others on influenza virus (He, Draghi et al. 2004; Long, Michaelsson et al. 2008), suggest that NK cell activation is augmented as a consequence of T cell/IL-2 responses to infection. Moreover, I have validated (in collaboration with Dr. Anthony Fooks, rabies and wildlife zoonoses

group, VLA) the rabies vaccination model in mice (unpublished), observing very similar NK cell "recall" responses as in humans and essentially identical observations have very recently been reported for interactions between murine NK cells and T cells and the protozoan parasite *Leishmania major* (Bihl, Pecheur et al. 2010), indicating that this pathway of NK cell activation is conserved between humans and mice.

I hypothesize, therefore, that an important function of effector/memory CD4+ T cells may be to secrete IL-2, activating NK cells to secrete cytokines and become cytolytic. The size, speed and duration of this "recall" NK cell response suggests that it may represent a critical component of the immediate and the sustained effector response to reinfection or infection post-vaccination. However, despite interest in the phenotypic characteristics of "memory" NK cells (Sun, Beilke et al. 2009), the functional importance of NK cells during secondary immune responses is not known. Testing the hypothesis that T cell/IL-2-driven NK cell responses contribute to post-vaccination immunity and protection from development of disease will certainly warrant further investigation in a larger setting as well as by comparing numerous types of vaccines, i.e. whole organism versus DNA sub-unit as well as clinically effective vaccines versus vaccines which have not been shown to elicit protection from disease (e.g. HBV versus HIV).

References

Aagaard, C., T. T. Hoang, et al. (2009). "Protection and polyfunctional T cells induced by Ag85B-TB10.4/IC31 against Mycobacterium tuberculosis is highly dependent on the antigen dose." PLoS One **4**(6): e5930.

Ahlenstiel, G., M. P. Martin, et al. (2008). "Distinct KIR/HLA compound genotypes affect the kinetics of human antiviral natural killer cell responses." J Clin Invest **118**(3): 1017-1026.

Akuffo, H., A. Alexis, et al. (1999). "Natural killer cells in cross-regulation of IL-12 by IL-10 in Leishmania antigen-stimulated blood donor cells." Clin Exp Immunol **117**(3): 529-534.

Amadei, B., S. Urbani, et al. (2010). "Activation of natural killer cells during acute infection with hepatitis C virus." Gastroenterology **138**(4): 1536-1545.

Amino, R., D. Giovannini, et al. (2008). "Host cell traversal is important for progression of the malaria parasite through the dermis to the liver." Cell Host Microbe **3**(2): 88-96.

Aponte, J. J., D. Schellenberg, et al. (2009). "Efficacy and safety of intermittent preventive treatment with sulfadoxine-pyrimethamine for malaria in African infants: a pooled analysis of six randomised, placebo-controlled trials." Lancet **374**(9700): 1533-1542.

Arase, H., E. S. Mocarski, et al. (2002). "Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors." Science **296**(5571): 1323-1326.

Arina, A., O. Murillo, et al. (2007). "The combined actions of NK and T lymphocytes are necessary to reject an EGFP+ mesenchymal tumor through mechanisms dependent on NKG2D and IFN gamma." Int J Cancer **121**(6): 1282-1295.

Artavanis-Tsakonas, K., K. Eleme, et al. (2003). "Activation of a subset of human NK cells upon contact with Plasmodium falciparum-infected erythrocytes." J Immunol **171**(10): 5396-5405.

Artavanis-Tsakonas, K. and E. M. Riley (2002). "Innate immune response to malaria: rapid induction of IFN-gamma from human NK cells by live Plasmodium falciparum-infected erythrocytes." J Immunol **169**(6): 2956-2963.

Artavanis-Tsakonas, K., J. E. Tongren, et al. (2003). "The war between the malaria parasite and the immune system: immunity, immunoregulation and immunopathology." Clin Exp Immunol **133**(2): 145-152.

Baer, K., M. Roosevelt, et al. (2007). "Kupffer cells are obligatory for Plasmodium yoelii sporozoite infection of the liver." Cell Microbiol **9**(2): 397-412.

Ballou, W. R. (2009). "The development of the RTS,S malaria vaccine candidate: challenges and lessons." Parasite Immunol **31**(9): 492-500.

Baratin, M., S. Roetynck, et al. (2007). "Dissection of the role of PfEMP1 and ICAM-1 in the sensing of plasmodium falciparum-infected erythrocytes by natural killer cells." PLoS One **2**(2): e228.

Barbosa, A., D. Naniche, et al. (2009). "Plasmodium falciparum-specific cellular immune responses after immunization with the RTS,S/AS02D candidate malaria vaccine in infants living in an area of high endemicity in Mozambique." Infect Immun **77**(10): 4502-4509.

Barnes, K. I., D. N. Durrheim, et al. (2005). "Effect of artemether-lumefantrine policy and improved vector control on malaria burden in KwaZulu-Natal, South Africa." PLoS Med **2**(11): e330.

Behr, C., R. Poupot, et al. (1996). "Plasmodium falciparum stimuli for human gammadelta T cells are related to phosphorylated antigens of mycobacteria." Infect Immun **64**(8): 2892-2896.

Bejon, P., J. Lusingu, et al. (2008). "Efficacy of RTS,S/AS01E vaccine against malaria in children 5 to 17 months of age." N Engl J Med **359**(24): 2521-2532.
Berg, R. E., E. Crossley, et al. (2005). "Relative contributions of NK and CD8 T cells to IFN-gamma mediated innate immune protection against Listeria monocytogenes." J Immunol **175**(3): 1751-1757.
Berthoud, T. K., H. Fletcher, et al. (2009). "Comparing human T cell and NK cell responses in viral-based malaria vaccine trials." Vaccine **28**(1): 21-27.
Beutler, B. and E. T. Rietschel (2003). "Innate immune sensing and its roots: the story of endotoxin." Nat Rev Immunol **3**(2): 169-176.
Beveridge, N. E., D. A. Price, et al. (2007). "Immunisation with BCG and recombinant MVA85A induces long-lasting, polyfunctional Mycobacterium tuberculosis-specific CD4+ memory T lymphocyte populations." Eur J Immunol **37**(11): 3089-3100.
Bhattarai, A., A. S. Ali, et al. (2007). "Impact of artemisinin-based combination therapy and insecticide-treated nets on malaria burden in Zanzibar." PLoS Med **4**(11): e309.
Bihl, F., J. Pecheur, et al. (2010). "Primed antigen-specific CD4+ T cells are required for NK cell activation in vivo upon Leishmania major infection." J Immunol **185**(4): 2174-2181.
Biron, C. A., K. B. Nguyen, et al. (1999). "Natural killer cells in antiviral defense: function and regulation by innate cytokines." Annu Rev Immunol **17**: 189-220.
Breman, J. G. (2001). "The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden." Am J Trop Med Hyg **64**(1-2 Suppl): 1-11.
Bryceson, Y. T., M. E. March, et al. (2005). "Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells." J Exp Med **202**(7): 1001-1012.
Carding, S. R. and P. J. Egan (2002). "Gammadelta T cells: functional plasticity and heterogeneity." Nat Rev Immunol **2**(5): 336-345.
Carter, J. A., A. J. Ross, et al. (2005). "Developmental impairments following severe falciparum malaria in children." Trop Med Int Health **10**(1): 3-10.
Casares, S. and T. D. Brumeanu (2010). "The RTS,S malaria vaccine." Vaccine **28**(31): 4880-4894.
CDC (2006). "Malaria Life Cycle."
CDC (2008). "Rabies vaccines licensed and marketed in the United States, 2008."
Ceesay, S. J., C. Casals-Pascual, et al. (2008). "Changes in malaria indices between 1999 and 2007 in The Gambia: a retrospective analysis." Lancet **372**(9649): 1545-1554.
Celis, E. and T. J. Wiktor (1985). "Amplification of rabies virus-induced stimulation of human T cell lines and clones by antigen-specific antibodies." J Virol **56**(2): 426-433.
Cenna, J., G. S. Tan, et al. (2008). "Immune modulating effect by a phosphoprotein-deleted rabies virus vaccine vector expressing two copies of the rabies virus glycoprotein gene." Vaccine **26**(50): 6405-6414.
Chakravarty, S., I. A. Cockburn, et al. (2007). "CD8+ T lymphocytes protective against malaria liver stages are primed in skin-draining lymph nodes." Nat Med **13**(9): 1035-1041.
Chavez-Galan, L., M. C. Arenas-Del Angel, et al. (2009). "Cell death mechanisms induced by cytotoxic lymphocytes." Cell Mol Immunol **6**(1): 15-25.
Ciuffreda, D., D. Comte, et al. (2008). "Polyfunctional HCV-specific T-cell responses are associated with effective control of HCV replication." Eur J Immunol **38**(10): 2665-2677.
Claassen, I. J., A. D. Osterhaus, et al. (1998). "Antigen detection in vivo after immunization with different presentation forms of rabies virus antigen, II. Cellular, but not humoral, systemic immune responses against rabies virus immune-stimulating complexes are macrophage dependent." Immunology **94**(4): 455-460.

Cleaveland, S., E. M. Fevre, et al. (2002). "Estimating human rabies mortality in the United Republic of Tanzania from dog bite injuries." Bull World Health Organ **80**(4): 304-310.

Cleaveland, S., M. Kaare, et al. (2003). "A dog rabies vaccination campaign in rural Africa: impact on the incidence of dog rabies and human dog-bite injuries." Vaccine **21**(17-18): 1965-1973.

Clyde, D. F. (1975). "Immunization of man against falciparum and vivax malaria by use of attenuated sporozoites." Am J Trop Med Hyg **24**(3): 397-401.

Coban, C., Y. Igari, et al. (2010). "Immunogenicity of whole-parasite vaccines against Plasmodium falciparum involves malarial hemozoin and host TLR9." Cell Host Microbe **7**(1): 50-61.

Cooper, M. A., J. M. Elliott, et al. (2009). "Cytokine-induced memory-like natural killer cells." Proc Natl Acad Sci U S A **106**(6): 1915-1919.

Cox-Singh, J., T. M. Davis, et al. (2008). "Plasmodium knowlesi malaria in humans is widely distributed and potentially life threatening." Clin Infect Dis **46**(2): 165-171.

Currier, J., H. P. Beck, et al. (1995). "Antigens released at schizont burst stimulate Plasmodium falciparum-specific CD4+ T cells from non-exposed donors: potential for cross reactive memory T cells to cause disease." Int Immunol **7**(5): 821-833.

Currier, J., J. Sattabongkot, et al. (1992). "'Natural' T cells responsive to malaria: evidence implicating immunological cross-reactivity in the maintenance of TCR alpha beta+ malaria-specific responses from non-exposed donors." Int Immunol **4**(9): 985-994.

D'Ombrain, M. C., D. S. Hansen, et al. (2007). "gammadelta-T cells expressing NK receptors predominate over NK cells and conventional T cells in the innate IFN-gamma response to Plasmodium falciparum malaria." Eur J Immunol **37**(7): 1864-1873.

Darrah, P. A., S. T. Hegde, et al. (2010). "IL-10 production differentially influences the magnitude, quality, and protective capacity of Th1 responses depending on the vaccine platform." J Exp Med **207**(7): 1421-1433.

Darrah, P. A., D. T. Patel, et al. (2007). "Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major." Nat Med **13**(7): 843-850.

De Souza, J. B., K. H. Williamson, et al. (1997). "Early gamma interferon responses in lethal and nonlethal murine blood-stage malaria." Infect Immun **65**(5): 1593-1598.

Desai, M., F. O. ter Kuile, et al. (2007). "Epidemiology and burden of malaria in pregnancy." Lancet Infect Dis **7**(2): 93-104.

Desombere, I., F. Clement, et al. (2005). "The duration of in vitro stimulation with recall antigens determines the subset distribution of interferon-gamma-producing lymphoid cells: a kinetic analysis using the Interferon-gamma Secretion Assay." J Immunol Methods **301**(1-2): 124-139.

Di Santo, J. P. (2006). "Natural killer cell developmental pathways: a question of balance." Annu Rev Immunol **24**: 257-286.

Diallo, D. A., S. N. Cousens, et al. (2004). "Child mortality in a West African population protected with insecticide-treated curtains for a period of up to 6 years." Bull World Health Organ **82**(2): 85-91.

Dick, S., M. Waterfall, et al. (1996). "Naive human alpha beta T cells respond to membrane-associated components of malaria-infected erythrocytes by proliferation and production of interferon-gamma." Immunology **88**(3): 412-420.

Diebold, S. S., T. Kaisho, et al. (2004). "Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA." Science **303**(5663): 1529-1531.

Dodet, B., E. V. Adjogoua, et al. (2008). "Fighting rabies in Africa: the Africa Rabies Expert Bureau (AfroREB)." Vaccine **26**(50): 6295-6298.

Duffy, P. E. and M. Fried (2005). "Malaria in the pregnant woman." Curr Top Microbiol Immunol **295**: 169-200.

Eastman, R. T. and D. A. Fidock (2009). "Artemisinin-based combination therapies: a vital tool in efforts to eliminate malaria." Nat Rev Microbiol **7**(12): 864-874.

Ejigiri, I. and P. Sinnis (2009). "Plasmodium sporozoite-host interactions from the dermis to the hepatocyte." Curr Opin Microbiol **12**(4): 401-407.

Esin, S., G. Batoni, et al. (2008). "Direct binding of human NK cell natural cytotoxicity receptor NKp44 to the surfaces of mycobacteria and other bacteria." Infect Immun **76**(4): 1719-1727.

Etessami, R., K. K. Conzelmann, et al. (2000). "Spread and pathogenic characteristics of a G-deficient rabies virus recombinant: an in vitro and in vivo study." J Gen Virol **81**(Pt 9): 2147-2153.

Fehniger, T. A., M. A. Cooper, et al. (2003). "CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity." Blood **101**(8): 3052-3057.

Fell, A. H., J. Currier, et al. (1994). "Inhibition of Plasmodium falciparum growth in vitro by CD4+ and CD8+ T cells from non-exposed donors." Parasite Immunol **16**(11): 579-586.

Fernando, S. D., D. M. Gunawardena, et al. (2003). "The impact of repeated malaria attacks on the school performance of children." Am J Trop Med Hyg **69**(6): 582-588.

Ferre, A. L., P. W. Hunt, et al. (2009). "Mucosal immune responses to HIV-1 in elite controllers: a potential correlate of immune control." Blood **113**(17): 3978-3989.

Fletcher, J. M., H. G. Prentice, et al. (1998). "Natural killer cell lysis of cytomegalovirus (CMV)-infected cells correlates with virally induced changes in cell surface lymphocyte function-associated antigen-3 (LFA-3) expression and not with the CMV-induced down-regulation of cell surface class I HLA." J Immunol **161**(5): 2365-2374.

Forbes, E. K., C. R. Sander, et al. (2008). "Multifunctional, high-level cytokine-producing Th1 cells in the lung, but not spleen, correlate with protection against Mycobacterium tuberculosis aerosol challenge in mice." J Immunol **181**(7): 4955-4964.

Franklin, B. S., P. Parroche, et al. (2009). "Malaria primes the innate immune response due to interferon-gamma induced enhancement of toll-like receptor expression and function." Proc Natl Acad Sci U S A **106**(14): 5789-5794.

Frevert, U. (2004). "Sneaking in through the back entrance: the biology of malaria liver stages." Trends Parasitol **20**(9): 417-424.

Garnham, P. C. C. (1996). "Malaria parasites and other haemosporidia. Oxford, United Kingdom: Blackwell Scientific."

Gascoyne, D. M., E. Long, et al. (2009). "The basic leucine zipper transcription factor E4BP4 is essential for natural killer cell development." Nat Immunol **10**(10): 1118-1124.

Gaucher, D., R. Therrien, et al. (2008). "Yellow fever vaccine induces integrated multilineage and polyfunctional immune responses." J Exp Med **205**(13): 3119-3131.

Gowda, D. C. (2007). "TLR-mediated cell signaling by malaria GPIs." Trends Parasitol **23**(12): 596-604.

Greenwood, B. and T. Mutabingwa (2002). "Malaria in 2002." Nature **415**(6872): 670-672.

Guerra, N., Y. X. Tan, et al. (2008). "NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy." Immunity **28**(4): 571-580.

Guma, M., A. Angulo, et al. (2004). "Imprint of human cytomegalovirus infection on the NK cell receptor repertoire." Blood **104**(12): 3664-3671.

Hanlon, C. A., I. V. Kuzmin, et al. (2005). "Efficacy of rabies biologics against new lyssaviruses from Eurasia." Virus Res **111**(1): 44-54.

Harari, A., V. Dutoit, et al. (2006). "Functional signatures of protective antiviral T-cell immunity in human virus infections." Immunol Rev **211**: 236-254.

Hawkins, E. D., M. Hommel, et al. (2007). "Measuring lymphocyte proliferation, survival and differentiation using CFSE time-series data." Nat Protoc **2**(9): 2057-2067.

Hay, S. I., C. A. Guerra, et al. (2004). "The global distribution and population at risk of malaria: past, present, and future." Lancet Infect Dis **4**(6): 327-336.
He, X. S., M. Draghi, et al. (2004). "T cell-dependent production of IFN-gamma by NK cells in response to influenza A virus." J Clin Invest **114**(12): 1812-1819.
He, X. S., T. H. Holmes, et al. (2008). "Baseline levels of influenza-specific CD4 memory T-cells affect T-cell responses to influenza vaccines." PLoS One **3**(7): e2574.
Heil, F., H. Hemmi, et al. (2004). "Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8." Science **303**(5663): 1526-1529.
Hensmann, M. and D. Kwiatkowski (2001). "Cellular basis of early cytokine response to Plasmodium falciparum." Infect Immun **69**(4): 2364-2371.
Hershkovitz, O., B. Rosental, et al. (2009). "NKp44 receptor mediates interaction of the envelope glycoproteins from the West Nile and dengue viruses with NK cells." J Immunol **183**(4): 2610-2621.
Hooper, D. C., K. Morimoto, et al. (1998). "Collaboration of antibody and inflammation in clearance of rabies virus from the central nervous system." J Virol **72**(5): 3711-3719.
Horowitz, A., R. H. Behrens, et al. (2010). "Nk cells as effectors of acquired immune responses: Effector CD4+ T cell-dependent activation of NK cells following vaccination." J Immunol **185**(5): 2808-2818.
Horowitz, A., K. C. Newman, et al. (2010). "Cross-talk between T cells and NK cells generates rapid effector responses to Plasmodium falciparum-infected erythrocytes." J Immunol **184**(11): 6043-6052.
Horowitz, A. and E. M. Riley (2010). "Activation of human NK cells by malaria-infected red blood cells." Methods Mol Biol **612**: 429-446.
Ito, N., M. Sugiyama, et al. (2005). "Characterization of M gene-deficient rabies virus with advantages of effective immunization and safety as a vaccine strain." Microbiol Immunol **49**(11): 971-979.
Jackson, A. C., J. P. Rossiter, et al. (2006). "Expression of Toll-like receptor 3 in the human cerebellar cortex in rabies, herpes simplex encephalitis, and other neurological diseases." J Neurovirol **12**(3): 229-234.
Johnson, N., A. F. Cunningham, et al. (2010). "The immune response to rabies virus infection and vaccination." Vaccine **28**(23): 3896-3901.
Jones, K. R., J. K. Hickling, et al. (1990). "Polyclonal in vitro proliferative responses from nonimmune donors to Plasmodium falciparum malaria antigens require UCHL1+ (memory) T cells." Eur J Immunol **20**(2): 307-315.
Kamath, A. T., A. F. Rochat, et al. (2008). "Adult-like anti-mycobacterial T cell and in vivo dendritic cell responses following neonatal immunization with Ag85B-ESAT-6 in the IC31 adjuvant." PLoS One **3**(11): e3683.
Kanzler, H., F. J. Barrat, et al. (2007). "Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists." Nat Med **13**(5): 552-559.
Karre, K., H. G. Ljunggren, et al. (1986). "Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy." Nature **319**(6055): 675-678.
Kester, K. E., J. F. Cummings, et al. (2009). "Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection." J Infect Dis **200**(3): 337-346.
Khakoo, S. I., C. L. Thio, et al. (2004). "HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection." Science **305**(5685): 872-874.
Kiessling, R., E. Klein, et al. (1975). ""Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell." Eur J Immunol **5**(2): 117-121.

Korbel, D. S. (2006). "The human natural killer cell response to *Plasmodium falciparum*." PhD Thesis. University of London.
Korbel, D. S., K. C. Newman, et al. (2005). "Heterogeneous human NK cell responses to Plasmodium falciparum-infected erythrocytes." J Immunol **175**(11): 7466-7473.
Korbel, D. S., P. J. Norman, et al. (2009). "Killer Ig-like receptor (KIR) genotype predicts the capacity of human KIR-positive CD56dim NK cells to respond to pathogen-associated signals." J Immunol **182**(10): 6426-6434.
Lafon, M. (2005). "Rabies virus receptors." J Neurovirol **11**(1): 82-87.
Lalvani, A., P. Moris, et al. (1999). "Potent induction of focused Th1-type cellular and humoral immune responses by RTS,S/SBAS2, a recombinant Plasmodium falciparum malaria vaccine." J Infect Dis **180**(5): 1656-1664.
Lang, J., E. Feroldi, et al. (2009). "Pre-exposure purified vero cell rabies vaccine and concomitant routine childhood vaccinations: 5-year post-vaccination follow-up study of an infant cohort in Vietnam." J Trop Pediatr **55**(1): 26-31.
Le-Barillec, K., J. G. Magalhaes, et al. (2005). "Roles for T and NK cells in the innate immune response to Shigella flexneri." J Immunol **175**(3): 1735-1740.
Lees, C. Y., D. J. Briggs, et al. (2002). "Induction of protective immunity by topic application of a recombinant adenovirus expressing rabies virus glycoprotein." Vet Microbiol **85**(4): 295-303.
Leiden, J. M., B. A. Karpinski, et al. (1989). "Susceptibility to natural killer cell-mediated cytolysis is independent of the level of target cell class I HLA expression." J Immunol **142**(6): 2140-2147.
Lengeler, C. (2004). "Insecticide-treated bed nets and curtains for preventing malaria." Cochrane Database Syst Rev(2): CD000363.
Lertmemongkolchai, G., G. Cai, et al. (2001). "Bystander activation of CD8+ T cells contributes to the rapid production of IFN-gamma in response to bacterial pathogens." J Immunol **166**(2): 1097-1105.
Lindblade, K. A., T. P. Eisele, et al. (2004). "Sustainability of reductions in malaria transmission and infant mortality in western Kenya with use of insecticide-treated bednets: 4 to 6 years of follow-up." JAMA **291**(21): 2571-2580.
Lindenstrom, T., E. M. Agger, et al. (2009). "Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional CD4 memory T cells." J Immunol **182**(12): 8047-8055.
Litwin, V., J. Gumperz, et al. (1993). "Specificity of HLA class I antigen recognition by human NK clones: evidence for clonal heterogeneity, protection by self and non-self alleles, and influence of the target cell type." J Exp Med **178**(4): 1321-1336.
Ljunggren, H. G. and K. Karre (1990). "In search of the 'missing self': MHC molecules and NK cell recognition." Immunol Today **11**(7): 237-244.
Lodmell, D. L. and L. C. Ewalt (2000). "Rabies vaccination: comparison of neutralizing antibody responses after priming and boosting with different combinations of DNA, inactivated virus, or recombinant vaccinia virus vaccines." Vaccine **18**(22): 2394-2398.
Lodmell, D. L. and L. C. Ewalt (2001). "Post-exposure DNA vaccination protects mice against rabies virus." Vaccine **19**(17-19): 2468-2473.
Lodmell, D. L., M. J. Parnell, et al. (2001). "One-time gene gun or intramuscular rabies DNA vaccination of non-human primates: comparison of neutralizing antibody responses and protection against rabies virus 1 year after vaccination." Vaccine **20**(5-6): 838-844.
Lodmell, D. L., M. J. Parnell, et al. (2002). "Rabies DNA vaccination of non-human primates: post-exposure studies using gene gun methodology that accelerates induction of

neutralizing antibody and enhances neutralizing antibody titers." Vaccine **20**(17-18): 2221-2228.

Lodmell, D. L., N. B. Ray, et al. (1998). "Gene gun particle-mediated vaccination with plasmid DNA confers protective immunity against rabies virus infection." Vaccine **16**(2-3): 115-118.

Lodmell, D. L., N. B. Ray, et al. (1998). "DNA immunization protects nonhuman primates against rabies virus." Nat Med **4**(8): 949-952.

Lodmell, D. L., N. B. Ray, et al. (2000). "DNA vaccination of mice against rabies virus: effects of the route of vaccination and the adjuvant monophosphoryl lipid A (MPL)." Vaccine **18**(11-12): 1059-1066.

Loewenberg, S. (2007). "The US President's Malaria Initiative: 2 years on." Lancet **370**(9603): 1893-1894.

Long, B. R., J. Michaelsson, et al. (2008). "Elevated frequency of gamma interferon-producing NK cells in healthy adults vaccinated against influenza virus." Clin Vaccine Immunol **15**(1): 120-130.

Macete, E. V., J. Sacarlal, et al. (2007). "Evaluation of two formulations of adjuvanted RTS, S malaria vaccine in children aged 3 to 5 years living in a malaria-endemic region of Mozambique: a Phase I/IIb randomized double-blind bridging trial." Trials **8**: 11.

Malek, T. R. (2008). "The biology of interleukin-2." Annu Rev Immunol **26**: 453-479.

Mandelboim, O., N. Lieberman, et al. (2001). "Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells." Nature **409**(6823): 1055-1060.

Manning, S. E., C. E. Rupprecht, et al. (2008). "Human rabies prevention--United States, 2008: recommendations of the Advisory Committee on Immunization Practices." MMWR Recomm Rep **57**(RR-3): 1-28.

Mansfield, K. L., N. Johnson, et al. (2008). "Up-regulation of chemokine gene transcripts and T-cell infiltration into the central nervous system and dorsal root ganglia are characteristics of experimental European bat lyssavirus type 2 infection of mice." J Neurovirol **14**(3): 218-228.

Maroof, A., L. Beattie, et al. (2008). "Posttranscriptional regulation of Il10 gene expression allows natural killer cells to express immunoregulatory function." Immunity **29**(2): 295-305.

McCall, M. B., M. G. Netea, et al. (2007). "Plasmodium falciparum infection causes proinflammatory priming of human TLR responses." J Immunol **179**(1): 162-171.

McGregor, I. A. (1984). "Epidemiology, malaria and pregnancy." Am J Trop Med Hyg **33**(4): 517-525.

McKimmie, C. S., N. Johnson, et al. (2005). "Viruses selectively upregulate Toll-like receptors in the central nervous system." Biochem Biophys Res Commun **336**(3): 925-933.

Mebatsion, T., M. Konig, et al. (1996). "Budding of rabies virus particles in the absence of the spike glycoprotein." Cell **84**(6): 941-951.

Miller, L. H., D. I. Baruch, et al. (2002). "The pathogenic basis of malaria." Nature **415**(6872): 673-679.

Mohan, K., P. Moulin, et al. (1997). "Natural killer cell cytokine production, not cytotoxicity, contributes to resistance against blood-stage Plasmodium chabaudi AS infection." J Immunol **159**(10): 4990-4998.

Monteiro, J. M., C. Harvey, et al. (1998). "Role of interleukin-12 in primary influenza virus infection." J Virol **72**(6): 4825-4831.

Moore, R. N., K. J. Goodrum, et al. (1976). "Mediation of an endotoxic effect by macrophages." J Reticuloendothel Soc **19**(3): 187-197.

Moorthy, V. S. and W. R. Ballou (2009). "Immunological mechanisms underlying protection mediated by RTS,S: a review of the available data." Malar J **8**(1): 312.

Moorthy, V. S., C. Diggs, et al. (2009). "Report of a consultation on the optimization of clinical challenge trials for evaluation of candidate blood stage malaria vaccines, 18-19 March 2009, Bethesda, MD, USA." Vaccine **27**(42): 5719-5725.

Moretta, A., C. Bottino, et al. (2002). "What is a natural killer cell?" Nat Immunol **3**(1): 6-8.

Morimoto, K., Y. Shoji, et al. (2005). "Characterization of P gene-deficient rabies virus: propagation, pathogenicity and antigenicity." Virus Res **111**(1): 61-67.

Mota, M. M., G. Pradel, et al. (2001). "Migration of Plasmodium sporozoites through cells before infection." Science **291**(5501): 141-144.

Mota, M. M. and A. Rodriguez (2004). "Migration through host cells: the first steps of Plasmodium sporozoites in the mammalian host." Cell Microbiol **6**(12): 1113-1118.

Mufunda, J., P. Nyarango, et al. (2007). "Roll back malaria--an African success story in Eritrea." S Afr Med J **97**(1): 46-50.

Mung'Ala-Odera, V., R. W. Snow, et al. (2004). "The burden of the neurocognitive impairment associated with Plasmodium falciparum malaria in sub-saharan Africa." Am J Trop Med Hyg **71**(2 Suppl): 64-70.

Murphy, F. A. and S. P. Bauer (1974). "Early street rabies virus infection in striated muscle and later progression to the central nervous system." Intervirology **3**(4): 256-268.

Nadin-Davis, S. A. and C. Fehlner-Gardiner (2008). "Lyssaviruses: current trends." Adv Virus Res **71**: 207-250.

Nebbia, G., F. M. Mattes, et al. (2008). "Polyfunctional cytomegalovirus-specific CD4+ and pp65 CD8+ T cells protect against high-level replication after liver transplantation." Am J Transplant **8**(12): 2590-2599.

Neves, P. C., D. C. Matos, et al. (2009). "TLR expression and NK cell activation after human yellow fever vaccination." Vaccine **27**(41): 5543-5549.

Newman, K. C., D. S. Korbel, et al. (2006). "Cross-talk with myeloid accessory cells regulates human natural killer cell interferon-gamma responses to malaria." PLoS Pathog **2**(12): e118.

Newman, K. C. and E. M. Riley (2007). "Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens." Nat Rev Immunol **7**(4): 279-291.

Nishimura, M. I., I. Stroynowski, et al. (1988). "H-2Kb antigen expression has no effect on natural killer susceptibility and tumorigenicity of a murine hepatoma." J Immunol **141**(12): 4403-4409.

Niwa, R., S. Hatanaka, et al. (2004). "Enhancement of the antibody-dependent cellular cytotoxicity of low-fucose IgG1 Is independent of FcgammaRIIIa functional polymorphism." Clin Cancer Res **10**(18 Pt 1): 6248-6255.

Nyarango, P. M., T. Gebremeskel, et al. (2006). "A steep decline of malaria morbidity and mortality trends in Eritrea between 2000 and 2004: the effect of combination of control methods." Malar J **5**: 33.

O'Leary, J. G., M. Goodarzi, et al. (2006). "T cell- and B cell-independent adaptive immunity mediated by natural killer cells." Nat Immunol **7**(5): 507-516.

O'Meara, W. P., P. Bejon, et al. (2008). "Effect of a fall in malaria transmission on morbidity and mortality in Kilifi, Kenya." Lancet **372**(9649): 1555-1562.

Okell, L. C., C. J. Drakeley, et al. (2008). "Reduction of transmission from malaria patients by artemisinin combination therapies: a pooled analysis of six randomized trials." Malar J **7**: 125.

Olotu, A., J. Lusingu, et al. (2010). "Efficacy of RTS,S/ASO1E in 5-17 months African children: Single blinded extended follow up." ms submitted.

Omer, F. M., J. B. de Souza, et al. (2003). "Activation of transforming growth factor beta by malaria parasite-derived metalloproteinases and a thrombospondin-like molecule." J Exp Med **198**(12): 1817-1827.

Omer, F. M., J. A. Kurtzhals, et al. (2000). "Maintaining the immunological balance in parasitic infections: a role for TGF-beta?" Parasitol Today **16**(1): 18-23.
Ono, M., H. Okada, et al. (1997). "Deletion of SHIP or SHP-1 reveals two distinct pathways for inhibitory signaling." Cell **90**(2): 293-301.
Parroche, P., F. N. Lauw, et al. (2007). "Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9." Proc Natl Acad Sci U S A **104**(6): 1919-1924.
Pashine, A., N. M. Valiante, et al. (2005). "Targeting the innate immune response with improved vaccine adjuvants." Nat Med **11**(4 Suppl): S63-68.
Pena, J., C. Alonso, et al. (1990). "Natural killer susceptibility is independent of HLA class I antigen expression on cell lines obtained from human solid tumors." Eur J Immunol **20**(11): 2445-2448.
Perona-Wright, G., K. Mohrs, et al. (2009). "Systemic but not local infections elicit immunosuppressive IL-10 production by natural killer cells." Cell Host Microbe **6**(6): 503-512.
Perussia, B. (1998). "Fc receptors on natural killer cells." Curr Top Microbiol Immunol **230**: 63-88.
Pinheiro da Silva, F., M. Aloulou, et al. (2008). "Inhibitory ITAMs: a matter of life and death." Trends Immunol **29**(8): 366-373.
Pombo, D. J., G. Lawrence, et al. (2002). "Immunity to malaria after administration of ultra-low doses of red cells infected with Plasmodium falciparum." Lancet **360**(9333): 610-617.
Ponnudurai, T., A. H. Lensen, et al. (1991). "Feeding behaviour and sporozoite ejection by infected Anopheles stephensi." Trans R Soc Trop Med Hyg **85**(2): 175-180.
Querec, T. D., R. S. Akondy, et al. (2009). "Systems biology approach predicts immunogenicity of the yellow fever vaccine in humans." Nat Immunol **10**(1): 116-125.
Ravetch, J. V. and L. L. Lanier (2000). "Immune inhibitory receptors." Science **290**(5489): 84-89.
Reyburn, H., R. Mbatia, et al. (2005). "Association of transmission intensity and age with clinical manifestations and case fatality of severe Plasmodium falciparum malaria." JAMA **293**(12): 1461-1470.
Riley, E. M. (1999). "Is T-cell priming required for initiation of pathology in malaria infections?" Immunol Today **20**(5): 228-233.
Robinson, L. J., M. C. D'Ombrain, et al. (2009). "Cellular tumor necrosis factor, gamma interferon, and interleukin-6 responses as correlates of immunity and risk of clinical Plasmodium falciparum malaria in children from Papua New Guinea." Infect Immun **77**(7): 3033-3043.
Rochman, Y., R. Spolski, et al. (2009). "New insights into the regulation of T cells by gamma (c) family cytokines." Nat Rev Immunol **9**(7): 480-490.
Rodrigues, A., J. A. Schellenberg, et al. (2008). "Changing pattern of malaria in Bissau, Guinea Bissau." Trop Med Int Health **13**(3): 410-417.
Roestenberg, M., M. McCall, et al. (2009). "Protection against a malaria challenge by sporozoite inoculation." N Engl J Med **361**(5): 468-477.
Romero, V., J. Azocar, et al. (2008). "Interaction of NK inhibitory receptor genes with HLA-C and MHC class II alleles in Hepatitis C virus infection outcome." Mol Immunol **45**(9): 2429-2436.
Rosenberg, R., R. A. Wirtz, et al. (1990). "An estimation of the number of malaria sporozoites ejected by a feeding mosquito." Trans R Soc Trop Med Hyg **84**(2): 209-212.
Sachs, J. and P. Malaney (2002). "The economic and social burden of malaria." Nature **415**(6872): 680-685.

Sadasivaiah, S., Y. Tozan, et al. (2007). "Dichlorodiphenyltrichloroethane (DDT) for indoor residual spraying in Africa: how can it be used for malaria control?" Am J Trop Med Hyg **77**(6 Suppl): 249-263.

Saute, F., J. Aponte, et al. (2003). "Malaria in southern Mozambique: malariometric indicators and malaria case definition in Manhica district." Trans R Soc Trop Med Hyg **97**(6): 661-666.

Schellenberg, D., B. Cisse, et al. (2006). "The IPTi Consortium: research for policy and action." Trends Parasitol **22**(7): 296-300.

Schijns, V. E. (2000). "Immunological concepts of vaccine adjuvant activity." Curr Opin Immunol **12**(4): 456-463.

Schnell, M. J., J. P. McGettigan, et al. (2010). "The cell biology of rabies virus: using stealth to reach the brain." Nat Rev Microbiol **8**(1): 51-61.

Seixas, E., J. F. Moura Nunes, et al. (2009). "The interaction between DC and Plasmodium berghei/chabaudi-infected erythrocytes in mice involves direct cell-to-cell contact, internalization and TLR." Eur J Immunol **39**(7): 1850-1863.

Shanbag, P., N. Shah, et al. (2008). "Protecting Indian schoolchildren against rabies: pre-exposure vaccination with purified chick embryo cell vaccine (PCECV) or purified verocell rabies vaccine (PVRV)." Hum Vaccin **4**(5): 365-369.

Shanker, A., G. Verdeil, et al. (2007). "CD8 T cell help for innate antitumor immunity." J Immunol **179**(10): 6651-6662.

Sidjanski, S. and J. P. Vanderberg (1997). "Delayed migration of Plasmodium sporozoites from the mosquito bite site to the blood." Am J Trop Med Hyg **57**(4): 426-429.

Sinden, R. E. (1999). "Plasmodium differentiation in the mosquito." Parassitologia **41**(1-3): 139-148.

Singh, B., L. Kim Sung, et al. (2004). "A large focus of naturally acquired Plasmodium knowlesi infections in human beings." Lancet **363**(9414): 1017-1024.

Sinnis, P. and A. Coppi (2007). "A long and winding road: the Plasmodium sporozoite's journey in the mammalian host." Parasitol Int **56**(3): 171-178.

Siren, J., T. Sareneva, et al. (2004). "Cytokine and contact-dependent activation of natural killer cells by influenza A or Sendai virus-infected macrophages." J Gen Virol **85**(Pt 8): 2357-2364.

Sivori, S., M. Falco, et al. (2004). "CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: induction of cytokine release and cytotoxicity against tumors and dendritic cells." Proc Natl Acad Sci U S A **101**(27): 10116-10121.

Snow, R. W., E. L. Korenromp, et al. (2004). "Pediatric mortality in Africa: plasmodium falciparum malaria as a cause or risk?" Am J Trop Med Hyg **71**(2 Suppl): 16-24.

Soares, I. S. and M. M. Rodrigues (1998). "Malaria vaccine: roadblocks and possible solutions." Braz J Med Biol Res **31**(3): 317-332.

Stevenson, M. M. and E. M. Riley (2004). "Innate immunity to malaria." Nat Rev Immunol **4**(3): 169-180.

Stewart, V. A., S. M. McGrath, et al. (2006). "Pre-clinical evaluation of new adjuvant formulations to improve the immunogenicity of the malaria vaccine RTS,S/AS02A." Vaccine **24**(42-43): 6483-6492.

Stoute, J. A., M. Slaoui, et al. (1997). "A preliminary evaluation of a recombinant circumsporozoite protein vaccine against Plasmodium falciparum malaria. RTS,S Malaria Vaccine Evaluation Group." N Engl J Med **336**(2): 86-91.

Strengell, M., S. Matikainen, et al. (2003). "IL-21 in synergy with IL-15 or IL-18 enhances IFN-gamma production in human NK and T cells." J Immunol **170**(11): 5464-5469.

Sun, J. C., J. N. Beilke, et al. (2009). "Adaptive immune features of natural killer cells." Nature **457**(7229): 557-561.

Sun, P., R. Schwenk, et al. (2003). "Protective immunity induced with malaria vaccine, RTS,S, is linked to Plasmodium falciparum circumsporozoite protein-specific CD4+ and CD8+ T cells producing IFN-gamma." J Immunol **171**(12): 6961-6967.

Sutlu, T. and E. Alici (2009). "Natural killer cell-based immunotherapy in cancer: current insights and future prospects." J Intern Med **266**(2): 154-181.

Takeda, K., T. Suzuki, et al. (2006). "Interleukin-12 is involved in the enhancement of human natural killer cell activity by Lactobacillus casei Shirota." Clin Exp Immunol **146**(1): 109-115.

Tortorella, D., B. E. Gewurz, et al. (2000). "Viral subversion of the immune system." Annu Rev Immunol **18**: 861-926.

Trinchieri, G. (1989). "Biology of natural killer cells." Adv Immunol **47**: 187-376.

Urban, B. C., D. J. Ferguson, et al. (1999). "Plasmodium falciparum-infected erythrocytes modulate the maturation of dendritic cells." Nature **400**(6739): 73-77.

van Eijk, A. M., J. G. Ayisi, et al. (2004). "Effectiveness of intermittent preventive treatment with sulphadoxine-pyrimethamine for control of malaria in pregnancy in western Kenya: a hospital-based study." Trop Med Int Health **9**(3): 351-360.

Vitale, M., C. Bottino, et al. (1998). "NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis." J Exp Med **187**(12): 2065-2072.

Vos, A., A. Neubert, et al. (2001). "Immunogenicity of an E1-deleted recombinant human adenovirus against rabies by different routes of administration." J Gen Virol **82**(Pt 9): 2191-2197.

Walther, M., J. E. Tongren, et al. (2005). "Upregulation of TGF-beta, FOXP3, and CD4+CD25+ regulatory T cells correlates with more rapid parasite growth in human malaria infection." Immunity **23**(3): 287-296.

Walther, M., J. Woodruff, et al. (2006). "Innate immune responses to human malaria: heterogeneous cytokine responses to blood-stage Plasmodium falciparum correlate with parasitological and clinical outcomes." J Immunol **177**(8): 5736-5745.

Walzer, T. and M. Blery (2007). "Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46." Proc Natl Acad Sci U S A **104**(9): 3384-3389.

Warrell, D. A. (1976). "The clinical picture of rabies in man." Trans R Soc Trop Med Hyg **70**(3): 188-195.

Waterfall, M., A. Black, et al. (1998). "Gammadelta+ T cells preferentially respond to live rather than killed malaria parasites." Infect Immun **66**(5): 2393-2398.

Watson, H. D., G. H. Tignor, et al. (1981). "Entry of rabies virus into the peripheral nerves of mice." J Gen Virol **56**(Pt 2): 372-382.

White, N. J. (2004). "Antimalarial drug resistance." J Clin Invest **113**(8): 1084-1092.

WHO (1998). "World Survey of Rabies No. 32 for the year 1996." Geneva: WHO; WHO document EMC/ZDI/98.4.

WHO (2008). "Rabies Fact Sheet No. 99." WHO.

WHO (2008). "World Malaria Report 2008. Geneva, WHO/GMP."

Wu, X., N. M. Gowda, et al. (2010). "Protein-DNA complex is the exclusive malaria parasite component that activates dendritic cells and triggers innate immune responses." J Immunol **184**(8): 4338-4348.

Yamauchi, L. M., A. Coppi, et al. (2007). "Plasmodium sporozoites trickle out of the injection site." Cell Microbiol **9**(5): 1215-1222.

Yoshida, O., S. M. Akbar, et al. (2010). "Regulatory natural killer cells in murine liver and their immunosuppressive capacity." Liver Int **30**(6): 906-912.

Appendix 1

Cross-Talk between T Cells and NK Cells Generates Rapid Effector Responses to *Plasmodium falciparum*-Infected Erythrocytes

Amir Horowitz,* Kirsty C. Newman,* J. Henry Evans,† Daniel S. Korbel,* Daniel M. Davis,† and Eleanor M. Riley*

Rapid cell-mediated immune responses, characterized by production of proinflammatory cytokines, such as IFN-γ, can inhibit intraerythrocytic replication of malaria parasites and thereby prevent onset of clinical malaria. In this study, we have characterized the kinetics and cellular sources of the very early IFN-γ response to *Plasmodium falciparum*-infected RBCs among human PBMCs. We find that NK cells dominate the early (12–18 h) IFN-γ response, that NK cells and T cells contribute equally to the response at 24 h, and that T cells increasingly dominate the response from 48 h onward. We also find that although γδ T cells can produce IFN-γ in response to *P. falciparum*-infected RBCs, they are greatly outnumbered by αβ T cells, and thus, the majority of the IFN-γ$^+$ T cells are αβ T cells and not γδ T cells; γδ T cells are, however, an important source of TNF. We have previously shown that NK cell responses to *P. falciparum*-infected RBCs require cytokine and contact-dependent signals from myeloid accessory cells. In this study, we demonstrate that NK cell IFN-γ responses to *P. falciparum*-infected RBCs are also crucially dependent on IL-2 secreted by CD4$^+$ T cells in an MHC class II-dependent manner, indicating that the innate response to infection actually relies upon complex interactions between NK cells, T cells, and accessory cells. We conclude that activation of NK cells may be a critical function of IL-2–secreting CD4$^+$ T cells and that standard protocols for evaluation of Ag-specific immune responses need to be adapted to include assessment of NK cell activation as well as T cell-derived IL-2. *The Journal of Immunology*, 2010, 184: 6043–6052.

The optimal immune response to a malaria infection likely comprises rapid induction of inflammatory antiparasitic responses followed by equally rapid resolution of inflammation (mediated by anti-inflammatory cytokines) to prevent immunopathology (1). Rapid and robust cell-mediated immune responses can inhibit intraerythrocytic replication of malaria parasites and thereby prevent onset of clinical malaria (2). This process can be primed by ultra-low–dose infection/vaccination (3, 4) but has yet to be mimicked by subunit vaccines. Understanding the cellular and molecular pathways of this very early cellular response may allow the design of new approaches to vaccination, but there is still considerable debate over the precise sequence of events. In particular, the timing and magnitude of IFN-γ secretion are thought to be pivotal in determining the outcome of disease, and it is thus of importance to identify the major cellular sources of IFN-γ, the kinetics of its production, and the pathways by which it is induced and regulated.

IFN-γ can be produced by both innately activated cells and cells of the adaptive immune system. We have previously found that αβ T cells, γδ T cells, and NK cells all contribute to the IFN-γ response of PBMCs from malaria-naive donors cocultured for 18–24 h with live *Plasmodium falciparum*-infected RBCs (5), but we also found that the magnitude of the response, as well as the proportion of responding cells in each subset, varies among individuals (5, 6). In some individuals, there is little or no evidence of an NK cell response to *P. falciparum*-infected RBCs, whereas in other individuals, NK cells comprise ~70% of IFN-γ–producing cells (5). We have identified variation in the strength of costimulatory signals from myeloid accessory cells (7) as well as polymorphism among NK cell regulatory receptors (8) as two components of this variation. Others, however, have concluded that γδ T cells are the major population of IFN-γ–producing lymphocytes when PBMCs from naive individuals are incubated with *P. falciparum*-infected RBCs (9, 10) and that NK cells make no major contribution to the innate response to *P. falciparum* (9), although comparison of data from different studies is complicated by substantial differences in experimental protocols.

In this study, we have carefully compared the magnitude and the timing of T cell and NK cell responses to *P. falciparum*-infected RBCs in a large cohort of malaria-naive donors. We find that NK cells dominate the early (12–18 h) IFN-γ response, that NK cells and T cells contribute approximately equally to the response at 24 h, and that T cells then increasingly dominate the response from 48 h onward. We also find that although γδ T cells can produce IFN-γ in response to *P. falciparum*-infected RBCs, they are greatly outnumbered by αβ T cells, and thus, contrary to previous reports, the majority of the IFN-γ$^+$ T cells are αβ T cells and not γδ T cells; γδ T cells are, however, an important source of TNF. Importantly, however, we find that the NK cell IFN-γ response to *P. falciparum*-infected RBCs is crucially dependent on IL-2 secreted, in an MHC class II-dependent manner, by CD4$^+$ T cells. These data corroborate our recent observations that NK cells may be recruited by Ag-specific IL-2–secreting CD4$^+$ T cells to act as effector cells during

*Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine; and †Department of Biological Sciences, Imperial College London, London, United Kingdom

Received for publication January 14, 2010. Accepted for publication March 26, 2010.

This work was supported by the United Kingdom Medical Research Council (GO 400225 and G0500563) and a Wellcome Trust Ph.D. studentship (to J.H.E.).

Address correspondence and reprint requests to Dr. Eleanor M. Riley, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, U.K. E-mail address: eleanor.riley@lshtm.ac.uk

Abbreviations used in this paper: CI, confidence interval; GM, growth medium; MFI, mean fluorescence intensity; rh, recombinant human.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1000106

the recall response to vaccine Ags (A. Horowitz, R.H. Behrens, L. Okell, A.R. Fooks, and E.M. Riley, submitted for publication) and indicate that the innate response to infection actually relies upon complex interactions between NK cells, T cells, and accessory cells.

Materials and Methods

Blood donors

Adult blood donors were recruited at the London School of Hygiene and Tropical Medicine through an anonymous blood donation system. All donors were malaria naive and healthy and gave fully informed consent for their blood to be used in this study. Ethical approval was given by the London School of Hygiene and Tropical Medicine Ethics Committee (application number 805).

PBMC preparation and culture

Venous blood was collected into sodium heparin (10 IU/ml blood; CP Pharmaceuticals, London, U.K.), and PBMCs were isolated by Histopaque 1077 (Sigma-Aldrich, St. Louis, MO) density gradient centrifugation as described previously (11). Cells were resuspended at a concentration of 2×10^6 cells/ml and cultured in flat-bottom 24-well plates or U-bottom 96-well plates for periods of up to 6 d.

Recombinant human IL-12 (rhIL-12; PeproTech, Rocky Hill, NJ) and rhIL-18 (MBL International, Woburn, MA) were each used at 0.1 μg/ml.

P. falciparum *parasites*

P. falciparum parasites (strain 3D7) were grown in O^{Rh-} human erythrocytes (National Blood Service, London, U.K.) in RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 25 mM HEPES (Sigma-Aldrich), 28 mM sodium bicarbonate (BDH Prolabo Chemicals, London, U.K.), 20 μg/l hypoxanthine (Sigma-Aldrich), and 10% normal human AB serum (National Blood Service). Cultures were gassed with 3% O_2, 4% CO_2, and 93% N_2 and incubated at 37°C. Parasite cultures were routinely shown to be free from *Mycoplasma/Acholeplasma* species contamination using an ELISA-based Mycoplasma Detection Kit (Roche, Basel, Switzerland) incorporating polyclonal Abs against *Mycoplasma arginini*, *Mycoplasma hyorhinis*, *Acholeplasma laidlawii*, and *Mycoplasma orale*. Highly pure (>95%) mature schizonts were harvested from cultures of 5–8% parasitaemia by adherence to an LD separation column (Miltenyi Biotec, Auburn, CA). Columns were washed thoroughly with PBS to remove uninfected erythrocytes pre-elution. Schizont-infected (*P. falciparum*-infected RBC) or uninfected erythrocytes were added at a ratio of 3 RBC/mononuclear cell.

Depletion of T cell subsets

PBMC were stained for CD3-expressing cells using a T cell selection kit (StemCell Technologies, Vancouver, British Columbia, Canada); CD4-expressing cells using a Dynal bead-conjugated mAb to CD4 (Invitrogen, Carlsbad, CA); CD8-expressing cells using a PE-conjugated mAb to CD8 (Caltag Laboratories, Burlingame, CA) and anti-PE MicroBeads (Miltenyi Biotec); γδ TCR-expressing cells using PE-conjugated mAb to γδ TCR (Caltag Laboratories) and anti-PE MicroBeads (Miltenyi Biotec); and αβ TCR-expressing cells using a PE-conjugated mAb to αβ TCR (BD Biosciences, San Jose, CA) and anti-PE MicroBeads (Miltenyi Biotec). Depletion of CD4 cells was carried out using a Dynal Magnetic Particle Concentrator-2 (Invitrogen). All other depletions were carried out using LD separation columns (Miltenyi Biotec) according to the manufacturer's instructions. Postdepletion, the PBMC concentration was readjusted to 2×10^6, and cells were cultured with *P. falciparum*-infected RBCs at a ratio of 1:3 (as previously) and also at a ratio of 1:10 to ensure that changing the ratio of NK cells and accessory cells to *P. falciparum*-infected RBCs did not adversely affect the outcome of the experiments. No significant differences were seen between the 1:3 and 1:10 cultures, so only data from the 1:3 cultures are presented.

Blocking experiments

Two million PBMCs were cultured overnight in 24-well flat-bottom plates in the presence of purified blocking/neutralizing Abs or appropriate isotype control Abs for the following cytokines, cytokine receptors, and MHC molecules (all BD Biosciences): anti–IL-12 (p40/p70; clone C11.5), anti–IL-18α-chain (clone H44), anti–IL-2 (clone MQ1 17H12), anti–IFNR-α [2b] (clone 7N4-1), anti-TNF (clone MAb1), anti-MHC class I (HLA-A, -B, -C; clone W6/32), and anti-MHC class II (HLA-DR, DP, DQ; clone Tü-39).

Cell surface and intracellular staining for flow cytometry

Surface and intracellular staining was performed as described previously (6). The Abs/reagents used were: anti-CD3 PerCP, anti-αβ TCR-FITC, anti-γδ TCR (biotinylated), streptavidin PerCP, anti–IFN-γ APC, anti-CD4 APC-Cy7, anti-CD8 Pacific Blue, and anti-CD3 PE-Texas Red (BD Biosciences); anti-CD56 APC (Beckman Coulter, Fullerton, CA), anti–IFN-γ FITC, anti–IL-2 APC (BD Biosciences), and anti-CD56 PE-Cy7 (BD Biosciences). All Abs were mouse monoclonals. Flow cytometric analyses were performed using a BD FACSCalibur or BD Cyan flow cytometer (BD Biosciences) and FlowJo analysis software (TreeStar, Ashland, OR). Anti-CD3 Abs were included in the intracellular staining mixes to ensure that T cells that downregulated CD3 upon stimulation were not misclassified.

Multiplex assay

Secreted IL-2 and TNF-α were measured, according to the manufacturer's instructions, by cytometric bead array (CBA Th1/Th2/Th17 kit; BD Biosciences). All samples were acquired using an FACSCalibur flow cytometer, and analysis was performed using FCAP Array software (Soft Flow, St. Louis Park, MN).

Statistical analysis

All statistical analyses were performed using Prism 5 software (GraphPad, San Diego, CA).

Results

T cells and NK cells both contribute to early IFN-γ response to P. falciparum

We have previously demonstrated that both NK cells and T cells can produce IFN-γ following incubation for 18–24 h with *P. falciparum*-infected RBCs (5), but others have concluded that γδT cells are the only significant source of early IFN-γ (9, 10). This discrepancy may reflect, in part, heterogeneity between human blood donors in the timing and magnitude of the response and in the source of IFN-γ (6). We sought, therefore, to characterize more systematically the early IFN-γ response to *P. falciparum*-infected RBCs (Fig. 1*A–E*). Lymphocytes were identified as $CD3^+$ T cells or as $CD3^-CD56^+$ NK cells (Fig. 1*A*). After 21 h coculture with *P. falciparum*-infected RBCs, the proportion of NK cells that contained intracellular IFN-γ ranged from 0–81% with a median of 4.65%. The proportion of T cells that were positive for IFN-γ was significantly lower, ranging from 0–7% with a median of 0.82% (Fig. 1*B*). However, because T cells outnumber NK cells by ~10:1 among PBMCs, when we gate on all IFN-γ^+ cells (Fig. 1*C*), we find that T cells and NK cells contribute approximately equally to the population of IFN-γ^+ cells (Fig. 1*D*). On the other hand, IFN-γ staining intensity (mean fluorescence intensity [MFI]) is significantly higher for NK cells than for T cells ($p < 0.0001$) (Fig. 1*E*), indicating that NK cells are producing more IFN-γ per cell than T cells.

To determine whether NK and T cells responded to *P. falciparum*-infected RBCs with similar kinetics, we determined the proportion of all IFN-γ^+ cells that were either NK cells or T cells after coculturing for periods from 6 h to 6 d (Fig. 1*F*). Although there is heterogeneity in the response among donors, the distinct trend was for IFN-γ^+ cells to be almost exclusively NK cells after 6 h of coculture and for NK cells to continue to dominate the IFN-γ response at 12 h. However, by 24 h, the response was evenly split between NK cells and T cells, and T cell responses gradually came to dominate the IFN-γ response over the next 5 d. This observation, that the NK cell IFN-γ response is very rapid but transient, is not unexpected but does likely explain many of the apparent discrepancies in the literature in which innate responses tend to have been assayed at a single point in time and frequently as long as 72 h postinitiation of *P. falciparum*-infected RBC stimulation.

Because potent Ag-specific T cell responses to *P. falciparum*-infected RBCs might not be expected among malaria-naive donors, we next sought to identify which subsets of T cells were

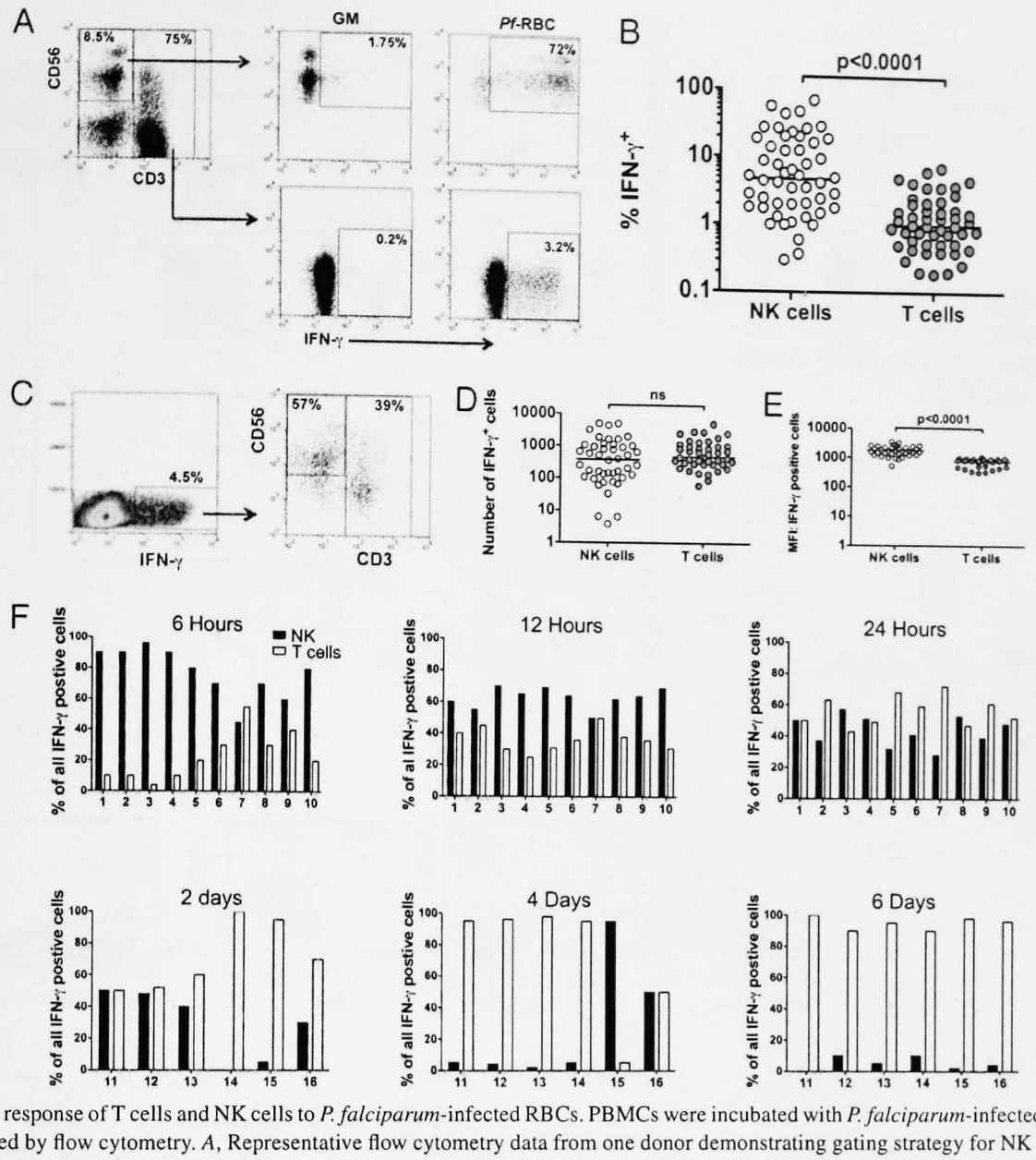


FIGURE 1. IFN-γ response of T cells and NK cells to *P. falciparum*-infected RBCs. PBMCs were incubated with *P. falciparum*-infected RBC for periods of 6 h to 6 d and analyzed by flow cytometry. *A*, Representative flow cytometry data from one donor demonstrating gating strategy for NK cells ($CD56^+$ $CD3^-$ lymphocytes) and T cells (all $CD3^+$ lymphocytes) and for detection of intracellular IFN-γ after 24 h coculture with (*right column*) or without (*left column*; GM *P. falciparum*-infected RBCs. *B*, The proportion of all NK cells or all T cells expressing intracellular IFN-γ after 24 h coculture with *P. falciparum*-infected RBCs. $n = 50$ malaria-naive donors. *C*, Gating strategy for identification of all IFN-γ^+ lymphocytes and their subsequent identification as either NK cells or T cells. *D*, The number of IFN-γ^+ NK cells or T cells per 100,000 lymphocytes after 24 h coculture with *P. falciparum*-infected RBCs. $n = 50$ naive donors. *E*, The MFI of staining for intracellular IFN-γ in NK cells and T cells after 24 h coculture with *P. falciparum*-infected RBCs. $n = 50$ naive donors. *F*, The percentage of all IFN-γ^+ cells that are either NK cells (black bars) or T cells (white bars) after 6, 12, or 24 h or 2, 4, or 6 d coculture with The *P. falciparum*-infected RBCs. Numbers on *x*-axis identify individual blood donors. *B*, *D*, and *E*, Horizontal lines represent medians. The *p* values are for two-tailed paired Wilcoxon tests with 95% CIs comparing NK cells with T cells from the same donor. CI, confidence interval; GM, growth medium.

producing IFN-γ. PBMCs from 14 donors were incubated with *P. falciparum*-infected RBCs for 21 h and gated as NK, $CD3^+$ $\gamma\delta TCR^+$, or $CD3^+$ $\alpha\beta TCR^+$ T cells; at some key time points, αβ T cells were gated as either $CD4^+$ or $CD8^+$ T cells (Fig. 2*A*). When each lymphocyte population was separately analyzed for IFN-γ expression, we again found that, on average, a higher proportion of NK cells than T cells produce IFN-γ, but among the T cell populations, a significantly higher proportion of $\gamma\delta TCR^+$ T cells were IFN-γ^+ than either of the $\alpha\beta TCR^+$ T cell subsets (Fig. 2*B*). However, because the majority of circulating T cells are $CD4^+$ and $\alpha\beta TCR^+$, when we gate on all IFN-γ^+ cells (Fig. 2*C*), we find that the majority of IFN-γ^+ T cells are $CD4^+$ $\alpha\beta TCR^+$ and that $CD4^+$ $\alpha\beta TCR^+$ T cells and NK cells contribute equally to the total IFN-γ^+ population, with $CD8^+$ $\alpha\beta TCR^+$ and $\gamma\delta TCR^+$ cells making only a minor contribution (Fig. 2*D*). To make sure that we had not missed the optimal timing for responses of any particular T cell subset, we repeated the kinetic analysis over 6 d (Fig. 2*E*) and found that $\alpha\beta^+$ T cells were the major contributing T cell population throughout the response.

NK cell and T cell responses to P. falciparum*-infected RBCs are both cytokine and MHC class II dependent*

We have previously shown that NK cell responses to *P. falciparum*-infected RBCs are absolutely dependent upon myeloid cell-derived IL-12 (12) and partially dependent upon IL-18, IFN-α, and IL-2 (7). Moreover, we, and others, have previously shown that memory $CD4^+$ T cells from malaria-naive individuals are able to respond to *P. falciparum*-infected RBCs in a classical MHC class II-restricted manner (13–15), and it is now generally accepted that these cells have been primed by cross-reacting Ags (13, 16). To confirm and extend these observations, PBMCs from 10 malaria-naive donors (selected to represent a range of NK responses from nonresponders to high responders) were cultured with *P. falciparum*-infected RBCs for 24 h in the presence of blocking or neutralizing Abs to IL-12, IL-18, IL-2, IFN-αR, MHC class I, MHC class II, or the relevant isotype control Abs. A representative data set for one donor is shown in Fig. 3; data for all 10 donors are shown in Fig. 4. As expected, NK cell IFN-γ

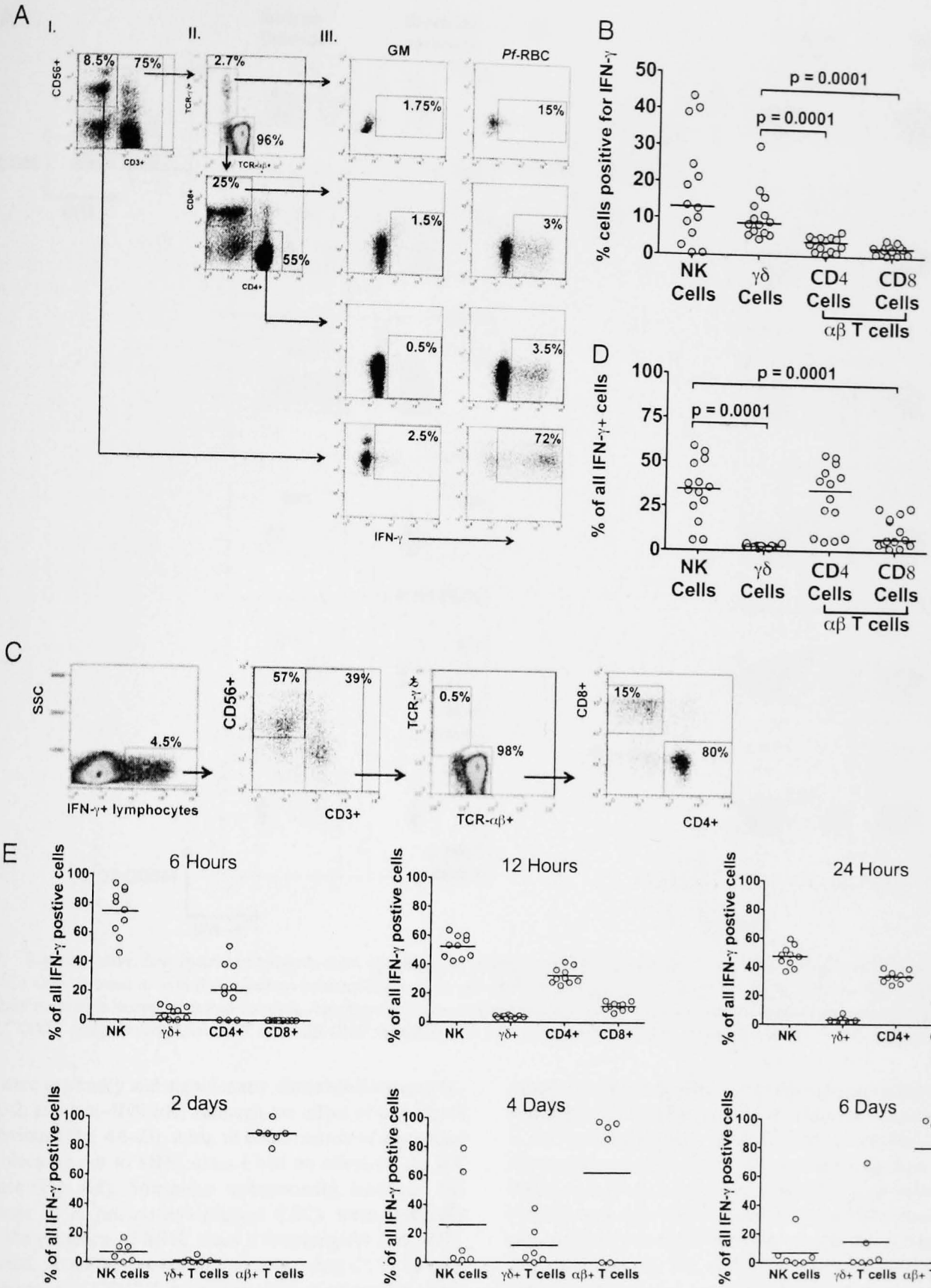


FIGURE 2. IFN-γ response of NK cells and T cell subsets. PBMCs were incubated with *P. falciparum*-infected RBC for periods of 6 h to 6 d and analyzed by flow cytometry. *A*, Representative flow cytometry data from one donor demonstrating gating strategy for NK cells ($CD56^+$ $CD3^-$ lymphocytes) and T cells (all $CD3^+$ lymphocytes) (*I*), which are then further classified as TCR-$\gamma\delta^+$ or TCR-$\alpha\beta^+$ T cells (*II*) and then all TCR-$\alpha\beta^+$ T cells are identified as being $CD4^+$ or $CD8^+$ T cells. Each population of lymphocytes can then be gated for detection of intracellular IFN-γ after 24 h coculture with *P. falciparum*-infected RBC (*III*, *right column*) or without (*III*, *left column*; GM). *B*, The percentage of all NK cells or all $\gamma\delta^+$ T cells, $CD4^+$ T cells, or $CD8^+$ T cells expressing intracellular IFN-γ after 24 h coculture with *P. falciparum*-infected RBCs. $n = 14$ naive donors. *C*, Gating strategy for identification of all IFN-γ^+ lymphocytes and their subsequent identification as either NK cells or $\gamma\delta^+$ T cells, $CD4^+$ T cells or $CD8^+$ T cells. *D*, The percentage of IFN-γ^+ cells that are NK cells or $\gamma\delta^+$ T cells, $CD4^+$ T cells or $CD8^+$ T cells per 100,000 lymphocytes after 24 h coculture with *P. falciparum*-infected RBCs. $n = 14$ naive donors. *E*, The percentage of all IFN-γ^+ cells that are either NK cells or T cells after 6, 12, or 24 h or 2, 4, or 6 d coculture with *P. falciparum*-infected RBCs. Numbers on *x*-axis identify individual blood donors. *B*, *D*, and *E*, Horizontal lines represent medians. The *p* values are for two-tailed paired Wilcoxon tests with 95% CIs comparing NK cells with T cells from the same donor. GM, growth medium.

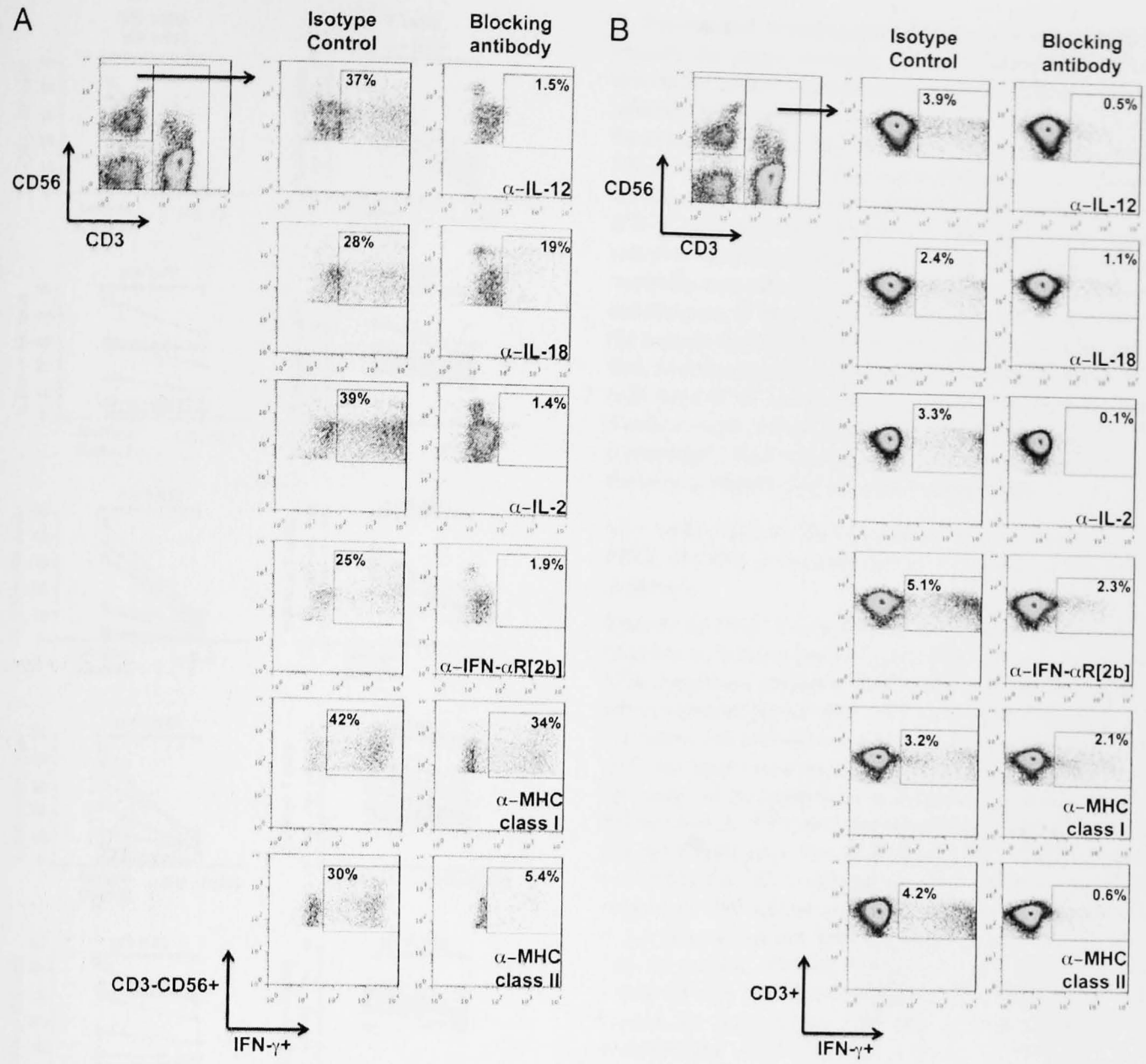


FIGURE 3. Representative flow cytometry plots to show the effects of blocking Abs on NK cell and T cell IFN-γ responses to *P. falciparum*-infected RBCs. PBMCs were cultured in with *P. falciparum*-infected RBCs in the presence of blocking Abs to IL-12, IL-18, IL-2, IFN-αR[2b], MHC class 1, MHC class II, or their respective isotype-matched controls. Representative flow cytometry data are shown for one donor, demonstrating the gating strategy for NK cells ($CD56^+$ $CD3^-$ lymphocytes) (*A*) and T cells (all $CD3^+$ lymphocytes) (*B*), which are then further classified as IFN-γ^+ (% shown) or IFN-γ^-.

responses were markedly and significantly diminished by anti–IL-12, anti–IL-2, and anti–IFN-αR, although the effect of anti–IL-18 was less obvious (Fig. 4*A–D*). Also, in confirmation of a previous study (8), blocking Ab to MHC class I had no effect on the NK cell response (Fig. 4*E*). Somewhat unexpectedly, however, NK cell responses to *P. falciparum*-infected RBCs were markedly reduced in the presence of MHC class II-blocking Ab (Fig. 4*F*).

As expected, given the previously published data (7, 8, 12–16), the preponderance of $CD4^+$ T cells in the IFN-γ–responding population, and the small but noticeable contribution of $CD8^+$ T cells, total T cell IFN-γ responses were markedly diminished in the presence of anti-MHC class II Abs (Fig. 4*F*) and significantly, but less markedly, diminished by MHC class I blockade (Fig. 4*E*). Total T cell responses were also significantly inhibited by neutralizing Abs to IL-12, IL-18, and IL-2, and there was a marginally significant effect of anti–IFN-αR (Fig. 4*A–D*), indicating that T cells are also dependent upon accessory cell-derived cytokines.

NK cell responses to P. falciparum*-infected RBCs are dependent upon T cell help*

The observation that NK cell responses to *P. falciparum*-infected RBCs were highly dependent upon both IL-2 and MHC class II raised the possibility that, as previously described for human NK cell responses to influenza (17, 18), NK cells require signals (such as IL-2) from Ag-specific $CD4^+$ T cells to respond optimally to *P. falciparum*-infected RBCs. To test this hypothesis, we depleted PBMCs of various lymphocyte populations and tested the remaining PBMCs for their ability to make IFN-γ in 24-h cultures (Fig. 5). NK cells among CD3-depleted PBMCs made strong responses to rhIL-12 plus IL-18 (Fig. 5*A*), indicating that these two cytokines (at optimal concentrations) are sufficient for NK cell activation. Conversely, NK cells among CD3-depleted PBMCs were completely unable to make IFN-γ in response to *P. falciparum*-infected RBCs (Fig. 5*B*), but NK cells among CD20 (B cell)-depleted PBMCs made a robust IFN-γ response to *P. falciparum*-infected RBCs (Fig. 5*C*). To determine which subset(s) of T cells provide help to NK cells, we depleted PBMCs of just $CD4^+$ T cells, just $CD8^+$ T cells, all $\alpha\beta TCR^+$ T cells, or all $\gamma\delta TCR^+$ T cells and cultured the remaining PBMCs with *P. falciparum*-infected RBCs for 24 h (Fig. 5*D–G*). It was clear that the NK cell response to *P. falciparum*-infected RBCs was only completely ablated when all T cells were removed but that removal of any T cell subset significantly reduced the NK cell IFN-γ response. Finally, to rule out any nonspecific effects of magnetic bead treatment, unlabeled PBMCs were incubated with anti-PE

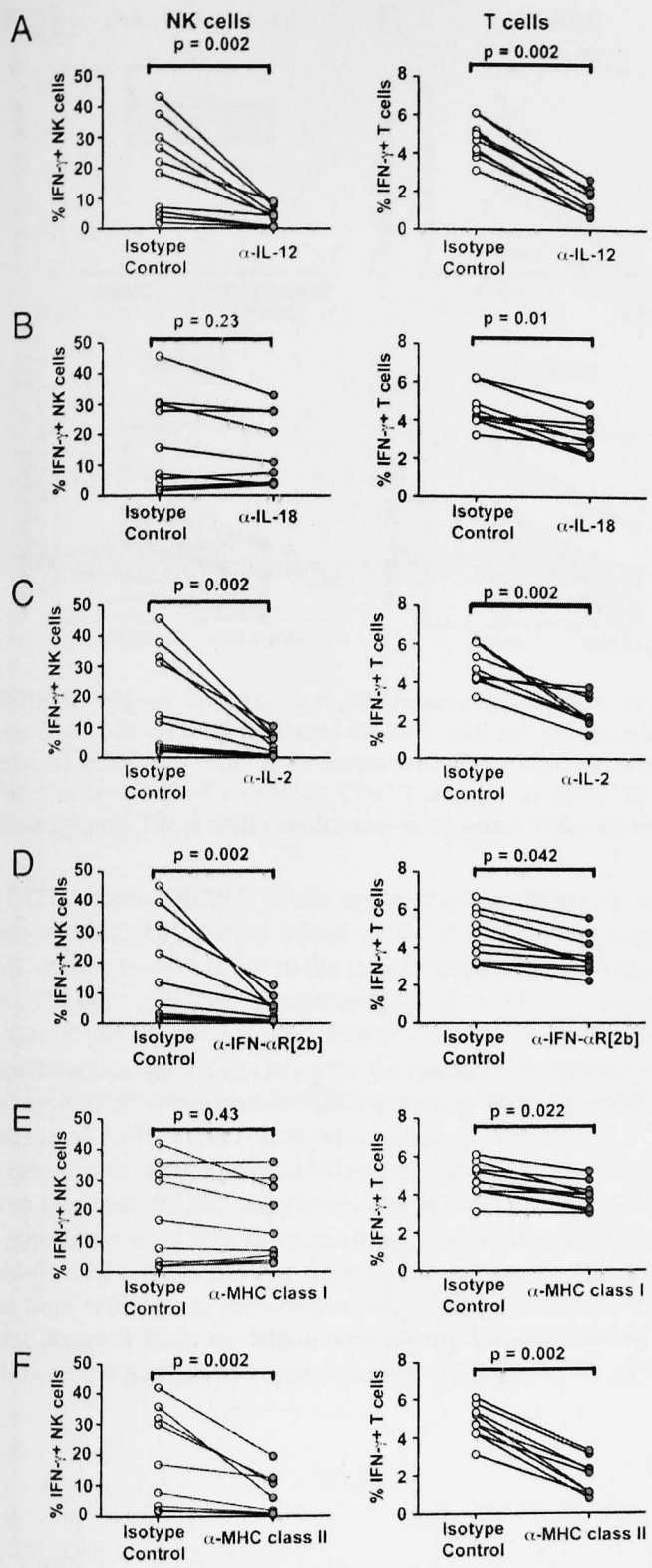


FIGURE 4. NK cell and T cell responses to *P. falciparum*-infected RBCs are both cytokine and MHC class II dependent. PBMCs from 10 donors were cultured with *P. falciparum*-infected RBCs for 24 h in the presence of blocking Abs to cytokines, cytokine receptors, MHC molecules, or with isotype-matched control Abs and the percentages of NK cells (*left columns*) and T cells (*right columns*) expressing intracellular IFN-γ determined by flow cytometry. The *p* values are for two-tailed paired Wilcoxon test with 95% CI. *A*, Anti–IL-12. *B*, Anti–IL-18. *C*, Anti–IL-2. *D*, Anti–IFN-αR. *E*, Anti-MHC class I. *F*, Anti-MHC class II.

microbeads and passed through the magnetic column (Fig. 5*H*); a robust IFN-γ response was seen.

CD4$^+$ T cells produce IL-2 within 12 h of coculture with P. falciparum*-infected RBCs*

Because NK cells require both T cell help and IL-2 to respond to *P. falciparum*-infected RBCs, it seemed likely that T cells are an essential source of IL-2. We sought to determine the kinetics of IL-2 production in response to *P. falciparum*-infected RBCs and to identify the major source(s) of this cytokine (Fig. 6). IL-2 was detected in culture supernatants within 3 h of PBMC-*P. falciparum*-infected RBC coculture, peaked at 4 h, and remained detectable for at least 24 h (Fig. 6*A*). In parallel cultures, intracellular IL-2 in NK cells and T cell subsets was analyzed by flow cytometry (Fig. 6*B–D*). All IL-2$^+$ cells were gated (Fig. 6*B*) and counted (Fig. 6*C*). IFN-γ$^+$ cells were first detected at 3 h postinitiation of coculture, and their numbers peaked at 12 h; the discrepancy in timing between the accumulation of soluble IL-2 in culture medium (Fig. 6*A*) and the peak of IL-2–secreting cells (Fig. 6*C*) presumably reflects the balance between secretion and consumption of IL-2 in the culture. At all time points, the vast majority (>80%) of IL-2–producing cells were CD4$^+$ T cells with only minor contributions from CD8$^+$ T cells at 6 and 12 h and from γδT cells and NK cells at 3 h (Fig. 6*D*). Interestingly, IL-2 responses were much less heterogeneous among the various donors than were IFN-γ responses.

γδ T cells produce TNF in response to P. falciparum*-infected RBCs, but TNF is not required for NK cell or T cell IFN-γ responses*

Because γδ TCR$^+$ T cells seemed to be necessary for an optimal NK response to *P. falciparum*-infected RBCs (Fig. 5) but did not seem to be significant producers of IL-2 (Fig. 6), we considered whether other cytokines produced by γδ T cells might contribute to NK cell activation. As Hensmann et al. (10) have previously shown that γδT cells represent a significant source of intracellular TNF during the early (18 h) response to *P. falciparum*-infected RBCs, and as Robinson et al. (19) concluded (from T cell subset depletion assays) that γδ T cells are a significant source of TNF, we considered the possibility that γδ T cell-derived TNF might potentiate NK cell responses. TNF was detected in supernatants within 4 h of PBMC-*P. falciparum*-infected RBC coculture and peaked at ~12 h (Fig. 7*A*). In parallel, PBMCs from eight malaria-naive donors were incubated with *P. falciparum*-infected RBCs for up to 24 h and then stained for intracellular TNF and surface markers to identify macrophages (CD14$^+$ CD68$^+$) and γδ T cells (Fig. 7*B*). When gating on all macrophages (Fig. 7*D*), TNF$^+$ macrophages were detectable within 2 h of *P. falciparum*-infected RBC coculture, the response peaking (with >35% of all macrophages being TNF$^+$) at 4 h and declining to barely detectable levels by 24 h. In comparison, TNF$^+$ γδ T cells were detected from ~5 to 6 h postinitiation of *P. falciparum*-infected RBC stimulation, and the response was maximal (with ~30% of all γδT cells being TNF$^+$) at 12–24 h. However, we found no evidence that TNF was required for induction of an early IFN-γ response, because when we stimulated PBMCs from the same donors with *P. falciparum*-infected RBCs in the presence of neutralizing Abs to TNF, the NK cell and T cell IFN-γ responses were unaffected (Fig. 7*E*).

T cell help for NK cell activation by P. falciparum*-infected RBCs does not require NK cell-T cell contact*

We have shown that T cell-derived signals are required for NK cell activation by *P. falciparum*-infected RBCs. One of these signals is clearly IL-2, emanating principally from CD4$^+$ αβ T cells, but T cell depletion studies suggest that there may also be an as yet undefined role for γδ T cells in NK cell activation. We have previously shown that direct contact between NK cells and myeloid accessory cells is required for optimal NK cell responses to *P. falciparum*-infected RBCs (7), and we therefore considered the possibility that T cells might also provide contact-dependent signals to NK cells (Fig. 8). Intact PBMCs and CD3-depleted PBMCs were cultured in separate compartments of a transwell plate separated by a semipermeable membrane; intact PBMCs in the lower chamber

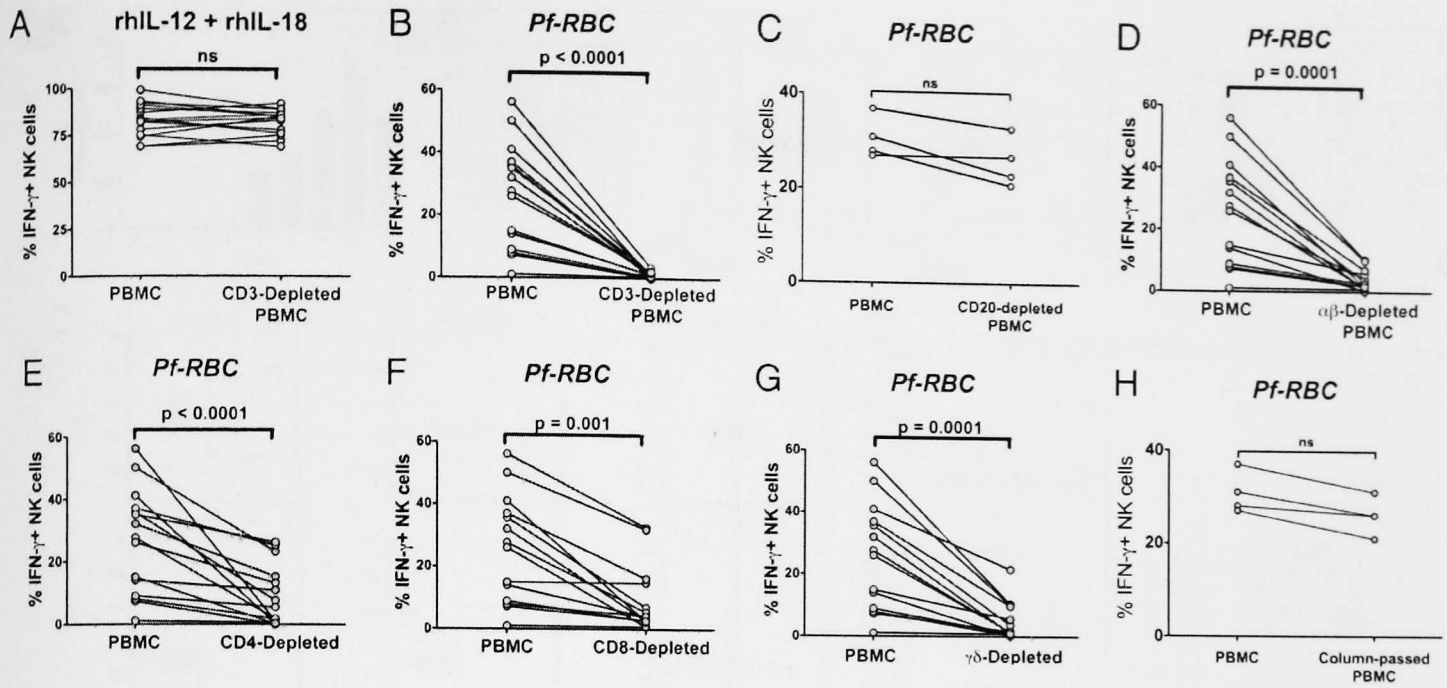


FIGURE 5. NK cell responses to *P. falciparum*-infected RBCs are dependent upon $CD4^+$ T cell help. Intact PBMCs or PBMCs depleted of B cells or various T cell subsets were incubated for 24 h with 100 ng/ml each of rhIL-12 plus rhIL-18 (*A*) or with *P. falciparum*-infected RBC (*B–H*), and the percentages of NK cells staining for intracellular IFN-γ were measured by flow cytometry. PBMC were depleted of all $CD3^+$ cells (*A*, *B*), $CD20^+$ B cells (*C*), αβ T cells (*D*), $CD4^+$ T cells (*E*), $CD8^+$ T cells (*F*), or γδ T cells (*G*), or incubated with anti-PE microbeads (without Ab) (*H*) and passed through the magnetic column. The *p* values are for two-tailed paired Wilcoxon tests, 95% CI, for 15 donors.

and CD3-depleted PBMCs in the upper chamber. When *P. falciparum*-infected RBCs were added to the CD3-depleted (upper) PBMC compartment but not to the intact PBMCs (lower compartment), NK cells in neither compartment produced IFN-γ, suggesting that *P. falciparum*-infected RBCs need to be in the same compartment as the T cells in order for NK cells to become activated, consistent with a need for APCs to take up Ag and present it to T cells in an MHC class II-dependent manner. However, if *P. falciparum*-infected RBCs were added to both compartments, NK cells in both the intact PBMC population and in the CD3-depleted NK cell population were able to respond fully, indicating that soluble T cell-derived signals passing through the semipermeable membrane were sufficient to activate the NK cells. To determine if this soluble signal is IL-2, we added neutralizing anti–IL-2 Ab to the CD3-depleted PBMCs in the upper chamber and added *P. falciparum*-infected RBCs to both compartments. In this case, the NK cell IFN-γ response was significantly inhibited in both compartments.

Discussion

Although there is a general consensus that a rapid, cell-mediated, inflammatory response is required to contain the initial stages of human blood-stage malaria infections, there has been considerable debate over many years as to the key cellular effectors of this response (2). We have therefore conducted a detailed kinetic analysis of all the likely sources of IFN-γ and find that the very early (<24 h) IFN-γ response to *P. falciparum*-infected RBCs is dominated, in most PBMC donors, by NK cells, that NK cells and T cells contribute more or less equally to the response at 24 h, and that T cells come to dominate the response from 48 h onward. This rapid NK cell response to *P. falciparum*-infected RBCs among PBMCs from

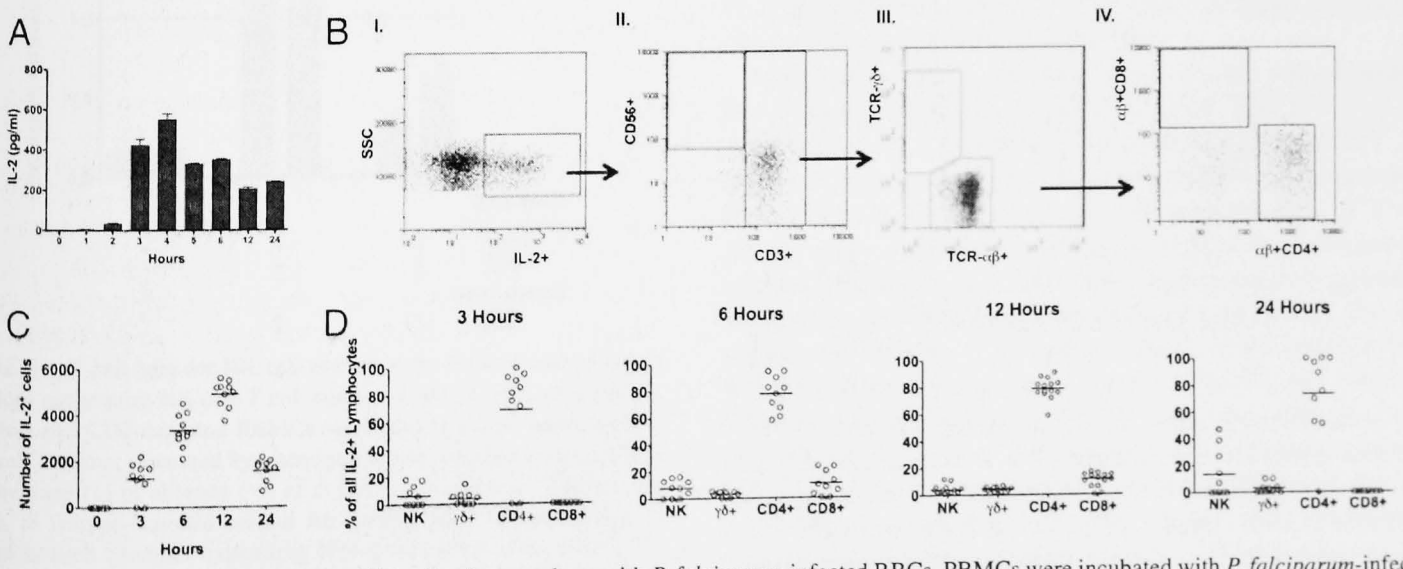


FIGURE 6. Rapid IL-2 production by $CD4^+$ T cells after coculture with *P. falciparum*-infected RBCs. PBMCs were incubated with *P. falciparum*-infected RBCs for up to 24 h. *A*, Median concentration of IL-2 in culture supernatants, determined by cytometric bead array, over time. $n = 5$ donors. *B*, Gating strategy for identification and phenotypic characterization of IL-2–producing cells. *C*, The total number of $IL\text{-}2^+$ lymphocytes at each time point. $n = 10$ donors. *D*, The proportion of all $IL\text{-}2^+$ lymphocytes that are either NK cells or are $\gamma\delta^+$ T cells, $CD4^+$ T cells, or $CD8^+$ T cells when comparing responses over 24 h. Data are presented for 10 donors at all time points except at 12 h for which data from 14 donors are shown. Horizontal lines indicate median values.

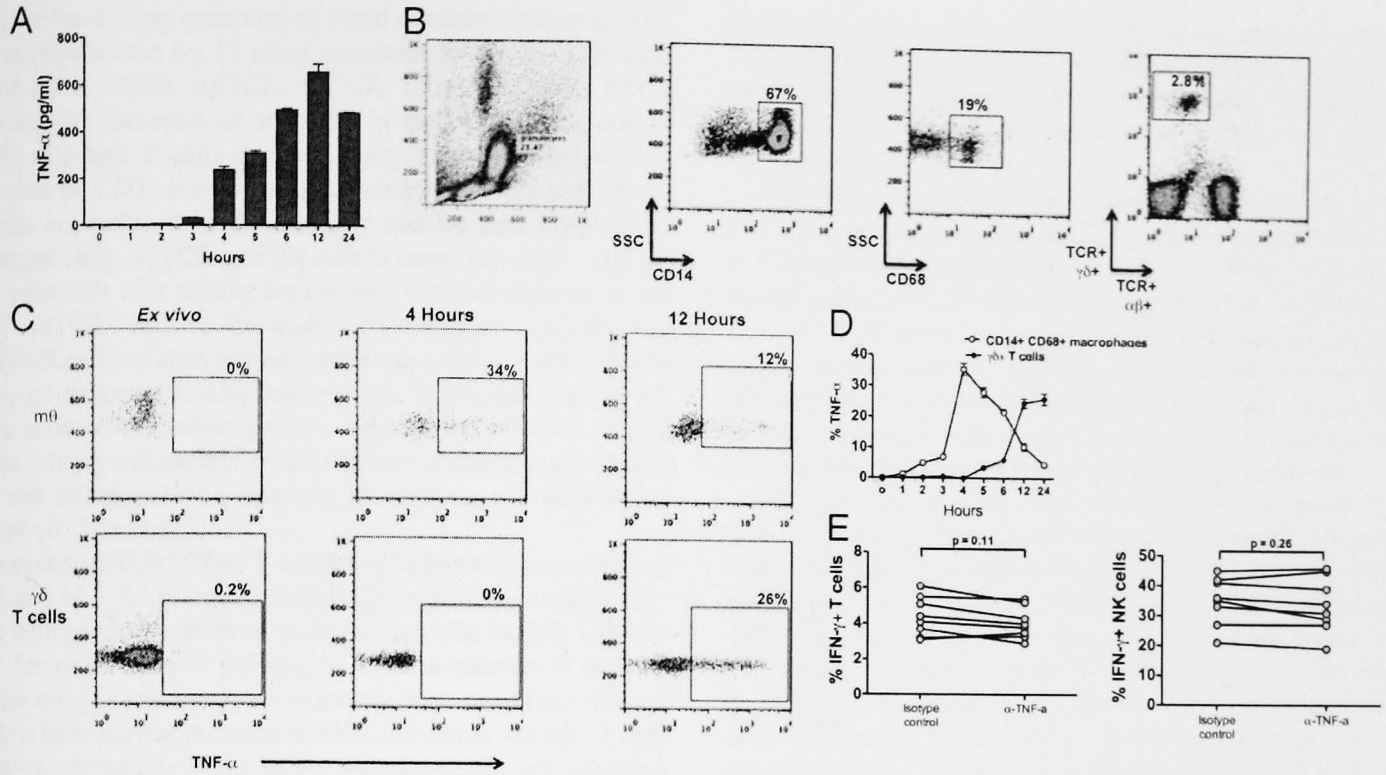


FIGURE 7. Rapid TNF production from macrophages and $\gamma\delta^+$ T cells in response to *P. falciparum*-infected RBCs. PBMCs were incubated with *P. falciparum*-infected RBCs for up to 24 h. *A*, Median concentration of TNF in culture supernatants, determined by cytometric bead array, over time. $n = 5$ donors. *B*, Gating strategy for phenotypic characterization of TNF-producing macrophages ($CD14^+CD68^+$) and $\gamma\delta^+$ T cells (TCR-$\gamma\delta^+$ TCR-$\alpha\beta^-$ when gated on all $CD3^+$ cells) after 12 h PBMC coculture with *P. falciparum*-infected RBCs. *C*, Representative flow cytometry data from one donor showing the percentages of macrophages (*top panels*) or $\gamma\delta^+$ T cells (*bottom panels*) expressing intracellular TNF after 4 or 12 h. *D*, PBMCs from eight donors were incubated for up to 24 h with *P. falciparum*-infected RBCs, and percentages of macrophages (light circles) and $\gamma\delta^+$ T cells (dark circles) producing TNF-α were measured by flow cytometry. Data represent medians (± SE). *E*, PBMCs from eight donors with previously documented high IFN-γ responses to *P. falciparum*-infected RBCs were incubated for 24 h with *P. falciparum*-infected RBCs in the presence of neutralizing Ab to TNF or with an isotype-matched control Ab and the percentages of T cells (*left panel*) and NK cells (*right panel*) expressing IFN-γ were measured. The *p* values are for two-tailed paired Wilcoxon tests, 95% CI.

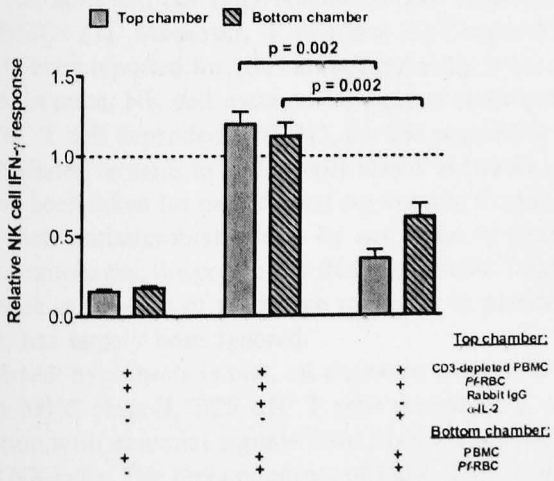


FIGURE 8. T cell help for NK cell activation by *P. falciparum*-infected RBCs does not require NK cell-T cell contact. PBMCs were placed in the lower well and CD3-depleted PBMCs were placed in the upper well of a transwell chamber separated by a semipermeable membrane and cultured in the presence (+) or absence (−) of *P. falciparum*-infected RBCs, anti–IL-2 Ab, or isotype-matched control Ab (rabbit IgG). The percentage of NK cells in each chamber expressing IFN-γ was determined after 24 h. Data for each donor were normalized to the response given by their own PBMCs cultured directly with *P. falciparum*-infected RBCs in the lower chamber (median 41%, interquartile ranges 6%) and are shown as medians and interquartile ranges for five donors. The *p* values are for two-tailed paired Wilcoxon tests, 95% CI.

malaria-naive donors is in full agreement with both of our own earlier studies (5) and others (20). D'Ombrain et al. (9) concluded that NK cells do not contribute significantly to the innate IFN-γ response to *P. falciparum*-infected RBCs; however, their studies did not include the very early time points (12–18 h) at which we observe maximal NK cell responses, suggesting that an NK response may have been present in their donors but was missed, although it is surprising that D'Ombrain et al. (9) report so few IFN-γ^+ NK cells at 24 h. Although Hensmann et al. (10) stained for CD3, CD56, and IFN-γ after 18 h coculture with *P. falciparum*-infected RBCs, they studied only four donors and did not explicitly report the NK cell IFN-γ response; from the data presented in their paper, it is possible that between 10% and 40% of IFN-γ^+ cells in their donors could have been NK cells, which is well within the range that we have observed in our much larger donor panel.

More problematic is our observation that the vast majority of IFN-γ–producing T cells (at all time points from 6 h to 6 d of *P. falciparum*-infected RBC coculture) are TCR $\alpha\beta^+$ and $CD4^+$; this finding is in direct contradiction to the findings of both Hensmann et al. (10) and D'Ombrain et al. (9), who suggested that $\gamma\delta^+$ T cells are the major producers of IFN-γ in response to *P. falciparum*-infected RBC stimulation. The reason for these conflicting results is unclear. It is possible that there are technical explanations. The three studies used different *P. falciparum* clones, which might differ in their production of the phosphorylated nonpeptidic Ags, which are believed to be the ligands for $V\gamma9^+$ T cells (21), although our observation of very robust TNF response to *P. falciparum*-infected RBCs by γδT cells tends to argue against this explanation. We have previously observed that γδT cells require quite considerable amounts of IL-2 to proliferate in response to *P. falciparum*-infected RBCs (22); we do not routinely add exogenous IL-2 or other costimulatory agents to our cultures, which may explain the lack of γδT cell IFN-γ responses in our experiments, but again the robust γδ T cell TNF response suggests that this is not the explanation.

Lastly, we have taken great care to avoid misclassification of lymphocyte populations by 1) using simultaneous seven-color analysis of CD3, CD56, αβTCR, γδTCR, CD4, CD8, and IFN-γ expression; 2) inclusion of anti-CD3 in the intracellular mix to identify activated T cells that may have downregulated surface expression of CD3; and 3) using a biotinylated anti-γδTCR Ab that does not suffer from the tendency that we have observed of some supposedly γδTCR-specific Abs to stain both $CD3^-$ and $\alpha\beta$ TCR^+ cells. We note with some concern that the majority of the IFN-γ^+ γδTCR^+ cells in the study by D'Ombrain et al. (9) also express NK cell markers, raising interesting questions as to the true identity of these cells. Alternatively, the differences seen in the various studies may reflect genuine heterogeneity in γδT cell responses among individuals or populations, reflecting the known inter- and intrapopulation diversity in numbers and TCR repertoires of $\gamma\delta^+$ T cells (23).

The strong αβTCR^+ $CD4^+$ T cell IFN-γ response that we observed after 2–6 d of *P. falciparum*-infected RBC activation is highly consistent with previous reports in naive humans (13, 14, 24), with evidence for cross-reactive priming of malaria-reactive T cells by a diverse array of commonly encountered micro-organisms (13) and with data from murine infection models, indicating that $\alpha\beta^+$ T cells can play a crucial role in the IFN-γ response to primary infections (25–27). It is difficult to predict whether such cross-reactive responses would be beneficial should these individuals become infected with malaria; naive adults show very variable responses to primary malaria infections with some making very rapid, and partially protective, proinflammatory responses, whereas others make little or no inflammatory response and develop rapidly escalating parasitemia (28, 29).

A somewhat unexpected finding of this study was the absolute dependency of NK cells on $CD4^+$ T cell-derived IL-2 for their response to *P. falciparum*-infected RBCs. In hindsight, this should not have been a surprise. We have previously shown that NK cell responses to *P. falciparum*-infected RBCs depend upon cytokine and contact-mediated signals from myeloid cells (7), and we did notice in those experiments that NK responses were diminished by anti–IL-2 and that adding purified adherent accessory cells to isolated NK cells did not fully restore the NK response seen in intact PBMCs (7). Moreover, T cell and IL-2 dependency has previously been reported for NK cell activation by influenza virus (18), and, in mice, NK cell cytotoxicity against some tumor cells is αβTCR^+ T cell dependent (30, 31), but the possibility that this is IL-2 mediated remains to be formally tested. However, although it has long been taken for granted that Ag-specific T cells mediate some of their antimicrobial effects by activation of innate cells, such as macrophages, the possibility that Ag-specific T cells might interact with other cells of the innate response, in particular with NK cells, has largely been ignored.

Our current hypothesis is that, on exposure to cognate Ag presented by MHC class II, TCR $\alpha\beta^+$ T cells secrete IL-2, which, in combination with essential signals from myeloid accessory cells, activates NK cells. The preponderance of $CD4^+$ T cells among the IL-2^+ cell population is consistent with the very marked effect on the NK cell response of MHC class II blockade. The transwell experiments indicate that the IL-2 is secreted from the T cells and diffuses in solution toward the NK cells; there is no apparent requirement for contact between the T cell and the NK cell or for the focal secretion of IL-2 into an immune synapse. However, it is likely that T cells also provide other NK cell-potentiating signals. For example, although we found no evidence that *P. falciparum*-infected RBC-activated γδ T cells produced significant amounts of IL-2, depletion of γδ T cells from PBMCs substantially reduced the NK cell IFN-γ response. We do not believe that this is simply due to a reduction in the proportion of T cells in the cultures because γδ T cells represent a very small proportion of the total T cell pool and an equally small proportion of the IL-2^+ T cells. We have not yet identified the γδ T cell contribution to the NK response, but it does not appear to be TNF.

Our data suggest that full reactivation of IFN-γ–producing T cells also requires IL-2–mediated signals because the peak of the T cell IL-2 response preceded the peak of the T cell IFN-γ response, and neutralization of IL-2 markedly reduced IFN-γ responses of T cells as well as NK cells. However, NK cell IFN-γ responses peaked within 6 h of the peak IL-2 response, whereas T cell IFN-γ responses did not peak until ~36 h after the peak IL-2 response, suggesting that T cells may require higher concentrations of IL-2 or more sustained IL-2 signaling to become fully activated than do NK cells.

Our observations imply that enhancing T cell responses to malaria (i.e., by vaccination) should also enhance the NK cell response. Given its speed (which precedes the bulk of the T cell IFN-γ response by hours or days), the NK IFN-γ response may represent an important determinant of vaccine efficacy, and the ability of vaccine-induced T cells to support NK cell effector function might be an important biomarker of an effective T cell response to the vaccine. Finally, our demonstration that NK cell responses to *P. falciparum*-infected RBCs are so highly dependent on T cell IL-2 may necessitate reconsideration of data based simply on T cell depletion of PBMCs; the possibility that the IFN-γ–producing cells might be NK cells, and that the main role of T cells might be to secrete IL-2, needs to be considered, and IFN-γ ELISA, cytometric bead array, or ELISPOT data need to be confirmed by intracellular cytokine staining and flow cytometry.

Acknowledgments

We thank Carolynne Stanley for subject recruitment and sampling, Sarah Nogaro for screening potential donors for IFN-γ expression in response to *P. falciparum*-infected RBC, and Patrick Corran and Jackie Cook for statistical advice.

Disclosures

The authors have no financial conflicts of interest.

References

1. Artavanis-Tsakonas, K., J. E. Tongren, and E. M. Riley. 2003. The war between the malaria parasite and the immune system: immunity, immunoregulation and immunopathology. *Clin. Exp. Immunol.* 133: 145–152.
2. Stevenson, M. M., and E. M. Riley. 2004. Innate immunity to malaria. *Nat. Rev. Immunol.* 4: 169–180.
3. Pombo, D. J., G. Lawrence, C. Hirunpetcharat, C. Rzepczyk, M. Bryden, N. Cloonan, K. Anderson, Y. Mahakunkijcharoen, L. B. Martin, D. Wilson, et al. 2002. Immunity to malaria after administration of ultra-low doses of red cells infected with *Plasmodium falciparum*. *Lancet* 360: 610–617.
4. Roestenberg, M., M. McCall, J. Hopman, J. Wiersma, A. J. Luty, G. J. van Gemert, M. van de Vegte-Bolmer, B. van Schaijk, K. Teelen, T. Arens, et al. 2009. Protection against a malaria challenge by sporozoite inoculation. *N. Engl. J. Med.* 361: 468–477.
5. Artavanis-Tsakonas, K., and E. M. Riley. 2002. Innate immune response to malaria: rapid induction of IFN-gamma from human NK cells by live *Plasmodium falciparum*-infected erythrocytes. *J. Immunol.* 169: 2956–2963.
6. Korbel, D. S., K. C. Newman, C. R. Almeida, D. M. Davis, and E. M. Riley. 2005. Heterogeneous human NK cell responses to *Plasmodium falciparum*-infected erythrocytes. *J. Immunol.* 175: 7466–7473.
7. Newman, K. C., D. S. Korbel, J. C. Hafalla, and E. M. Riley. 2006. Cross-talk with myeloid accessory cells regulates human natural killer cell interferon-gamma responses to malaria. *PLoS Pathog.* 2: e118.
8. Korbel, D. S., P. J. Norman, K. C. Newman, A. Horowitz, K. Gendzekhadze, P. Parham, and E. M. Riley. 2009. Killer Ig-like receptor (KIR) genotype predicts the capacity of human KIR-positive CD56dim NK cells to respond to pathogen-associated signals. *J. Immunol.* 182: 6426–6434.
9. D'Ombrain, M. C., D. S. Hansen, K. M. Simpson, and L. Schofield. 2007. gammadelta-T cells expressing NK receptors predominate over NK cells and conventional T cells in the innate IFN-gamma response to *Plasmodium falciparum* malaria. *Eur. J. Immunol.* 37: 1864–1873.
10. Hensmann, M., and D. Kwiatkowski. 2001. Cellular basis of early cytokine response to *Plasmodium falciparum*. *Infect. Immun.* 69: 2364–2371.

11. Horowitz, A., and E. M. Riley. 2010. Activation of human NK cells by malaria-infected red blood cells. *Methods Mol. Biol.* 612: 429–446.
12. Artavanis-Tsakonas, K., K. Eleme, K. L. McQueen, N. W. Cheng, P. Parham, D. M. Davis, and E. M. Riley. 2003. Activation of a subset of human NK cells upon contact with *Plasmodium falciparum*-infected erythrocytes. *J. Immunol.* 171: 5396–5405.
13. Currier, J., J. Sattabongkot, and M. F. Good. 1992. 'Natural' T cells responsive to malaria: evidence implicating immunological cross-reactivity in the maintenance of TCR alpha beta+ malaria-specific responses from non-exposed donors. *Int. Immunol.* 4: 985–994.
14. Dick, S., M. Waterfall, J. Currie, A. Maddy, and E. Riley. 1996. Naive human alpha beta T cells respond to membrane-associated components of malaria-infected erythrocytes by proliferation and production of interferon-gamma. *Immunology* 88: 412–420.
15. Jones, K. R., J. K. Hickling, G. A. Targett, and J. H. Playfair. 1990. Polyclonal in vitro proliferative responses from nonimmune donors to *Plasmodium falciparum* malaria antigens require UCHL1+ (memory) T cells. *Eur. J. Immunol.* 20: 307–315.
16. Riley, E. M. 1999. Is T-cell priming required for initiation of pathology in malaria infections? *Immunol. Today* 20: 228–233.
17. Fehniger, T. A., M. A. Cooper, G. J. Nuovo, M. Cella, F. Facchetti, M. Colonna, and M. A. Caligiuri. 2003. CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. *Blood* 101: 3052–3057.
18. He, X. S., M. Draghi, K. Mahmood, T. H. Holmes, G. W. Kemble, C. L. Dekker, A. M. Arvin, P. Parham, and H. B. Greenberg. 2004. T cell-dependent production of IFN-gamma by NK cells in response to influenza A virus. *J. Clin. Invest.* 114: 1812–1819.
19. Robinson, L. J., M. C. D'Ombrain, D. I. Stanisic, J. Taraika, N. Bernard, J. S. Richards, J. G. Beeson, L. Tavul, P. Michon, I. Mueller, and L. Schofield. 2009. Cellular tumor necrosis factor, gamma interferon, and interleukin-6 responses as correlates of immunity and risk of clinical *Plasmodium falciparum* malaria in children from Papua New Guinea. *Infect. Immun.* 77: 3033–3043.
20. Baratin, M., S. Roetynck, B. Pouvelle, C. Lemmers, N. K. Viebig, S. Johansson, P. Bierling, A. Scherf, J. Gysin, E. Vivier, and S. Ugolini. 2007. Dissection of the role of PfEMP1 and ICAM-1 in the sensing of Plasmodium-falciparum-infected erythrocytes by natural killer cells. *PLoS ONE* 2: e228.
21. Behr, C., R. Poupot, M. A. Peyrat, Y. Poquet, P. Constant, P. Dubois, M. Bonneville, and J. J. Fournie. 1996. *Plasmodium falciparum* stimuli for human gammadelta T cells are related to phosphorylated antigens of mycobacteria. *Infect. Immun.* 64: 2892–2896.
22. Waterfall, M., A. Black, and E. Riley. 1998. Gammadelta+ T cells preferentially respond to live rather than killed malaria parasites. *Infect. Immun.* 66: 2393–2398.
23. Carding, S. R., and P. J. Egan. 2002. Gammadelta T cells: functional plasticity and heterogeneity. *Nat. Rev. Immunol.* 2: 336–345.
24. Fell, A. H., J. Currier, and M. F. Good. 1994. Inhibition of *Plasmodium falciparum* growth in vitro by CD4+ and CD8+ T cells from non-exposed donors. *Parasite Immunol.* 16: 579–586.
25. Berg, R. E., E. Crossley, S. Murray, and J. Forman. 2005. Relative contributions of NK and CD8 T cells to IFN-gamma mediated innate immune protection against *Listeria monocytogenes*. *J. Immunol.* 175: 1751–1757.
26. Le-Barillec, K., J. G. Magalhaes, E. Corcuff, A. Thuizat, P. J. Sansonetti, A. Phalipon, and J. P. Di Santo. 2005. Roles for T and NK cells in the innate immune response to *Shigella flexneri*. *J. Immunol.* 175: 1735–1740.
27. Lertmemongkolchai, G., G. Cai, C. A. Hunter, and G. J. Bancroft. 2001. Bystander activation of CD8+ T cells contributes to the rapid production of IFN-gamma in response to bacterial pathogens. *J. Immunol.* 166: 1097–1105.
28. Walther, M., J. E. Tongren, L. Andrews, D. Korbel, E. King, H. Fletcher, R. F. Andersen, P. Bejon, F. Thompson, S. J. Dunachie, et al. 2005. Upregulation of TGF-beta, FOXP3, and CD4+CD25+ regulatory T cells correlates with more rapid parasite growth in human malaria infection. *Immunity* 23: 287–296.
29. Walther, M., J. Woodruff, F. Edele, D. Jeffries, J. E. Tongren, E. King, L. Andrews, P. Bejon, S. C. Gilbert, J. B. De Souza, et al. 2006. Innate immune responses to human malaria: heterogeneous cytokine responses to blood-stage *Plasmodium falciparum* correlate with parasitological and clinical outcomes. *J. Immunol.* 177: 5736–5745.
30. Arina, A., O. Murillo, S. Hervás-Stubbs, A. Azpilikueta, J. Dubrot, I. Tirapu, E. Huarte, C. Alfaro, J. L. Pérez-Gracia, G. González-Aseguinolaza, et al. 2007. The combined actions of NK and T lymphocytes are necessary to reject an EGFP+ mesenchymal tumor through mechanisms dependent on NKG2D and IFN gamma. *Int. J. Cancer* 121: 1282–1295.
31. Shanker, A., G. Verdeil, M. Buferne, E. M. Inderberg-Suso, D. Puthier, F. Joly, C. Nguyen, L. Leserman, N. Auphan-Anezin, and A. M. Schmitt-Verhulst. 2007. CD8 T cell help for innate antitumor immunity. *J. Immunol.* 179: 6651–6662.

Appendix 2

IK Cells as Effectors of Acquired Immune Responses: ffector CD4$^+$ T Cell-Dependent Activation of NK Cells ollowing Vaccination

mir Horowitz,* Ron H. Behrens,*,† Lucy Okell,* Anthony R. Fooks,‡ and leanor M. Riley*

e characterized vaccine-induced cellular responses to rabies virus in naive adult volunteers. Contrary to current paradigms, we served potent and prolonged in vitro NK cell cytokine production and degranulation responses after restimulation of PBMCs with activated rabies virus in vaccinated, but not in unvaccinated, individuals. This "recall" NK cell response was absolutely dendent on Ag-specific IL-2 from CD45RO$^+$ CD4$^+$ T cells as well as IL-12 and IL-18 from accessory cells. Importantly, NK cells presented over 70% of all IFN-γ–secreting and degranulating cells in the first 12–18 h after virus rechallenge indicating they ay be required for rapid control of infection after vaccination. Activation of NK cells may be a critical function of IL-2–secreting ector memory T cells. Although IL-2–dependent postvaccination NK cell activation has been reported previously, this is the first ne the magnitude of this effect and its contribution to the overall vaccine-induced response has been appreciated and the echanisms of NK activation postvaccination have been elucidated. Our data will allow standard protocols for evaluating vaccine-duced immunity to be adapted to assess NK cell effector responses. *The Journal of Immunology*, 2010, 185: 2808–2818.

he innate immune system is designed to provide rapid but generic responses to foreign organisms. Conversely, adaptive responses are slow (requiring clonal expansion of ive precursors to generate sufficiently large populations of ef-ctor and memory cells) but highly specific. Bidirectional inter-tions between innate and adaptive immune systems are essential immunity to infection and for successful vaccination. Innate ector cells amplify and direct the subsequent adaptive response -3), whereas effector T cells activate innate cells to kill pathogens pathogen-infected cells (4).

NK cells are innate immune effectors that, by cytokine pro-ction or cytotoxicity, help to contain an infection until an ef-tive adaptive response is mounted. NK cells become activated en the balance of activating and inhibitory signals that they eive is disturbed (5). Direct NK cell activation follows in-action with cells that (due to mutation or infection) lack ligands inhibitory NK cell surface receptors for self-MHC (the "missing-f" phenomenon) and/or express stress-induced ligands for NK ll-activating receptors. Indirect NK cell activation occurs after microbial ligation of pattern recognition receptors on myeloid accessory cells and is mediated by cytokines (IL-12, IL-18, and IFN-α) and by contact-dependent stimuli from myeloid cells (e.g., ICAM-1/LFA-1) (6).

Our investigations of the pathways of NK cell activation by *Plasmodium falciparum* (malaria)-infected RBCs revealed essential roles for IL-12 and accessory cell contact as well as subsidiary roles for IL-18 and IFN-α (7–9). However, optimal NK cell responses to malaria-infected cells were also dependent on IL-2 (7) and although myeloid cells were essential (7), we were never able to fully recapitulate the NK cell response of mixed PBMCs by addition of myeloid cells (plastic adherent monocytes, macrophages, and dendritic cells) to purified NK cells (A. Horowitz, unpublished data). These observations (A. Horowitz, unpublished data), bolstered by reports of T cell and IL-2–dependent activation of human NK cells (8, 9) and of an increase in the frequency of IFN-γ–producing NK cells among restimulated PBMCs after influenza vaccination (10) led us to speculate that IL-2 from Ag-specific T cells might allow NK cells to contribute to the effector arm of adaptive immune responses and thus NK cells may make a significant contribution to the cellular effector response to vaccination.

In this study, we tested the hypothesis that NK cell responses are specifically enhanced, after vaccination, by IL-2 emanating from Ag-specific T cells. Using rabies virus vaccination as our model system (as inapparent exposure to rabies virus is likely to be extremely rare and our volunteers are thus expected to be fully naive prior to vaccination), we demonstrate that NK cells are the major contributors to the immediate vaccine-specific cytokine and cytotoxic recall response, that this response is dependent on IL-2 from Ag-specific memory T cells and is associated with extensive NK cell proliferation. We conclude that NK cells represent an important, and underappreciated, component of the adaptive immune response; that activation of NK cells may be a critical function of IL-2 secreting effector memory T cells; and that protocols for evaluation of vaccine-induced immune responses need to include assessment of NK cell activation as well as T cell IL-2 secretion.

epartment of Infectious and Tropical Diseases, London School of Hygiene and pical Medicine; †Department of Travel Medicine, Hospital for Tropical Diseases, ndon; and ‡Rabies and Wildlife Zoonoses Group, World Health Organization laborating Centre of Rabies and Rabies-Related Viruses, Veterinary Laboratories ency, Surrey, United Kingdom

eived for publication March 15, 2010. Accepted for publication July 1, 2010.

s work was supported by the United Kingdom Medical Research Council (Grant 400225) and the University College London Hospitals' Comprehensive Biomed-Research Centre.

dress correspondence and reprint requests to Prof. Eleanor M. Riley, London ool of Hygiene and Tropical Medicine, Room 236, Keppel Street, London, United gdom WC1E 7HT. E-mail address: eleanor.riley@lshtm.ac.uk

online version of this article contains supplemental material.

reviations used in this paper: GM, growth medium; MCMV, murine CMV; MFI, n fluorescence intensity; PRR, pattern recognition receptor; rNCP, recombinant es virus nucleocapsid protein.

w.jimmunol.org/cgi/doi/10.4049/jimmunol.1000844

Materials and Methods

Vaccination of study participants

Thirty healthy adult volunteers (median age = 28 y; intraquartile range = 26–31 y) received three doses (days 0, 7, and 21) of 2.5 IU heat-inactivated rabies virus (Flury LEP strain) (RABIPUR, Novartis vaccines) by i.m. injection. All subjects gave fully informed, written consent, and the study was approved by the London School of Hygiene and Tropical Medicine Ethics Committee.

Blood sampling, PBMC preparation, and cell culture

PBMC collected immediately before the first and third vaccinations (i.e., day 0, day 21) were cryopreserved in liquid nitrogen so that pre- and postvaccination samples could be analyzed side-by-side at the end of the study. PBMCs (2×10^6 cells/ml) were cultured in 96-well U-bottom plates for up to 7 d (with or without 25 ng/ml PMA plus 1 μg/ml ionomycin [both Sigma-Aldrich, St. Louis, MO] [P/I], rhIL-12, rhIL-18, or rhIL-2 [0.1 μg/ml] or 200 arbitrary units/ml inactivated rabies virus 07/162 [5th International Standard; National Institute for Biological Standards and Control, Potters Bar, U.K.]). Neutralizing anti–IL-2 (MQ1-17H12; BD Biosciences, San Jose, CA), or anti–IL-12 and anti–IL-18 (R&D Systems, Minneapolis, MN) were each used at final concentrations of 10 μg/ml. Recombinant rabies virus nucleocapsid protein (rNCP) (M. Juozapaitis, Institute of Biotechnology, Vilnius, Lithuania) was used at a final concentration of 10 μg/ml. Brefeldin A (3 μg/ml) and monensin (1.33 μg/ml) were added to all cultures for the last 5 h of the culture.

$CD3^+$ cells were positively selected using FlowComp human CD3 Dynabeads and a Dynal Magnetic Particle Concentrator-2 (both Invitrogen, Carlsbad, CA), according to the manufacturer's instructions and purity confirmed with anti-human CD3 Ab (clone UCHT-1; BD Biosciences). Purity of both $CD3^+$ and $CD3^-$ cell populations was >97%. T cell subsets were depleted on LD separation columns (Miltenyi Biotec, Auburn, CA) using mAbs to CD4 (BD Biosciences) or CD8 (Caltag Laboratories, Burlingame, CA) or anti-CD45RO–coated microbeads (Miltenyi Biotec).

PBMCs labeled with 10 μM CFSE (Invitrogen) were cultured at 37°C with 5% CO_2 for up to 7 d with or without rabies virus 07/162 and analyzed by flow cytometry.

Cell surface and intracellular staining for flow cytometry

Surface and intracellular staining was performed as previously described previously (11). Abs used were as follows: CD56 PE (N901), CD56 APC (N901) (both Beckman Coulter, Fullerton, CA), CD56 PE-Cy7 (B159), CD45RO PE-Cy7 (UCHL1), CD3-PerCP (SK7), CD4 APC-Cy7 (RPA-T4), CD4 PE (RPA-T4),CD8 PE (SK1), LAMP-1 biotin (H4A3), Perforin PE (δG9), IL-2 APC (MQ1-17H12), streptavidin-PerCP (all BD Biosciences), anti-CD69 PE (CH/4), and CD3 PE-Texas Red (S4.1) (both Caltag/Invitrogen), CD8 Pacific Blue (LT8; eBiosciences, San Diego, CA) and IFN-γ FITC (D9D10; Ab Serotec).

Data analysis

Data analyses were performed using prism5 (GraphPad, San Diego, CA) or Stata10 (StataCorp, College Station, TX). To estimate the proportion of the precursor lymphocyte population proliferating through each division, the number of CFSE-diluted cells in each division was divided by $2^{(\text{number of divisions} + 0.5)}$ (12).

Results

T cells and NK cells upregulate CD69 and produce IFN-γ in response to rabies Ag, after rabies vaccination

PBMCs isolated from vaccinees immediately before their first and third vaccinations (day 0 and day 21) were cultured for 21 h without stimulation (growth medium [GM]), with P/I (positive control) or with inactivated rabies virus (07/162) as the specific recall Ag. Cell surface expression of CD69 and intracellular expression of IFN-γ were analyzed in $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T cells and in $CD56^+$ $CD3^-$ NK cells (Fig. 1*A*). A representative example of IFN-γ and CD69 expression before (*left panels*) or after (*right panels*) two doses of rabies vaccine is shown in Fig. 1*B*, and data from five individual vaccinees are shown in Fig. 1*C* and 1*D*. In the prevaccination samples, P/I induced upregulation of both IFN-γ and CD69 in $CD4^+$ and $CD8^+$ T cells and in NK cells but none of these cells responded to rabies virus 07/162. By contrast, in the postvaccination (day 21) samples, rabies virus 07/162 induces highly significant upregulation of IFN-γ and CD69 in $CD4^+$ and $CD8^+$ T cells *and* in NK cells. Moreover, among NK cells the IFN-γ responses are as high as those induced by the polyclonal stimulator P/I (with at least 30% of all NK cells staining positively for IFN-γ) and the mean fluorescence intensity (MFI) for IFN-γ staining is significantly higher among NK cells than among T cells (Fig. 1*B*, 1*D*). Both $CD56^{bright}$ and $CD56^{dim}$ NK cells produce IFN-γ in response to rabies virus 07/162 (data not shown) and although a higher percentage of $CD56^{bright}$ NK cells than $CD56^{dim}$ cells make IFN-γ (~50 versus ~20%), because $CD56^{bright}$ cells make up only a small proportion of the NK cell population, the majority (>75%) of the IFN-γ^+ NK cells are in fact $CD56^{dim}$.

Postvaccination, NK cells make both early and sustained IFN-γ responses to vaccine Ags

Having found that vaccination primes NK cells to make a robust and immediate IFN-γ recall response to vaccine Ag, we sought to determine how quickly this NK response develops and for how long it is sustained, by sampling cultured PBMCs at intervals over a 7-d period (Fig. 2). Restimulation of postvaccination PBMCs with rabies virus 07/162 leads to an initial, modest peak in IFN-γ production from both $CD4^+$ and $CD8^+$ T cells at 18–24 h, but there is a second much more robust response that begins on day 5 and which is still increasing on day 7 (Fig. 2*A*). By contrast, a very robust NK cell recall response is clearly underway within 12 h and peaks at 18 h; however, there was also a second wave of NK cell IFN-γ production, coinciding with the secondary wave of the T cell response, such that ~30% of NK cells were still making IFN-γ 7 d into the recall response. This biphasic NK cell and T cell response may reflect an initial responding population giving rise to a second generation of responsive cells or providing essential stimuli for further cell activation. In either case, it is apparent that NK cells are not only the earliest contributors of IFN-γ to the postvaccination recall response but also contribute to this response for a protracted period.

Although the proportion of NK cells producing IFN-γ in the restimulated postvaccination samples was much higher than the proportion of T cells producing IFN-γ, circulating T cells outnumber NK cells by ~10:1. We therefore calculated the contribution of NK cells to the total pool of IFN-γ–producing cells (Fig. 2*B*). Twelve and 18 h after in vitro restimulation with rabies virus 07/162, >70% of all IFN-γ^+ cells were NK cells. Although this dropped to ~40% by 24 h, NK cells continued to represent 30–50% of all IFN-γ^+ cells until at least 7 d after restimulation. Thus, not only are NK cells readily activated postvaccination, but they contribute very significantly to the total effector cell pool during the first 7 d of the recall response. When taken together with the very high MFI for IFN-γ staining among NK cells (Fig. 1) these data suggest that the overwhelming majority of the early IFN-γ emanates from NK cells.

NK cells degranulate and release perforin in response to rabies Ag after rabies vaccination

One of the hallmarks of NK cells is their ability to rapidly detect and kill (within minutes) infected, transformed, or nonself cells by releasing preformed cytolytic molecules, such as perforin and granzymes, stored in secretory granules. Although $CD8^+$ T cells kill in a similar manner, their stores of cytolytic granules are very limited and $CD8^+$ T cells thus need time, after reactivation, to synthesize cytolytic granules and become fully cytotoxic (13). To determine whether NK cytotoxic responses are enhanced postvaccination and whether NK cells might thus represent the first wave of actively cytotoxic cells during the recall response, we cultured pre- and postvaccination PBMCs with P/I or rabies virus

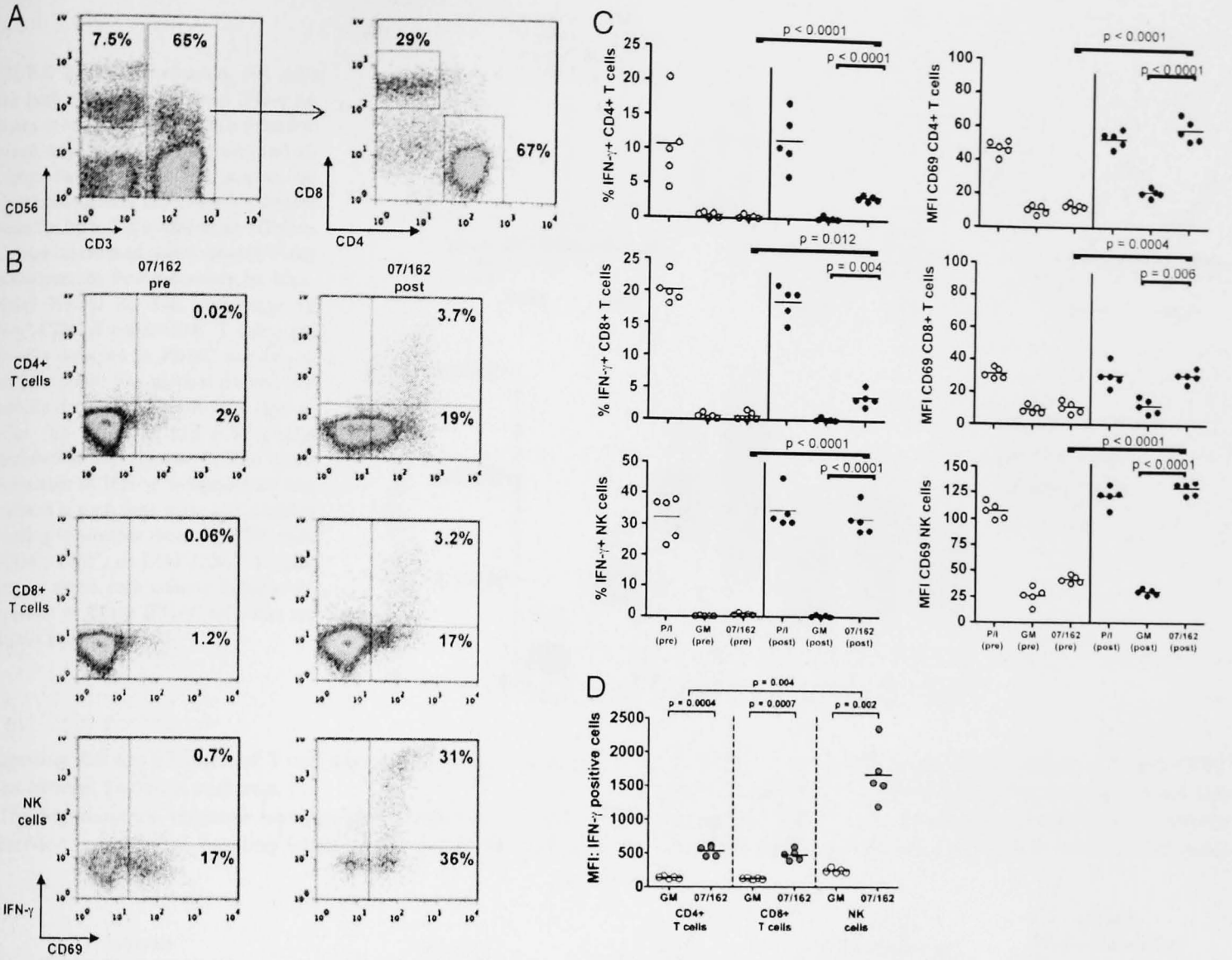


FIGURE 1. After rabies vaccination, T cells and NK cells upregulate CD69 and produce IFN-γ in response to rabies virus. PBMCs were isolated before (pre) and after (post) two doses of rabies vaccine, cultured in vitro for 21 h in GM alone (GM) or with PMA and Ionomycin (P/I) or heat-inactivated rabies virus (07/162) and analyzed by flow cytometry for surface expression of CD69 and intracellular IFN-γ. *A*, Lymphocytes (identified by characteristic forward light scatter and side light scatter) were identified as NK cells ($CD56^+$ $CD3^-$), $CD4^+$ $CD3^+$ T cells, or $CD8^+CD3^+$ T cells. *B*, Representative flow cytometry plots for PBMCs obtained before or after rabies vaccination from one donor, showing IFN-γ and CD69 expression in $CD4^+$ T cells, $CD8^+$ T cells, and NK cells after restimulation in vitro with rabies virus 07/162. *C*, Percentages of IFN-γ^+ cells (*left plots*) and MFI of CD69 expression (*right plots*) among $CD4^+$ T cells, $CD8^+$ T cells, and NK cells isolated from five subjects, before (open circles) and after (filled circles) vaccination, and restimulated in vitro. *D*, MFI of IFN-γ^+ $CD4^+$ T cells, $CD8^+$ T cells, and NK cells among postvaccination PBMCs from five subjects cultured with (07/162; filled circles) or without (GM; open circles) inactivated rabies virus). The *p* values are derived from two-tailed paired Wilcoxon test.

07/162 and measured surface expression of CD107a/LAMP-1 (as an indicator of degranulation) and intracellular levels of perforin.

Representative flow cytometry plots for CD107a/LAMP-1 and perforin staining of $CD8^+$ T cells and NK cells from pre- and postvaccination PBMCs are shown in Fig. 3; data from five individual vaccinees are shown in Fig. 4*A*. As expected, resting (GM) NK cells do not express surface CD107a/LAMP-1 but resting $CD56^{dim}$ (but not $CD56^{bright}$) NK cells do contain preformed perforin; 12 h polyclonal (P/I) stimulation results in increased surface expression of LAMP-1 and complete loss of intracellular perforin, consistent with NK cell degranulation. In contrast, resting $CD8^+$ T cells do not contain perforin but do synthesize perforin and upregulate LAMP-1 in response to P/I stimulation. Importantly, stimulation with rabies virus 07/162 also causes marked degranulation (upregulation of LAMP-1 and loss of perforin) of $CD56^{dim}$ NK cells in the postvaccination but not in the prevaccination samples. Degranulation in 07/162-restimulated postvaccination $CD8^+$ T cells is less marked, but there is clear induction of perforin synthesis.

To determine whether NK cells or $CD8^+$ T cells were the major contributors to the early cytotoxic response we analyzed the degranulation response of the two cell populations over time (up to 72 h) (Fig. 4*B*) and calculated the absolute number of degranulating cells that were either NK cells or $CD8^+$ T cells at each time point (Fig. 4*C*). There was rapid (within 6 h) and sustained (at least 72 h) upregulation of surface LAMP-1 expression in rabies virus restimulated, postvaccination, NK cells and $CD8^+$ T cells. In NK cells, LAMP-1 upregulation was evident by 6 h, peaked (at >25% of all NK cells) at 12 h and then gradually declined, whereas in $CD8^+$ T cells, LAMP-1 expression was maximal by 6 h (at ~4% of all $CD8^+$ T cells) and was sustained at this level for the duration of the experiment. The kinetics of the perforin response in postvaccination NK cells mirrored the LAMP-1 response, with the proportion of perforin$^+$ cells falling significantly within 6 h and reaching its lowest point at 12 h, indicating perforin release by degranulation in postvaccination NK cells within 6 h of re-exposure to the vaccine Ag. However, perforin took up to 12 h to accumulate in restimulated postvaccination $CD8^+$ T cells

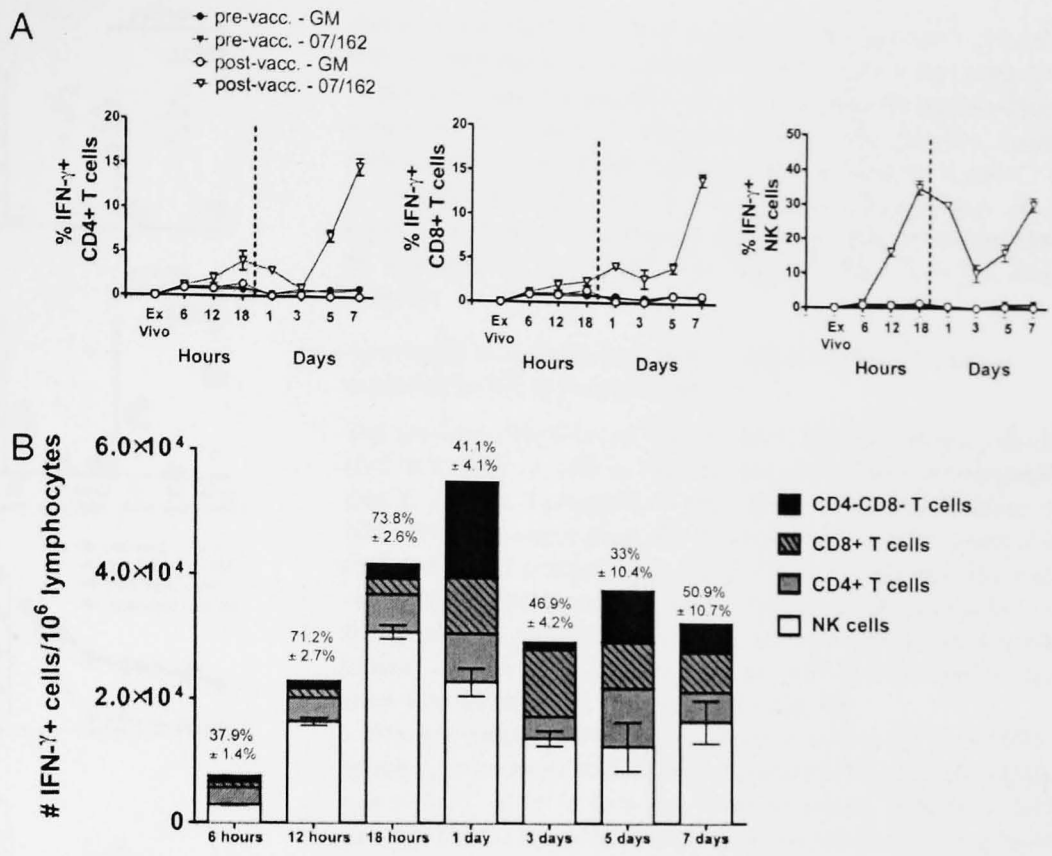


FIGURE 2. Postvaccination, NK cells make both early and sustained IFN-γ responses to vaccine Ags. PBMCs from five subjects were isolated before (pre) and after (post) two doses of rabies vaccine, analyzed immediately (ex vivo) or cultured in vitro for up to 7 d in GM alone (GM) or with heat-inactivated rabies virus (07/162) and analyzed by flow cytometry for intracellular IFN-γ. *A*, The percentage of IFN-γ^+ $CD4^+$ T cells, $CD8^+$ T cells, and NK cells detected in PBMC cultures at each time point. The vertical dashed line separates data from a short-term restimulation (up to 18 h) and a long-term restimulation experiment. *B*, The absolute number of IFN-γ^+ lymphocytes was calculated at each time point and stratified according to whether these were NK cells or $CD4^+$, $CD8^+$, or $CD4^-CD8^-$ T cells. Numbers above each column indicate the proportion of all the IFN-γ^+ cells that are NK cells (mean ± SE).

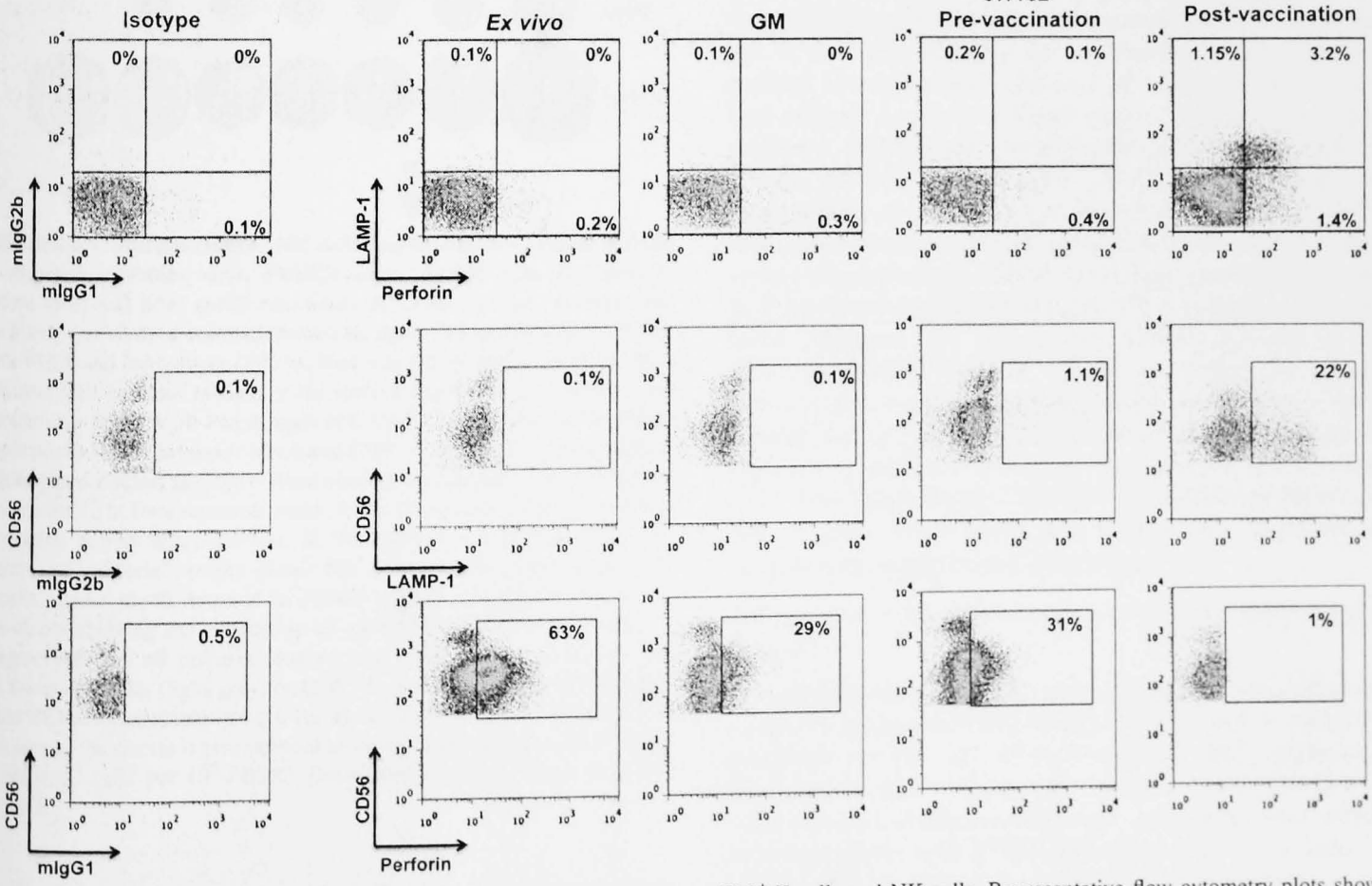


FIGURE 3. Representative staining of LAMP-1 and perforin expression in $CD8^+$ T cells and NK cells. Representative flow cytometry plots showing $CD8^+$ T cell expression of LAMP-1 and perforin (*upper panels*) and LAMP-1 (*middle panels*) and perforin (*lower panels*) expression in NK cells isolated from one donor data before and after rabies vaccination (ex vivo) and after culture in vitro in GM (GM) or with rabies virus 07/162 for 12 h. Isotype matched control Abs (mIgG2b or mIgG1; *left panels*) were used to establish background levels of staining.

suggesting that the 6 h burst of T cell degranulation may not lead to an optimal cytotoxic response.

The degranulation response was Ag-specific because LAMP-1 expression remained at baseline levels in all unstimulated cells and in rabies virus restimulated prevaccination NK and $CD8^+$ T cells. Perforin levels remained at baseline in unstimulated and prevaccination $CD8^+$ T cells. There was a gradual loss of perforin over time in prevaccination NK cells and in unstimulated post-

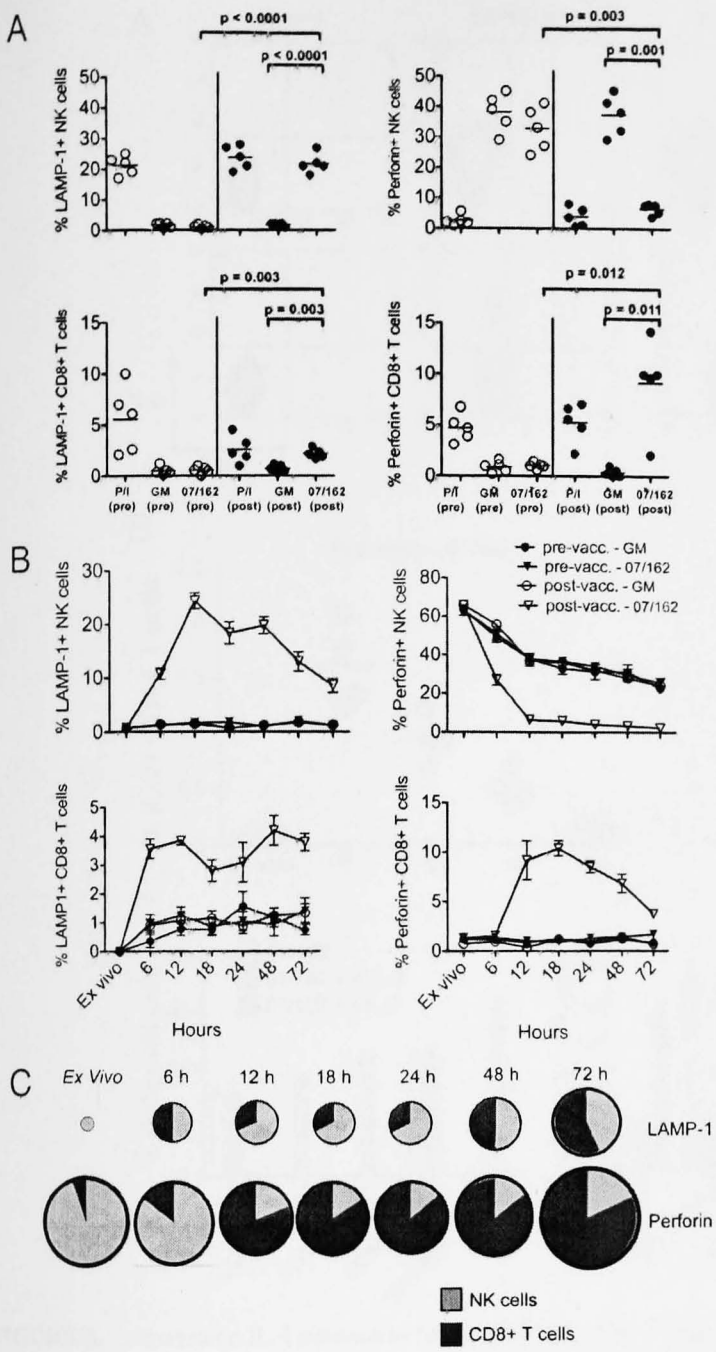


FIGURE 4. Postvaccination, NK cells degranulate and release perforin in response to rabies virus. PBMCs were isolated from five subjects before (pre) and after (post) two doses of rabies vaccine, analyzed immediately (ex vivo) or cultured in vitro for up to 72 h in GM alone (GM) or with PMA and ionomycin (P/I) or heat-inactivated rabies virus (07/162) and analyzed by flow cytometry for surface expression of LAMP-1 and intracellular perforin. *A*, Percentages of LAMP-1$^+$ (*left plots*) and perforin$^+$ (*right plots*) NK cells (*upper plots*) and CD8$^+$ T cells (*lower plots*) isolated before (open circles) and after (filled circles) vaccination, and restimulated in vitro for 12 h. Data represent mean ± SE. The *p* values are derived from two-tailed paired Wilcoxon test. *B*, The percentages of LAMP-1$^+$ (*left plots*) and perforin$^+$ (*right plots*) NK cells (*upper plots*) and CD8$^+$ T cells (*lower plots*) detected in PBMC cultures at each time point. *C*, Pie charts showing the proportion of all LAMP-1 (*top row*) expressing lymphocytes and all perforin (*bottom row*) expressing lymphocytes that are either NK cells (light gray) or CD8$^+$ T cells (black) among postvaccinated PBMCs stimulated in vitro for up to 72 h with rabies virus 07/162. The size of the circles is proportional to the absolute numbers of LAMP-1$^+$ or perforin$^+$ cells per 10^6 PBMC. Data represent mean values from five subjects.

vaccination NK cells, presumably reflecting nonspecific leakage of perforin.

The relative contributions of NK cells and CD8$^+$ T cells to the pool of cytotoxic effectors are shown in Fig. 4*C*. NK cells represent the majority of both LAMP-1$^+$ and perforin$^+$ cells 6 h after Ag stimulation. The marked decrease at 12 h in the proportion of perforin$^+$ cells that are NK cells, together with the increased proportion of the LAMP-1$^+$ cells that are NK cells, provides further evidence of a wave of NK cell degranulation between 6 and 12 h. In contrast, although CD8$^+$ T cells represent the majority of all perforin$^+$ cells from 12 h onward, they do not represent the majority of degranulating, LAMP-1$^+$ cells, until 48–72 h into the recall response.

Ag-specific IL-2 production from CD45RO$^+$ CD4$^+$ T cells is required for NK cell recall responses

Our previous data (7) and that of others (9) indicating a role for IL-2 and CD4$^+$ T cells in NK cell activation, led us to speculate that IL-2 from Ag-specific T cells drives the recall response of NK cells after vaccination. We therefore analyzed the kinetics of IL-2 and IFN-γ production by CD4$^+$ T cells among pre- and postvaccination PBMCs restimulated in vitro with rabies virus 07/162 for up to 24 h. Representative flow cytometry plots for one vaccinated subject are shown in Fig. 5*A* and postvaccination data from four subjects are summarized in Fig. 5*B*.

Prevaccination, CD4$^+$ T cells produce neither IL-2 nor IFN-γ in response to rabies virus 01/162. Postvaccination, a clear population of IL-2$^+$ CD4$^+$ T cells can be distinguished, peaking as early as 6 h after re-exposure to rabies virus and declining to negligible values within 24 h. Very few of the IL-2$^+$ cells produced IFN-γ (Fig. 5*A*) suggesting that at this early stage they are Th0 rather than Th1 effector cells. In line with our hypothesis, the peak of T cell IL-2 production (6 h) preceded the onset of NK cell IFN-γ production (12 h; Fig. 2*A*).

To determine whether this CD4$^+$ T cell IL-2 response contributed to the postvaccination NK cell response, postvaccination PBMCs from five subjects were cultured overnight with rabies virus 07/162 in the presence or absence of rhIL-2 or a neutralizing Ab to IL-2 and analyzed for intracellular IFN-γ, intracellular perforin or cell surface expression of LAMP-1 (Fig. 5*C*; statistical analysis is shown in Supplemental Table I). In parallel experiments, PBMCs were depleted of all CD3$^+$ T cells, just CD4$^+$ T cells, just CD8$^+$ T cells or just CD45RO$^+$CD3$^+$ T cells. In the intact PBMC cultures, as before, there were potent NK cell IFN-γ and degranulation (decreased intracellular perforin and increased surface expression of LAMP-1) responses to the recall Ag. Anti–IL-2 Ab completely ablated both the IFN-γ response and degranulation. Moreover, NK cells among PBMCs depleted of CD3$^+$, CD4$^+$, or CD45RO$^+$CD3$^+$ T cells did not mount any significant IFN-γ or degranulation responses; depletion of CD8$^+$ T cells, in contrast, had no significant detrimental effect on the NK cell recall response. In support of our hypothesis, however, NK cells among CD4$^+$ T cell-depleted and CD45RO$^+$ T cell-depleted PBMCs were able to make robust IFN-γ and degranulation responses when cultures were supplemented with rhIL-2.

Extensive NK cell proliferation during postvaccination recall response

To determine whether IL-2 produced by T cells during the postvaccination recall response induced NK cell proliferation, which might potentiate the NK cell effector response and/or replenish the NK cell pool after activation-induced cell death of the first wave of the effector response, we labeled pre- and postvaccination PBMCs from four donors with CFSE, cultured them for 7 d without stimulation (GM) or with rabies virus 07/162, and then analyzed CFSE expression separately in CD4$^+$ T cells, CD8$^+$ T cells and NK cells (Fig. 6*A*) and in both CD45RO$^+$ and CD45RO$^-$ populations of T cells (Fig. 6*B*). Cells cultured without Ag did not proliferate

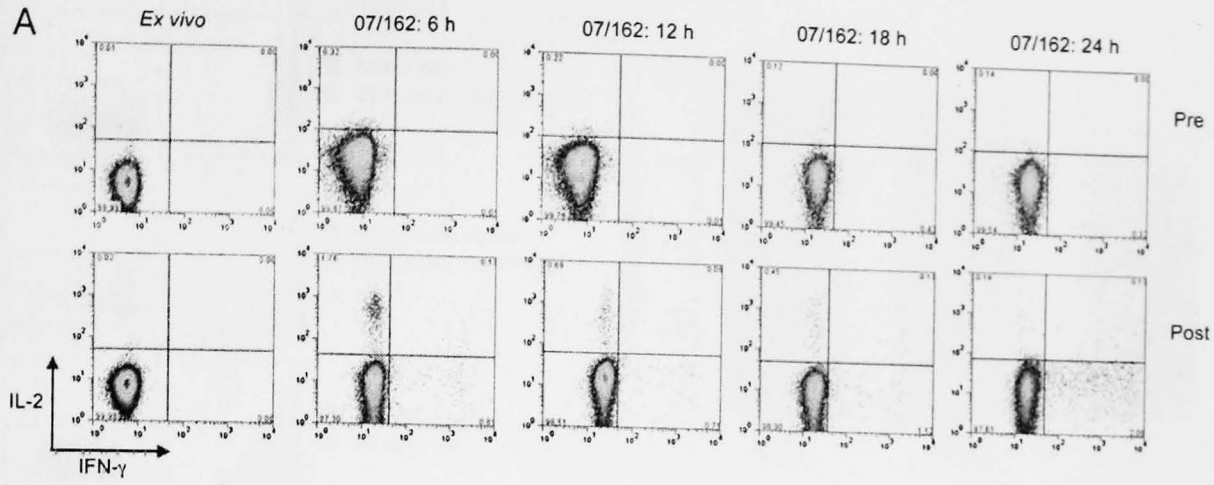


FIGURE 5. Ag-specific IL-2 production from CD45RO$^+$ CD4$^+$ T cells is required for NK cell recall responses. Pre- and postvaccination PBMCs from four subjects were cultured for periods of up to 24 h without stimulation (GM) or with rabies virus (07/162) and analyzed by flow cytometry for intracellular IL-2 and IFN-γ. *A*, Representative flow cytometry plots showing IL-2 and IFN-γ expression in pre- (*upper plots*) and post- (*lower plots*) vaccination CD4$^+$ T cells from one donor in response to 07/162 stimulation over 24 h. *B*, Percentages of CD4$^+$ T cells expressing intracellular IL-2 in response to 07/162 restimulation over time; postvaccination samples from four subjects. *C*, Postvaccination PBMCs from four subjects were cultured for 12 h (perforin and LAMP-1 expression) or 24 h (IFN-γ expression) with rabies virus 07/162 in the presence or absence of rhIL-2 or a neutralizing Ab to IL-2 and analyzed for intracellular IFN-γ (*left panel*), intracellular perforin (*middle panel*), or cell surface expression of LAMP-1 (*right panel*). Parallel experiments were carried out using PBMCs that had been depleted of all CD3$^+$ T cells, just CD4$^+$ T cells, just CD8$^+$ T cells, or just CD45RO$^+$CD3$^+$ T cells. Data represent mean ± SE. Statistical analysis is presented in Supplementary Table I.

(data not shown). Extensive proliferation was observed in postvaccination T cells and NK cells after 7 d restimulation with the recall Ag, rabies virus 07/162. The CFSE dilution patterns (Fig. 6*A*) suggested that a higher proportion of NK cells than T cells were proliferating and that NK cells were going through more rounds of proliferation than the T cells. Indeed, we were unable to see any undivided NK cells and we could discern at least seven distinct CFSE peaks among the NK cells, whereas a substantial proportion of T cells remained undivided and only approximately four peaks of divided cells could be seen; this was confirmed by detailed analysis of samples from four vaccinees (Figs. 6*C*, 6*D*). The average number of divisions undergone by NK cells (mean/SE: 2.55/0.01) was significantly higher than for CD8$^+$ T cells (mean/SE: 1.12/0.18; $p < 0.001$), CD4$^+$ T cells (mean/SE: 0.96/0.07; $p < 0.001$); $p = 0.31$) or CD45RO$^+$CD4$^+$ T cells (1.20/0.37; $p = 0.005$). Analysis of the proportion of the initial cell population that had gone through one or more divisions (12) confirmed that, after 7 d, all NK cells had divided at least once but only ~30% of all CD45RO$^+$ CD4$^+$ T cells and <10% of CD8$^+$ T cells or CD45RO$^-$ CD4 T cells had done so (Fig. 6*D*); however, the time to first cell division did not differ between NK cells and the CD4$^+$ and CD8$^+$ T cells (data not shown).

IL-12 and IL-18 synergize with IL-2 to activate NK cells after vaccination

To determine whether IL-2 alone is sufficient for NK cells to show a "recall" response to rabies virus after vaccination or whether other signals (e.g., from myeloid accessory cells) are also required for optimal NK cell responses we compared the recall response with structurally intact but heat-killed rabies virus (07/162) with

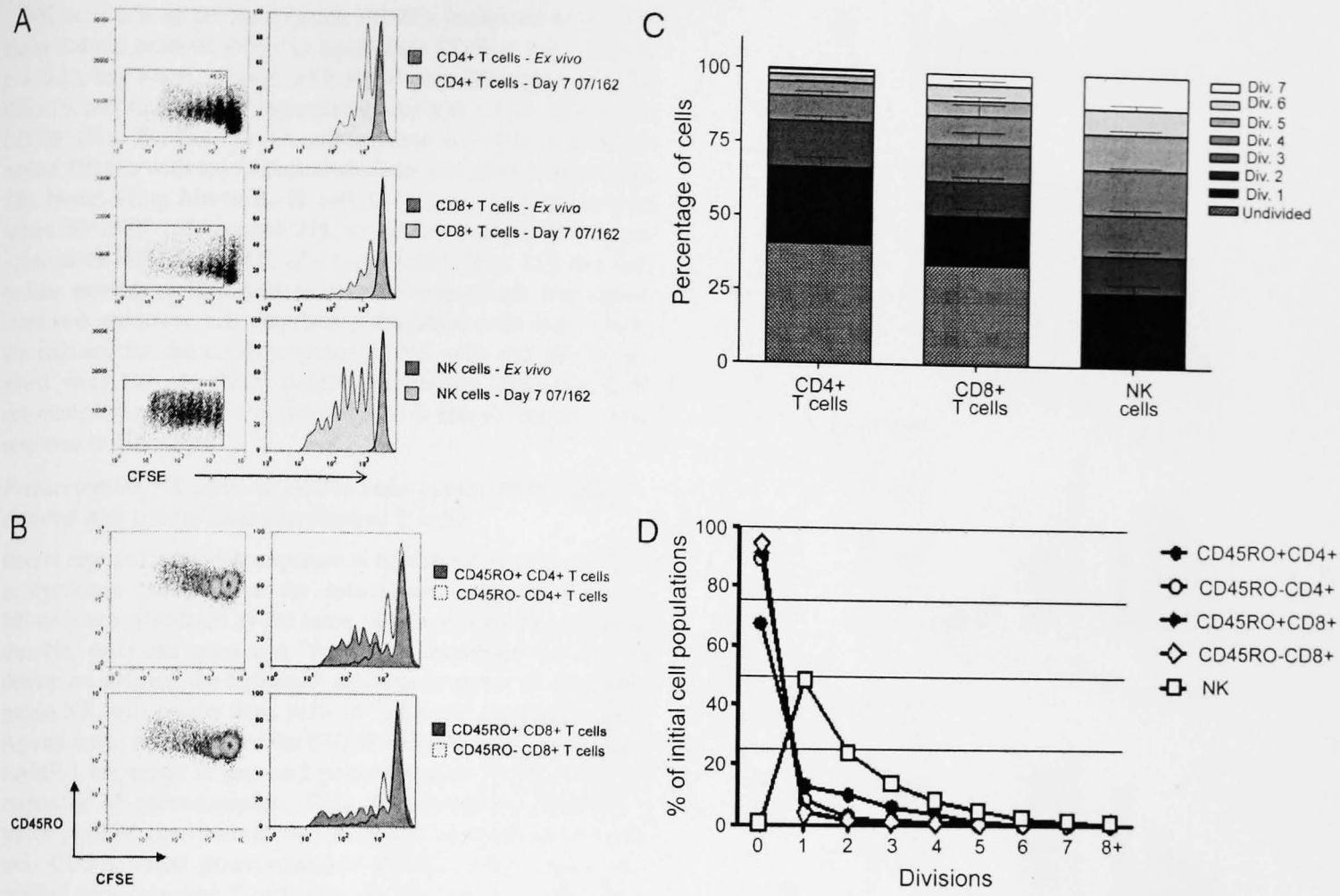


FIGURE 6. Extensive NK cell proliferation during postvaccination recall response. Pre- and postvaccination PBMCs from four donors were labeled with CFSE, cultured for 7 d with rabies virus (07/162) and analyzed by flow cytometry. *A*, Representative flow cytometry plots of CFSE dilution (*left plots*) in postvaccination $CD4^+$ T cells (*upper plots*), $CD8^+$ T cells (*middle plots*), and NK cells (*lower plots*) after 7 d incubation with 07/162. Histogram overlays (*right plots*) compare CFSE dilution in postvaccination cells either ex vivo (dark fill) or after 7 d restimulation with 07/162 (light fill). *B*, Representative flow cytometry plots of CFSE dilution and CD45RO expression (*left plots*) after 7 d restimulation with rabies virus (07/162) in postvaccination $CD4^+$ (*upper plots*) and $CD8^+$ (*lower plots*) T cells. Histogram overlays (*right plots*) compare CFSE dilution in $CD45RO^+$ (dark fill) or $CD45RO^-$ (no fill) $CD4^+$ or $CD8^+$ T cells. *C*, The percentage of $CD4^+$ T cells, $CD8^+$ T cells, or NK cells in the postvaccination, 7 d-restimulated PBMC population that had not divided, or had divided one or more times. Data represent mean + SE for four donors. *D*, Estimated proportions of the precursor populations of NK cells and $CD45RO^+$ or $CD45RO^-$ T cells that did not divide, or that divided one or more times during the 7 d restimulation assay. Data represent means for four donors.

that to purified rNCP and we investigated the effect on the NK cell recall response of adding or neutralizing IL-12 and IL-18 in the presence or absence of rIL-2 (Fig. 7). Suboptimal concentrations of rhIL-12, IL-18, and IL-2 that do not, alone or in combination, induce NK cell activation were determined by titration; for each cytokine a concentration of 0.01 ng/ml was selected.

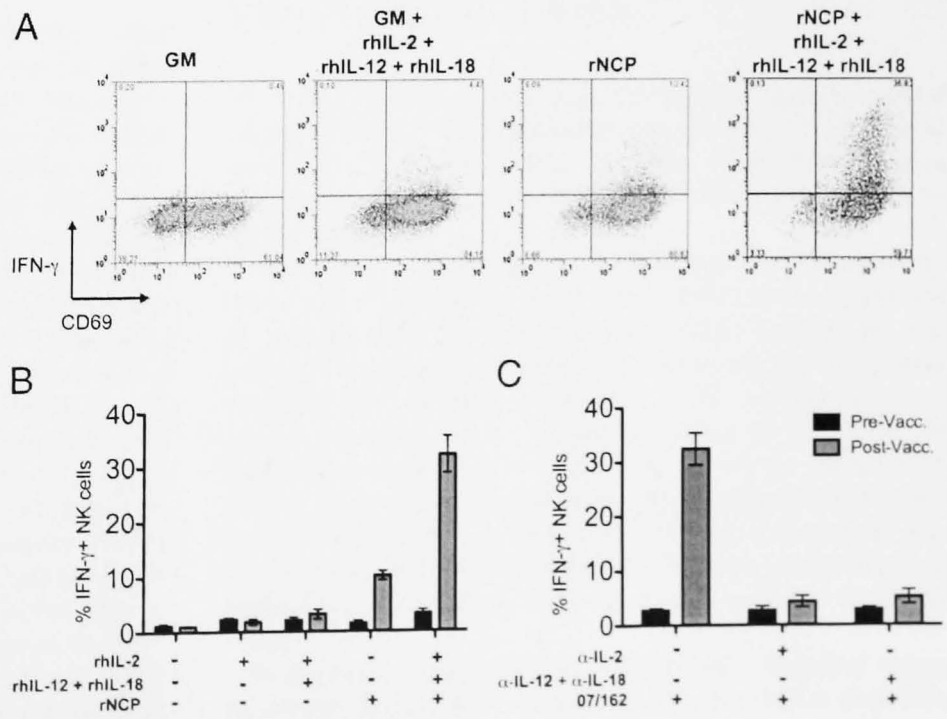


FIGURE 7. IL-12 and IL-18 synergize with IL-2 to activate NK cells after vaccination. Pre- and postvaccination PBMCs from five donors were restimulated in vitro with inactivated whole rabies virus (07/162) or with purified rNCP, in the presence or absence of recombinant human IL-2 or IL-12 plus IL-18, or in the presence or absence of neutralizing Abs to IL-2 or IL-12 plus IL-18, for 24 h and analyzed by flow cytometry for CD69 and IFN-γ expression. *A*, Representative flow cytometry plots showing IFN-γ production and CD69 expression in postvaccination NK cells cultured with or without rNCP in the presence or absence of rhIL-2, rhIL-12, and rhIL-18. *B*, Percentage of NK cells producing $IFN\text{-}\gamma^+$ after restimulation with rNCP in the presence or absence of recombinant cytokines. Data represent mean ± SE of five subjects. The *p* values are derived from two-tailed paired Student *t* test. *C*, Percentage of NK cells producing $IFN\text{-}\gamma^+$ after restimulation with rabies virus 07/162 in the presence or absence of neutralizing Abs to IL-2, IL-12, and IL-18. *B* and *C*, Data represent mean ± SE.

NK cells among postvaccination PBMCs incubated with rNCP alone did not produce IFN-γ or upregulate CD69 ($t = 2.1$, $df = 4$, $p = 0.6$), but when cultured with rNCP plus 0.01ng/ml rhIL-12, rhIL-18, and rhIL-2 they responded strongly ($t = 8.94$, $df = 4$, $p = 0.0009$) (Fig. 7*A*, 7*B*). No NK cell response was seen in prevaccination PBMCs with any of the combinations of stimuli tested (Fig. 7*B*). Neutralizing Abs to IL-12 and IL-18 completely ablated the rabies virus 07/162-induced NK cell IFN-γ response in postvaccination PBMCs ($t = 8.5$, $df = 4$, $p = 0.001$) (Fig. 7*C*), in a very similar manner to IL-2 neutralization. We conclude that signals from two accessory cell populations (myeloid cells and T cells) are required for the recall response of NK cells and that inactivated virus but not rNCP (which presumably lacks the TLR-activating ligands present in whole virus) is able to induce a recall response in NK cells.

Prevaccination NK cells respond to rabies virus 07/162 when cultured with primed (postvaccination) T cells

Recent reports suggest that exposure to haptenated proteins, MCMV, or cytokines can enhance the subsequent response of murine NK cells to reactivation by the same stimuli, raising the possibility that NK cells can acquire a "memory" phenotype (14–16). To determine whether the enhanced antiviral response of postvaccination NK cells results from such an "adaptive" response to prior Ag exposure, we compared the 07/162-induced IFN-γ, CD69, and LAMP-1 responses of pre- and postvaccination PBMCs with the responses of prevaccination, CD3 T cell-depleted PBMCs to which purified postvaccination T cells had been added; as a control, CD3-depleted postvaccination PBMCs were mixed with purified prevaccination T cells (Fig. 8). We observed that prevaccination NK cells responded vigorously to inactivated rabies virus 07/162 when cultured together with postvaccination T cells and the magnitude of the NK response was proportional to the number of T cells added to the culture. Conversely CD3-depleted postvaccination NK cells cultured with naive T cells were fully responsive to high-dose IL-12/18 but were unable to respond to rabies virus. These data suggest that there are no intrinsic differences between prevaccination and postvaccination NK cells and that postvaccination NK cells are simply responding to the high levels of cytokines emanating from rabies virus-specific $CD4^+$ T cells and myeloid accessory cells.

Discussion

The purpose of vaccination is to expand and differentiate small populations of naive, Ag-specific T and B lymphocytes into larger populations of memory cells with enhanced effector function, to accelerate the clearance of pathogenic microorganisms. Evaluation of vaccine-induced immune responses typically includes measurement of Ab titers and some assay of cell-mediated immunity, such as lymphocyte proliferation, cytokine secretion, or cytotoxicity. Although bulk assays (in which the responding cell type is not known) can now be supplemented by single-cell assays (such as flow cytometry, which allows both the number and phenotype of responding cells to be assessed), these assays are typically designed—and their outputs interpreted—on the assumption that differences in cell-mediated effector responses postvaccination, and especially after recall Ag stimulation of cells in vitro, are due solely to the actions of Ag-specific effector cells. Two recent examples, from otherwise groundbreaking studies, serve to illustrate the pervasiveness of these assumptions. In a study using functional genomics, polychromatic flow cytometry and systems biology to evaluate the global response to yellow fever vaccination, only the effector functions of $CD4^+$ T cells were characterized at the single-cell level and IFN-γ secreted by PBMC cultures was assumed to be derived from

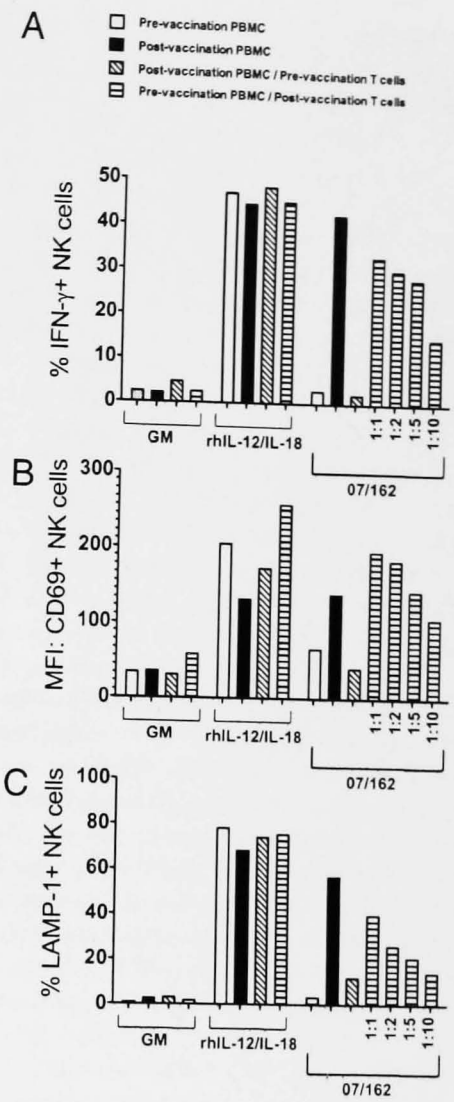


FIGURE 8. Prevaccination NK cells respond to rabies virus 07/162 when cultured with postvaccination T cells. PBMCs, collected either before (white bars) or after (black bars) vaccination, were cultured without stimulus (GM) or with rhIL-12 and rhIL-18, or with 07/162, for 24 h and stained for intracellular IFN-γ (*A*); CD69 (*B*); and LAMP-1 (*C*). Responses of whole PBMCs were compared with those of CD3-depleted postvaccination PBMCs to which naive (prevaccination) T cells had been added (diagonally cross-hatched bars) and with those of CD3-depleted prevaccination PBMCs to which varying numbers of memory (postvaccination) T cells had been added (horizontally cross-hatched bars); postvaccination T cells were added to prevaccination PBMCs at ratios of 1 T cell per PBMC, or 1 T cell per 2, 5, or 10 PBMCs.

Th1 cells, despite evidence that NK cells—a well-documented source of IFN-γ—were proliferating in the first 7 d after vaccination (17). A similar analysis of yellow fever vaccine-induced responses examined correlates only of $CD8^+$ T cell and Ab responses (18).

In this study, we compare, for the first time, pre- and postvaccination NK cell effector responses (IFN-γ production and exocytosis of cytotoxic granules), demonstrate that these responses are augmented in an Ag-specific manner by vaccination and demonstrate that NK cells contribute significantly to the postvaccination response, especially, but not exclusively, during the first hours and days after re-exposure to the vaccine Ag. Moreover, and again for the first time, we have elucidated the activation signals required for this postvaccination NK cell "recall" response. Our findings indicate that IL-2–mediated NK activation should be considered as an additional—and potentially very important—indicator of vaccine efficacy.

In many ways, our findings are not surprising. It has been known for several years that NK cells can represent a significant proportion

of IFN-γ^+ cells in the initial stages of a classical recall response (19), that IL-2 can augment NK cell responses (20, 21), that the NK cell response to influenza virus depends on IL-2 and T cells (9), and that increased numbers of IFN-γ–producing NK cells can be detected after influenza vaccination (10). However, the logical conclusion of these observations—that Ag-specific IL-2 secretion from memory T cells may recruit NK cells as effectors of adaptive immunity and, thus, that NK cell responses can be potentiated by vaccination—has not previously been made explicit and has not been formally tested. Indeed, our collective fixation on NK cells as cells that can mediate effector function without prior sensitization (22, 23) has blinded us to the notion that they may perform their effector functions even more effectively after sensitization.

By detailed analysis of the response to rabies vaccination, we have shown that Ag-specific, CD45RO$^+$ CD4$^+$ T cells secrete IL-2 within 6 h of re-exposure to Ag and that this IL-2—in combination with IL-12 and IL-18 induced by the interaction of whole virus with other accessory cells—activates NK cells to produce IFN-γ and to degranulate, releasing perforin. A proposed schematic for the NK recall response is shown in Fig. 9. Importantly, these NK effector responses occur extremely rapidly, starting within 6 h (i.e., significantly earlier than the equivalent T cell responses), and are very robust, with 30–40% of all NK cells responding. Consequently, in the first 12–18 h after re-exposure to Ag, >70% of all IFN-γ–producing cells are NK cells. Importantly, however, NK cells also show a marked and prolonged proliferative response to the vaccine Ag and they continue to contribute to the effector cell population for at least 7 d after Ag re-exposure; indeed the secondary peak in the NK IFN-γ response may well represent maturation and activation of cells that have divided during the first few days of the response. Lastly, the recall NK cell response is extremely durable; we have repeated this analysis >4 mo after the last vaccination without any noticeable decline in the response (data not shown).

The indirect route of NK cell activation has largely been overlooked until recently but it is now clear that inflammatory cytokines (IL-12, IL-18, and IFN-α) and costimulatory signals from myeloid accessory cells are essential for optimal NK cell responsiveness to a wide range of viral, bacterial, and protozoal infections (5). Our finding that intact rabies virus, but not purified recombinant protein, was able to activate NK cells after vaccination in an IL-12– and IL-18–dependent manner, is consistent with a requirement for myeloid accessory cell-derived as well as T cell-derived signals for induction of recall NK responses. Neither the rabies virus encoded ligands for pattern recognition receptors (PRRs) nor the PRRs themselves are known. Although TLR3 is upregulated in brains of rabies virus-infected mice (24, 25) and humans (26), TLR3 preferentially recognizes dsRNA rather than ssRNA and is thus not an obvious candidate for recognition of rabies virus. Human TLR8/murine TLR7s are receptors for some ssRNAs (27, 28) and may thus be more likely innate receptors for rabies virus. One practical implication of the need for myeloid acessory cell stimuli for induction of recall NK responses is that evaluation of vaccine-induced immune responses by restimulation of PBMCs with purified protein Ags or synthetic peptides may not reveal the full extent of the NK recall response that may occur after exposure to whole pathogens. It is likely that NK recall responses will be further enhanced, after vaccination, by the presence of specific Ab; Ag–Ab complexes binding to CD16 are a powerful route for NK cell activation and in preliminary experiments we have observed that rabies virus restimulation of postvaccination PBMCs in the presence of autologous serum (i.e., containing anti-rabies Abs) leads to even more florid NK cell responses than the ones shown here.

Our finding that, in the absence of T cells, rIL-2 is sufficient to restore NK recall responses indicates that IL-2 is the only T cell-derived signal that is essential for the NK recall response. In support of this scenario, we have shown that although T cells are required for optimal activation of NK cells by malaria-infected

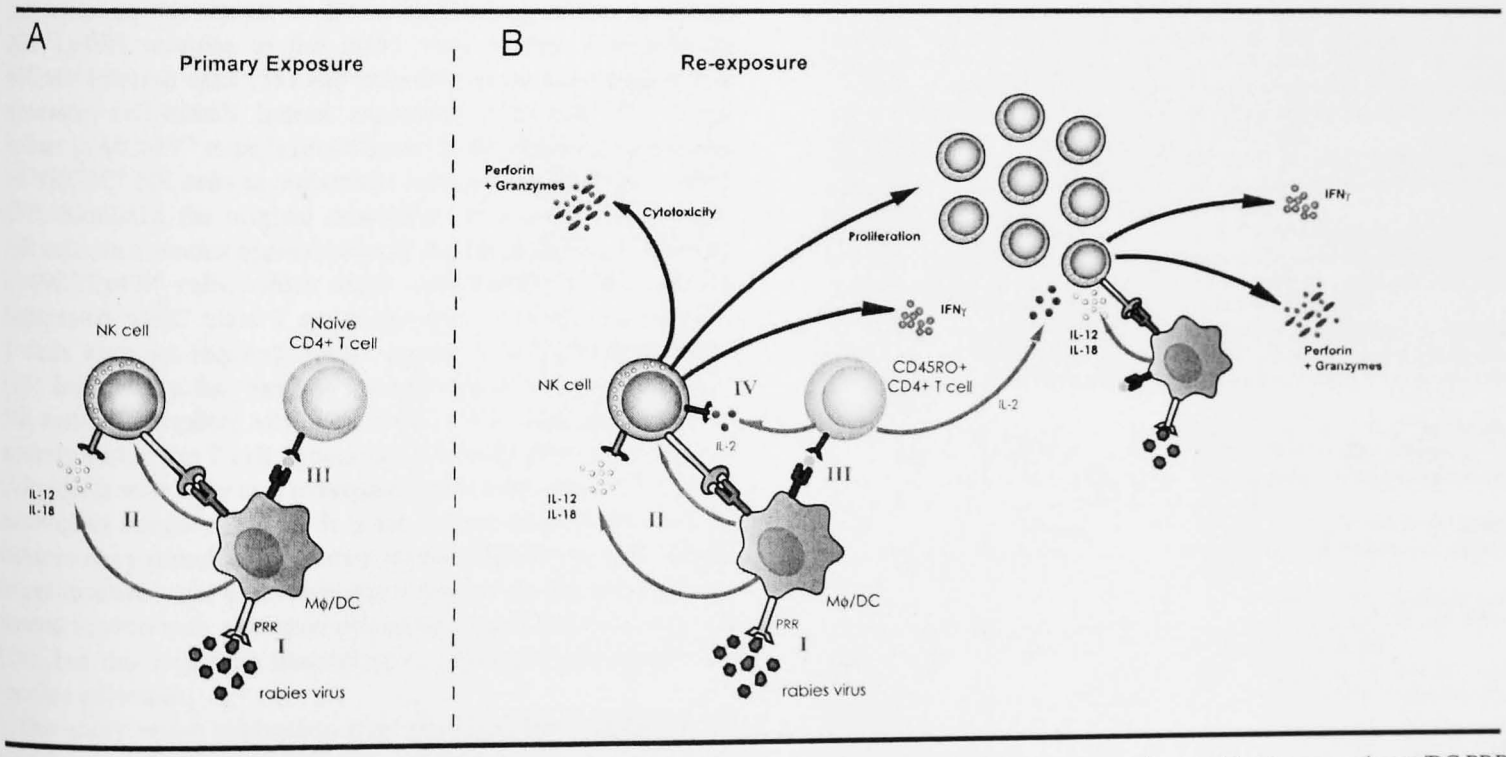


FIGURE 9. Schematic representation of suggested pathway for "recall" NK cell responses. *A*, On first exposure, pathogens bind to macrophages/DC PRR (either on the cell surface or within various intracellular compartments) (*I*), leading to cytokine secretion, and upregulation of costimulatory molecules (*II*). Pathogen uptake and peptide presentation by DCs will prime naive T cells (*III*). However, in the absence of a source of IL-2, NK cells will not become activated. *B*, On re-exposure to the same pathogen, PRR binding, cytokine secretion, and peptide Ag presentation will again take place (*I, II, III*). In addition, IL-2 from primed effector T cells (*IV*) provides signals, which allow NK cells to secrete cytokines, become cytotoxic and proliferate. In the presence of ongoing Ag presentation, a second wave of activation may provide for a sustained NK cell response.

RBCs, there is no requirement for NK cell–T cell contact and that the T cell-dependent signal can be delivered to the NK cells via a semipermeable membrane (29). Our data therefore reveal an important new role for vaccine-induced IL-2–secreting memory T cells and may, in part, explain the emerging consensus that polyfunctional T cells, which secrete IL-2 in addition to IFN-γ or TNF-α, are associated with positive outcomes of viral infection (30–32) and with particularly effective vaccination regimes (33, 34).

It was noticeable that whichever parameter we assayed (CD69 upregulation, IFN-γ production, degranulation), the NK cell response to rabies vaccination among the vaccinees was remarkably homogeneous. Overall, for different parts of the study, we assayed responses from 30 individuals and in every case there was a robust and persistent NK cell recall response. This is in marked contrast to our previous findings for NK cell responses to malaria-infected RBCs, *Mycobacterium bovis* bacillus Calmette-Guérin and bacterial LPS where NK IFN-γ responses are extremely heterogeneous, but similar (in homogeneity, if not magnitude) to responses we observed to high-dose rhIL-12+IL-18 (35). We have proposed that heterogeneity in NK cell IFN-γ responses to pathogens reflects both differences in the strength of accessory stimuli and variable expression of polymorphic NK cell receptors (which fine-tune the degree of activation) (35). The results of this vaccination study suggest that if the accessory cell stimulus is sufficiently strong (which may require synergism of signals from myeloid cells and T cells) then the effect of NK cell regulatory receptors may be overcome. If so, genetic diversity in NK cell regulatory receptors may not represent a major hurdle to effective vaccination.

Although there are superficial similarities between the "recall" NK response that we have described and the "memory-like" NK cells recently described in mice (14–16), there are important differences between the cytokine-driven response described in this study and some of the mouse studies. In mice infected with murine CMV (MCMV), NK cells proliferate, persist at higher than normal frequencies for several months and show enhanced cytokine and degranulation responses on reactivation (16); however, NK cell activation in this model is driven by binding of the activating NK Ly49H receptor to the m157 viral protein expressed on MCMV-infected cells (36) and may thus occur independently of accessory cell stimuli. Indeed, expansion of the Ly49H+ NK cell subset in MCMV+ mice is reminiscent of the expanded population of NKG2C+ NK cells in individuals seropositive for human CMV (37). Similarly, the original description of murine memory-like NK cells, in a contact hypersensitivity model, specifically involved Ly49C+/Ly49I+ cells, which might conceivably be activated by haptenated MHC class I molecules and, at least inasmuch as T cells were not required, would appear to be IL-2 independent (15). Importantly, the "memory" component of the postvaccination NK response to rabies virus described in this study appears to lie entirely within the T cell population: NK cells from unvaccinated individuals were fully able to respond to the virus when mixed with autologous memory T cells. It is not known whether nonspecific inflammatory stimuli can maintain human NK cells in a prolonged hyper-reactive state, as recently described for murine NK cells activated in vitro with a mixture of accessory cell-derived cytokines (14), but this might be interesting to explore in the context of vaccine adjuvants.

Our study raises interesting questions regarding the functional significance of enhanced NK cell responses after immunization. For infections where a protective role for NK cells is established, evaluation of NK responses postvaccination is likely to be a useful indicator of vaccine efficacy but for other infections the implications are less clear and further studies are required. In the case of rabies virus vaccination, it is widely accepted that neutralizing Abs are essential for protection (38) but experimental infections in mice suggest that cell-mediated immune responses (including signaling through the IFN-γR) are required for efficient viral clearance (39) and that proinflammatory cytokines (IFN-γ and IL-2) enhance vaccine immunogenicity, leading to significantly higher neutralizing Ab titers (40). The potential for NK cell IFN-γ responses to contribute to the efficacy of rabies vaccines thus deserves to be evaluated. The role of the degranulation response is less obvious. In these particular experiments, using killed virus, it is unlikely that NK cells are degranulating in response to infected cells and degranulation may simply be a marker of NK cell activation.

In summary, we have demonstrated that NK cells are major contributors to the effector lymphocyte population during the recall response to rabies vaccination. This should lead us to reconsider the precise roles of Ag-specific memory T cells in vaccine-induced immunity. Assays of CD4+ T cell IL-2 production, NK cell IFN-γ production and NK cytotoxicity need to be included in the arsenal of tools for evaluating correlates of vaccine-induced immunity.

Acknowledgments

We thank Carolynne Stanley for subject recruitment and sampling, Patrick Corran for statistical advice, and Karoline Schjetne (Invitrogen) and Dr. M. Juozapaitis (Institute of Biotechnology, Vilnius, Lithuania) for donation of essential reagents.

Disclosures

The authors have no finacial conflicts of interest.

References

1. Pashine, A., N. M. Valiante, and J. B. Ulmer. 2005. Targeting the innate immune response with improved vaccine adjuvants. *Nat. Med.* 11(4, Suppl)S63–S68.
2. Kanzler, H., F. J. Barrat, E. M. Hessel, and R. L. Coffman. 2007. Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. *Nat. Med.* 13: 552–559.
3. Schijns, V. E. 2000. Immunological concepts of vaccine adjuvant activity. *Curr. Opin. Immunol.* 12: 456–463.
4. Hoebe, K., E. Janssen, and B. Beutler. 2004. The interface between innate and adaptive immunity. *Nat. Immunol.* 5: 971–974.
5. Newman, K. C., and E. M. Riley. 2007. Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens. *Nat. Rev. Immunol.* 7: 279–291.
6. Baratin, M., S. Roetynck, B. Pouvelle, C. Lemmers, N. K. Viebig, S. Johansson, P. Bierling, A. Scherf, J. Gysin, E. Vivier, and S. Ugolini. 2007. Dissection of the role of PfEMP1 and ICAM-1 in the sensing of Plasmodium-falciparum-infected erythrocytes by natural killer cells. *PLoS ONE* 2: e228.
7. Newman, K. C., D. S. Korbel, J. C. Hafalla, and E. M. Riley. 2006. Cross-talk with myeloid accessory cells regulates human natural killer cell interferon-gamma responses to malaria. *PLoS Pathog.* 2: e118.
8. Fehniger, T. A., M. A. Cooper, G. J. Nuovo, M. Cella, F. Facchetti, M. Colonna, and M. A. Caligiuri. 2003. CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. *Blood* 101: 3052–3057.
9. He, X. S., M. Draghi, K. Mahmood, T. H. Holmes, G. W. Kemble, C. L. Dekker, A. M. Arvin, P. Parham, and H. B. Greenberg. 2004. T cell-dependent production of IFN-gamma by NK cells in response to influenza A virus. *J. Clin. Invest.* 114: 1812–1819.
10. Long, B. R., J. Michaelsson, C. P. Loo, W. M. Ballan, B. A. Vu, F. M. Hecht, L. L. Lanier, J. M. Chapman, and D. F. Nixon. 2008. Elevated frequency of gamma interferon-producing NK cells in healthy adults vaccinated against influenza virus. *Clin. Vaccine Immunol.* 15: 120–130.
11. Korbel, D. S., K. C. Newman, C. R. Almeida, D. M. Davis, and E. M. Riley. 2005. Heterogeneous human NK cell responses to *Plasmodium falciparum*-infected erythrocytes. *J. Immunol.* 175: 7466–7473.
12. Hawkins, E. D., M. Hommel, M. L. Turner, F. L. Battye, J. F. Markham, and P. D. Hodgkin. 2007. Measuring lymphocyte proliferation, survival and differentiation using CFSE time-series data. *Nat. Protoc.* 2: 2057–2067.
13. Chávez-Galán, L., M. C. Arenas-Del Angel, E. Zenteno, R. Chávez, and R. Lascurain. 2009. Cell death mechanisms induced by cytotoxic lymphocytes. *Cell. Mol. Immunol.* 6: 15–25.
14. Cooper, M. A., J. M. Elliott, P. A. Keyel, L. Yang, J. A. Carrero, and W. M. Yokoyama. 2009. Cytokine-induced memory-like natural killer cells. *Proc. Natl. Acad. Sci. USA* 106: 1915–1919.
15. O'Leary, J. G., M. Goodarzi, D. L. Drayton, and U. H. von Andrian. 2006. T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nat. Immunol.* 7: 507–516.
16. Sun, J. C., J. N. Beilke, and L. L. Lanier. 2009. Adaptive immune features of natural killer cells. *Nature* 457: 557–561.

17. Gaucher, D., R. Therrien, N. Kettaf, B. R. Angermann, G. Boucher, A. Filali-Mouhim, J. M. Moser, R. S. Mehta, D. R. Drake, III, E. Castro, et al. 2008. Yellow fever vaccine induces integrated multilineage and polyfunctional immune responses. *J. Exp. Med.* 205: 3119–3131.
18. Querec, T. D., R. S. Akondy, E. K. Lee, W. Cao, H. I. Nakaya, D. Teuwen, A. Pirani, K. Gernert, J. Deng, B. Marzolf, et al. 2009. Systems biology approach predicts immunogenicity of the yellow fever vaccine in humans. *Nat. Immunol.* 10: 116–125.
19. Desombere, I., F. Clement, H. Rigole, and G. Leroux-Roels. 2005. The duration of in vitro stimulation with recall antigens determines the subset distribution of interferon-gamma-producing lymphoid cells: a kinetic analysis using the Interferon-gamma Secretion Assay. *J. Immunol. Methods* 301: 124–139.
20. Malek, T. R. 2008. The biology of interleukin-2. *Annu. Rev. Immunol.* 26: 453–479.
21. Sutlu, T., and E. Alici. 2009. Natural killer cell-based immunotherapy in cancer: current insights and future prospects. *J. Intern. Med.* 266: 154–181.
22. Moretta, A., C. Bottino, M. C. Mingari, R. Biassoni, and L. Moretta. 2002. What is a natural killer cell? *Nat. Immunol.* 3: 6–8.
23. Trinchieri, G. 1989. Biology of natural killer cells. *Adv. Immunol.* 47: 187–376.
24. Mansfield, K. L., N. Johnson, A. Nunez, D. Hicks, A. C. Jackson, and A. R. Fooks. 2008. Up-regulation of chemokine gene transcripts and T-cell infiltration into the central nervous system and dorsal root ganglia are characteristics of experimental European bat lyssavirus type 2 infection of mice. *J. Neurovirol.* 14: 218–228.
25. McKimmie, C. S., N. Johnson, A. R. Fooks, and J. K. Fazakerley. 2005. Viruses selectively upregulate Toll-like receptors in the central nervous system. *Biochem. Biophys. Res. Commun.* 336: 925–933.
26. Jackson, A. C., J. P. Rossiter, and M. Lafon. 2006. Expression of Toll-like receptor 3 in the human cerebellar cortex in rabies, herpes simplex encephalitis, and other neurological diseases. *J. Neurovirol.* 12: 229–234.
27. Heil, F., H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner, and S. Bauer. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* 303: 1526–1529.
28. Diebold, S. S., T. Kaisho, H. Hemmi, S. Akira, and C. Reis e Sousa. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 303: 1529–1531.
29. Horowitz, A., K. C. Newman, J. H. Evans, D. S. Korbel, D. M. Davis, and E. M. Riley. 2010. Cross-talk between T cells and NK cells generates rapid effector responses to *Plasmodium falciparum*-infected erythrocytes. *J. Immunol.* 184: 6043–6052.
30. Ciuffreda, D., D. Comte, M. Cavassini, E. Giostra, L. Bühler, M. Perruchoud, M. H. Heim, M. Battegay, D. Genné, B. Mulhaupt, et al. 2008. Polyfunctional HCV-specific T-cell responses are associated with effective control of HCV replication. *Eur. J. Immunol.* 38: 2665–2677.
31. Ferre, A. L., P. W. Hunt, J. W. Critchfield, D. H. Young, M. M. Morris, J. C. Garcia, R. B. Pollard, H. F. Yee, Jr., J. N. Martin, S. G. Deeks, and B. L. Shacklett. 2009. Mucosal immune responses to HIV-1 in elite controllers: a potential correlate of immune control. *Blood* 113: 3978–3989.
32. Nebbia, G., F. M. Mattes, C. Smith, E. Hainsworth, J. Kopycinski, A. Burroughs, P. D. Griffiths, P. Klenerman, and V. C. Emery. 2008. Polyfunctional cytomegalovirus-specific CD4+ and pp65 CD8+ T cells protect against high-level replication after liver transplantation. *Am. J. Transplant.* 8: 2590–2599.
33. Aagaard, C., T. T. Hoang, A. Izzo, R. Billeskov, J. Troudt, K. Arnett, A. Keyser, T. Elvang, P. Andersen, and J. Dietrich. 2009. Protection and polyfunctional T cells induced by Ag85B-TB10.4/IC31 against *Mycobacterium tuberculosis* is highly dependent on the antigen dose. *PLoS One* 4: e5930.
34. Harari, A., V. Dutoit, C. Cellerai, P. A. Bart, R. A. Du Pasquier, and G. Pantaleo. 2006. Functional signatures of protective antiviral T-cell immunity in human virus infections. *Immunol. Rev.* 211: 236–254.
35. Korbel, D. S., P. J. Norman, K. C. Newman, A. Horowitz, K. Gendzekhadze, P. Parham, and E. M. Riley. 2009. Killer Ig-like receptor (KIR) genotype predicts the capacity of human KIR-positive CD56dim NK cells to respond to pathogen-associated signals. *J. Immunol.* 182: 6426–6434.
36. Arase, H., E. S. Mocarski, A. E. Campbell, A. B. Hill, and L. L. Lanier. 2002. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science* 296: 1323–1326.
37. Gumá, M., A. Angulo, C. Vilches, N. Gómez-Lozano, N. Malats, and M. López-Botet. 2004. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. *Blood* 104: 3664–3671.
38. Johnson, N., A. F. Cunningham, and A. R. Fooks. 2010. The immune response to rabies virus infection and vaccination. *Vaccine* 28: 3896–3901.
39. Hooper, D. C., K. Morimoto, M. Bette, E. Weihe, H. Koprowski, and B. Dietzschold. 1998. Collaboration of antibody and inflammation in clearance of rabies virus from the central nervous system. *J. Virol.* 72: 3711–3719.
40. Claassen, I. J., A. D. Osterhaus, M. Poelen, N. Van Rooijen, and E. Claassen. 1998. Antigen detection in vivo after immunization with different presentation forms of rabies virus antigen, II. Cellular, but not humoral, systemic immune responses against rabies virus immune-stimulating complexes are macrophage dependent. *Immunology* 94: 455–460.