TCR AND CO-RECEPTORS MEDIATED ACTIVATION OF Vγ9Vδ2 T CELLS

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades
der Bayerischen Julius-Maximilians-Universität Würzburg
vorgelegt von

Bladimiro Rincón Orozco

Bucaramanga, Kolumbien

Würzburg May 2007

Diese Dissertation wurde im Institut für Virologie und Immunbiologie der Bayerischen Julius-Maximilians-Universität Würzburg im Zeitraum von November 2003 bis Mai 2007 unterLeitung von Prof. Dr. Thomas Herrmann angefertigt.

Eingereicht am:

Vorsitzender: Prof. Dr. M. Müller

Gutachter : Prof. Dr. Thomas Herrmann

Prof. Dr. Roy Gross

Tag des Promotionskolloquiums:

Doktorurkunde ausgehändigt am:

Erklärung

Hiermit erkläre ich, dass ich die Dissertation

„ **TCR and co-receptors mediated activation of Vγ9Vδ2 T cells** “

in allen Teilen selbstständig angefertigt und keine anderen als die genannten Quellen und Hilfsmittel verwendet habe.

Weiterhin versichere ich, dass diese Dissertation weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen hat.

Ich habe bisher außer den mit dem Zulassungsgesuch urkundlich vorgelegten Graden keine weiteren akademischen Grade erworben oder zu erwerben versucht.

Würzburg, May 2007

Bladimiro Rincón Orozco

ERKLÄRUNG

Summary

A small percentage (1–5%) of the blood lymphocytes expresses alternative T-cell antigen receptor that uses γ and δ TCR rearranging genes. A subset of them expresses the Vγ9Vδ2 TCR. Those cells respond to self-nonpeptide and foreign antigens presented by unknown antigen-presenting molecules. Vγ9Vδ2 T cells also express Toll-like receptors and natural killer receptors that allow them to respond to other nonpeptide microbial components or to alterations in the expression of stress cell surface ligands such as NKG2D ligands.

Vγ9Vδ2 T cells frequently are regulated by the expression of activating and/or inhibitory NKRs (iNKRs) that can fine-tune their activation threshold and the activating NKG2D receptor is one of the most studied until now. NKG2D, a C-type lectin receptor directed against MICA/MICB and UL16-binding protein (ULBP) molecules, have been reported a powerful co-stimulus for Ag-mediated activation of CD8 and Vγ9Vδ2 T cells. Indeed, NKG2D is recruited within the Vγ9Vδ2 TCR immunological synapse and enhances recognition by Vγ9Vδ2 T cells of Mycobacteria-infected DCs and various MICA/MICB or ULBP hemopoietic and non-hemopoietic tumors. The level of NKG2D is upregulated by inflammatory cytokines (e.g. IL-15), and NKG2D ligands are induced after a physical or genotoxic stress and/or along infection by intracellular pathogens. Therefore, NKG2D is a key stress sensor that strongly enhances recognition of altered or infected self by human γδ T cells. Recent progress in the field supports the idea that γδ T cells fulfill a role in the innate and adaptative immune response in different way of the conventional αβ T cells.

We demonstrated direct activation of Vγ9Vδ2 T cells by NKG2D ligation through the association with DAP10 adapter molecules and independently of TCR-Ag recognition, similar to the NKG2D-mediated activation of NK cells. Culture of peripherical blood mononuclear cells with immobilized NKG2D mAb or NKG2D ligand MICA induces up-regulation of CD69 and CD25 in NK and Vγ9Vδ2 T cells but not in CD8 T cells. Additionally, the ligation of NKG2D induces in Vγ9Vδ2 T cells the up-regulation of molecules typical for antigen-presenting cells, such as co-stimulator molecules (CD86) antigen presenting molecules (CD1a, HLA-DR), adhesion molecules (CD54), and activation molecules (CD69).

Furthermore, NKG2D ligation in Vγ9Vδ2 T cells induces the production of cytokines such as TNF-α and chemokines such as, MIP-1α, but cannot induce the production of cytokines such

as IL-6 or IFN-γ and chemokines such as RANTES, MCP-1 and GM-CSF. In addition, NKG2D triggers the activation of the cytolytic machinery as efficient as CD3 stimulation as shown by measurement of the release of granules with esterase activity (BLT assay), perforin and the up-regulation of CD107a on the surface of Vγ9Vδ2 T cells. This NKG2D dependent cytolysis has been confirmed using purified Vγ9Vδ2 T cells, which kill MICA-transduced RMA cells but not the control cells. The TCR independence and NKG2D dependence of this killing is supported by mAb inhibition experiment. Finally, DAP 10, which mediates NKG2D signaling of human NK cells, is found in resting and activated Vγ9Vδ2 T cells. Moreover, data of intracellular signaling studies suggest an important role of Scr kinases in the NKG2D mediated killing and involvement of DAP-10-PI3K and PLCγ 1 pathways as mayor proteins implicated in target cell lysis, and shows remarkable difference with the TCR signaling.

The identification of these similarities in NKG2D function between NK and Vγ9Vδ2 T cells may be of interest for development of new strategies for Vγ9Vδ2 T cell-based immunotherapy in certain types of cancer and help to understand Vγ9Vδ2 T cell function in general.

Zusammenfassung

Ein geringer Prozentsatz (1-5%) der T-Lymphozyten (T-Zellen) besitzt einen alternativen T-Zellrezeptor (TCR), der aus der γ und δ Kette der rearrangierten Gene aufgebaut ist. Eine geringe Population dieser T-Zellen exprimiert den Vγ9Vδ2 TCR. Diese Zellen werden durch körpereigene nicht-Peptide und fremde Antigene, die von bisher unbekannten antigenpräsentierenden Molekülen präsentiert werden, aktiviert. Vγ9Vδ2 T-Zellen exprimieren zudem Toll-like Rezeptoren und NK-Rezeptoren die es ihnen ermöglichen auf weitere, mikrobielle nicht-Peptid Moleküle oder die veränderte Expression von stress-spezifischen Zelloberflächenmolekülen, wie dem NKG2D Liganden zu reagieren.

Vγ9Vδ2 T-Zellen werden häufig über die Expression von aktivierenden und/oder hemmenden NKRs (iNKRs) reguliert, die deren Aktivierungsschwelle fein einstellen können. Der bisher am Besten untersuchte NKR ist der aktivierende NKG2D Rezeptor. Es wurde gezeigt, dass NKG2D, ein C-Typ Lektinrezeptor, der sich gegen MICA/MICB und UL16-bindende Proteine (ULBP)-Moleküle richtet, als starker Kostimulus für die antigenvermittelte Aktivierung von CD8 und Vγ9Vδ2 T-Zellen dient. In der Tat wird NKG2D zu der Vγ9Vδ2 TCR immunologischen Synapse rekrutiert und stimuliert dort die Erkennung von mycobakteriell infizierten DCs und verschiedenen MICA/MICB oder ULBP hämopoetischen und nicht hämopoetischen Tumoren durch Vγ9Vδ2 T-Zellen. Die Expression von NKG2D wird durch inflammatorische Zytokine (wie z.B. IL-15) stimuliert, sowie nach physikalischem oder genotoxischem Stress und/oder während einer Infektion mit intrazellulären Pathogenen induziert. Daher gilt NKG2D als entscheidender Stress-Sensor, der eine verstärkte Identifikation von veränderten oder infizierten körpereigenen Antigenen durch menschliche γδ -Zellen bewirkt. Jüngste Fortschritte auf dem Gebiet stützen die Hypothese, dass γδ T-Zellen eine Rolle in der angeborenen, sowie der adaptiven Immunantwort spielen, allerdings auf andere Weise wirken wie die konventionellen αß T Zellen.

Wir haben gezeigt, dass die direkte Aktivierung von Vγ9Vδ2 T-Zellen durch die Bindung von NKG2D mittels Interaktion mit DAP10 Adaptermolekülen und unabhängig von TCR/Antigen Erkennung erfolgt, ähnlich der NKG2D vermittelten Aktivierung von NK-Zellen. Kulturen aus peripheren Blutzellen, die mit immobilisierten NKG2D monoklonalem Antikörper (mAb) oder dem NKG2D Liganden MICA behandelt wurden zeigten vermehrte Expression von CD69 und CD25 in NK und Vγ9Vδ2 T-Zellen, jedoch nicht in CD8-Zellen.

Desweiteren führte die Bindung von NKG2D in Vγ9Vδ2 T-Zellen zur Regulation von antigenpräsentierenden Molekülen nach NKG2D Stimulation, wie z.B. kostimulatorische Moleküle (CD80, CD68), antigenpräsentierende Moleküle (CD1a, HLA-DR), Adhäsionsmoleküle (CD54) und Aktivierungsmoleküle (CD69, CD95). Die Interaktion von NKG2D und Vγ9Vδ2 T-Zellen induzierte zudem die Produktion von Zytokinen, wie TNF-α, sowie Chemokinen wie MIP-1α, jedoch nicht von IL-6, IFN-γ oder RANTES , MCP-1 und GM-CSF. Desweiteren aktivierte NKG2D die zytolytischen Maschinerie ebenso effizient, wie CD3. Dies konnte durch Messung der Freisetzung von Granula mit Esterase-Aktivität (BLT-Assay) und von Perforin, sowie die verstärkte Expression von CD107a an der Zelloberfläche von Vγ9Vδ2 T-Zellen nachgewiesen werden. Die zytologische Aktivierung durch NKG2D konnte durch Vγ9Vδ2 T-Zellen, die MICA transduzierte RMA Zellen jedoch nicht die Kontrolzellen töten konnten, bestätigt werden. Die Tatsache, dass das Töten dieser Zellen unabhängig von TCR und abhängig von NKG2D erfolgt, wurde durch mAb Hemm-Experimente unterstützt. Schließlich wurde DAP10, das die Signalweiterleitung von NKG2D in menschlichen NK Zellen überträgt, sowohl in nicht aktivierten und activierten Vγ9Vδ2 Zellen nachgewiesen werden. Zudem lassen Daten von intrazellulären Signalstudien vermuten, dass Scr Kinase, wie auch DAP-10-PI3K und der PLCγ 1 eine wichtige Rolle beim Töten durch den NKG2D Signalweg spielen, der beachtliche Unterschiede zum TCR Signalweg aufweisen.

Die Entdeckung, dass NK-Zellen und Vγ9Vδ2 T-Zellen ähnlich auf die Bindung von NKG2D reagieren, könnte für die Entwicklung von einer Vγ9Vδ2 T-Zell-basierten Immuntherapie für die Behandlung bestimmter Krebsarten von Bedeutung sein und im Allgemeinen helfen die Vγ9Vδ2 T-Zell Funktion zu verstehen.

1 Introduction 1

1.1 The human γδ TCR repertoire 3

1.2 Structure of the human γδ T-cell receptor: studies on the Vγ9Vδ2 TCR G115 and data obtained with mouse TCR 6

1.3 Ligands recognized by human Vγ9Vδ2 T cells 7

1.3.1 Self-ligands of the Vγ9Vδ2TCR 8

1.3.2 Non-peptide ligands for Vγ9Vδ2 T Cells 9

1.3.2.1 The isoprenoid metabolism: pathways of production of non-peptide ligands for Vγ9Vδ2 T cells 10

1.3.2.2 Aminobisphosphonates: The metabolical link to phosphoantigen -production 12

1.3.2.3 Alkylamines: Vγ9Vδ2 T cell specific activators different from phosphoantigens 13

1.3.3 Modulation of Vγ9Vδ2 T cell activation by natural killer receptors (NKR) and Toll-like receptors 13

1.4 Antigen presentation by activated Vγ9Vδ2 T cells: an alternative type of professional APC 15

1.5 Expression of CD28 and NKG2D on Vγ9Vδ2 Tcells 16

1.6 NKG2D receptor signaling: data from mouse to human 17

1.7 Possible functions of Vγ9Vδ2 T Cells: implication on infections and tumor control 19

1.8 Vγ9Vδ2T cell response to infection 20

1.8.1 Immune regulation of Vγ9Vδ2 T cells: responses in vitro and in vivo in mycobacterial infections 21

1.9 Antiviral mechanisms of Vγ9Vδ2T cells 23

1.9.1 Non-cytolytic antiviral immunity mediated by Vγ9Vδ2 T cells 24

1.10 Role of Vγ9Vδ2 T cells in tumor regression 27

1.10.1 Role of Vγ9Vδ2 T cells in the immunosurveillance of lymphoid malignancies ..... **Error! Bookmark not defined.**

1.10.2 Anti-lymphoma activity of human γδ T cells in vivo 28

1.11 Aim of the work 30

2 Materials 31

2.1 Chemical reagents 31

2.2 Reagents (Media, Buffers and Solutions): 32

2.3 Cell lines: 35

2.4 Antibodies 36

2.5 Isotypes controls, secondary antibodies and recombinant proteins 37

2.6 Kits ... 38
2.7 PCR Primers ... 38
2.8 Enzymes and Inhibitors ... 38
2.9 Consumables ... 38
2.10 Apparatus ... 39

3 Methods ... 40

3.1 Cellular Biology ... 40
3.1.1 Isolation of peripheral blood mononuclear cells (PBLs) ... 40
3.1.2 Establishment of Vγ9Vδ2 T cell lines by stimulation of fresh Vγ9Vδ2 T cells from peripheral blood ... 40
3.1.3 Magnetic isolation of Vγ9Vδ2 T cells ... 41
3.1.4 In vitro stimulation with immobilized Abs or NKG2D ligand ... 42
3.1.5 Staining of cell surface molecules of Vγ9Vδ2 T cells ... 42
3.1.6 Staining of cell surface antigens of Vγ9Vδ2 T cells ... 42
3.1.7 Analysis of the production of cytokines (TNFα and IFNγ) by intracellular staining of Vγ9Vδ2 T cells ... 43
3.1.8 Analysis of the production of chemokines (GM-CSF, MIP1α, MCP-1, RANTES) and cytokines (IL-6) by intracellular staining of Vγ9Vδ2 T cells 43
3.1.9 Analysis of the release of perforin by Vγ9Vδ2 T cell by intracellular staining ... 44
3.1.10 CD107a upregulation after stimulation of Vγ9Vδ2 T cells ... 44
3.1.11 Measurements of granule release ... 44
3.1.12 Fluorometric assessment of T lymphocyte antigen specific lysis (FATAL) . 45
3.1.13 Determination of T lymphocyte specific lysis by ^{51}Chromium release assay 46
3.1.14 Determination of the modulation of costimulator molecules (CD40, CD80, CD86) antigen presenting molecules (CD1a, HLA-DR), adhesion molecules (CD54), and activation molecules (CD69, CD95) after the stimulation with NKG2D ligands. ... 47
3.1.15 Cytotoxicity inhibition assays using specific inhibitors of PLCγ (U-73122), PI3K (Ly294002), MEK1/2 (PD98059), p38 (SB203580), Src Kinases (PP2) and JNK2 (SP600125) ... 47
3.1.16 IFNγ ELISpot ... 48
3.1.17 Phosphospecific intracellular proteins by flow cytometry analysis ... 48
3.2 Molecular Biology and Biochemistry Methods ... 49
3.2.1 RNA extraction and quantification ... 49
3.2.2 RT-PCR amplification of human DAP 10 and β actin mRNA ... 49
3.2.3 Immunoblot of DAP 10 ... 50
3.3 Statistical analysis ... 51

4 Results 52

4.1 Expression of the activation markers CD95, CD69, CD25 and the memory marker CD45+RO expression on Vγ9Vδ2 T cells. 52

4.2 Analysis of NK associated receptors (CD16, CD56 and NKG2D) and costimulatory molecule (CD28) expression on Vγ9Vδ2 T cells from freshly isolated PBLs. 54

4.3 NKG2D ligation induces expression of activation markers on Vγ9Vδ2 T cells from PBLs 56

4.4 Analysis of activation markers (CD95, CD69 and CD25), memory markers (CD45RO, CD27), NK associated receptors (CD16, CD56 and NKG2D) and costimulatory molecule (CD28) expression on Vγ9Vδ2 T cells after IPP/IL-2 induced in vitro proliferation 58

4.5 IFN-γ and TNF-α production of Vγ9Vδ2 T-STL after NKG2D ligation 60

4.6 Analysis of chemokine (RANTES, MIP-1a, MCP-1 and GM-CSF) and (IL-6) production by Vγ9Vδ2 T cells after stimulation of NKG 2D. cytokine 61

4.7 Analysis of IFN-γ-secreting Vγ9Vδ2 T cells after TCR or NKG2D ligation. 63

4.8 Analysis of the regulation of Vγ9Vδ2 T cell surface molecules, which affect antigen-presentation after NKG2D stimulation. 64

4.9 Release of cytolytic granules by Vγ9Vδ2 T cells after NKG2D ligation 67

4.10 MICA and NKG2D-dependent lysis by in vitro-activated Vγ9Vδ2 T cells .. 69

4.11 NKG2D-dependent but not CD28-dependent cytotoxicity by in vitro-activated Vγ9Vδ2 T cells 72

4.12 DAP10 expression on Vγ9Vδ2 T cells 73

4.13 Analysis of the role of PLC-γ 1, Src kinases, P38, MEK 1/2, JNK and PI3K in NKG2D-mediated lysis by Vγ9Vδ2 T cells treated with specific pharmacological inhibitors 74

4.14 Analysis of phosphospecific intracellular proteins (ERK 1/2 and PLC-γ 1) in Vγ9Vδ2 T cells after stimulation with anti-NKG2D mAb or anti-CD3 mAb. 75

5 Discussion 77

5.1 Modulation of the memory/effector phenotype on Vγ9Vδ2 T cells after in vitro stimulation with TCR-specific antigen and regulation of activation markers (CD69 and CD25) on Vγ9Vδ2 T cells after NKG2D stimulation ... 77

5.2 NKG2D stimulation can modulate the antigen-presenting molecules on Vγ9Vδ2 T cells. 80

5.3 NKG2D stimulation induces the production of cytokines/chemokines by Vγ9Vδ2 T cells. 81

5.4 NKG2D mediated cytotoxicity 83

5.5 Analysis of the NKG2D signaling pathway in Vγ9Vδ2 T cells 85

5.6 Concluding remarks 87

6 References **89**

7 Abbreviations **97**

8 Curriculum Vitae **99**

PUBLICATIONS 101

PRESENTATIONS AND ABSTRACTS 101

9 Acknowledgements **104**

1 INTRODUCTION

In vertebrates, wide ranges of cells contribute to the immune response. Among those cells, lymphocytes occupy a central role because these cells determine the specificity of the immune response. Individual lymphocytes are specialized to respond to a limited set of structurally related antigens. Lymphocytes differ one from each other not only in the specificity of their receptors but also in their functions. B-lymphocytes are precursors of antibody-secreting cells, while T-lymphocytes express important regulatory functions, such as the ability to help or, in some cases, to inhibit the development of specific types of immune responses, including the production of antibodies by B cells and the increase of the microbicidial activity of macrophages. Other T-lymphocytes are involved in direct effector functions, such as the lysis of virus-infected cells or neoplastic cells.

While the vast majority of mature T lymphocytes carry a T cell receptor (TCR) molecule composed of α and β chains, a small percentage (1–5%) of the lymphocytes that circulate in the blood and peripheral organs express TCR heterodimers comprised of γ and δ chains (Hayday 2000; Das, Wang et al. 2001; Chen and Letvin 2003). γδ T cells constitute a minor portion of circulating CD3+ T lymphocytes (Hayday 2000); however, they represent the major T cells population in epithelial-rich tissues, such as the skin, reproductive tract (not in humans) and intestine, where they comprise up to 50 % of T cells (Dechanet, Merville et al. 1999). Nonetheless, conditions that lead to responses from γδ T cells are not fully understood and current concepts of γδ T cells as a “first line of defense,” as “regulatory cells” or as a “bridge between innate and adaptive responses” may not fully address of their complex behavior. In fact, γδ T cells form an entirely different lymphocyte-system, which develops along with the αβ T-cells in the thymus and in the periphery (Born, Reardon et al. 2006). γδ T cells recognize antigens independent of classical major histocompatibility complex (MHC) class I or class II presenting molecules and recognize soluble protein and non-protein antigens of endogenous origin, a property which distinguishes them from αβ T-cells (Hayday 2000; Kabelitz, Glatzel et al. 2000; Carding and Egan 2002). In addition to the classification according to TCR Vγ/Vδ (variable gamma chain/variable delta chain) usage, γδ T cells can be subdivided based on additional surface molecules such as CD4 and CD8. The vast majority of human peripheral blood γδ T cells lack CD4 and CD8 expression and thus display a ‘double-negative’ phenotype (Hayday 2000). The lack of CD8 and CD4 on the majority of human γδ cells correlates well with the lack of MHC restriction by classical MHC class I or class II

molecules (Morita, Beckman et al. 1995; Bukowski, Morita et al. 1998). The minor population expresses mostly Vδ1+ chain and comprises 20–30% of human γδ T cells. This population expresses CD8, whereas the presence of human CD4+ γδ T cells is extremely rare.

Recent evidences indicate that γδ T cells are implicated in the regulation of inflammation and autoimmunity (Carding and Egan 2000; Carding and Egan 2002). Other studies support both an effector and a regulatory role for γδ lymphocytes in biological responses *in vivo* (Hayday and Tigelaar 2003). Several data have demonstrated that γδ T lymphocytes are involved in tissue repair. In models of malignancy or infection, it has been shown that γδ T cells participate in the lysis of malignant or infected cells (Groh, Rhinehart et al. 1999; Girardi, Oppenheim et al. 2001; Kato, Tanaka et al. 2001; Carding and Egan 2002; Shen, Zhou et al. 2002; Chen and Letvin 2003; Gao, Yang et al. 2003; Wilhelm, Kunzmann et al. 2003). Interest in the development and function of γδ T cells has grown continuously since their discovery in 1986 (Weissman, Samelson et al. 1986), and recent data on the characteristics that define γδ T cells supports the idea that these cells play a distinct role from that of αß T cells in the immune response (Table 1).

Human γδ T cells can be distinguished from other lymphocyte lineages			
Characteristic	**γδ T cells**	**αβ T cells**	**B cells**
Antigen-receptor configuration	CD3 complex + γδ TCR	CD3 complex + αβ TCR	Ig
Theoretical receptor number	~10^{20}	~10^{15}	~10^{11}
Antigen recognition	Protein and non-protein	Peptide + MHC	Protein and non-protein
MHC restriction	Rare	Yes	No
Phenotype	Most are $CD4^-CD8^-$; iIELs are $CD8(\alpha\alpha)^+$	$CD4^+$ or $CD8^+$	$CD19^+CD20^+$
Frequency in blood	1–5% (25–60% in gut)	65–75%	5–10%
Distribution	Blood, epithelial and lymphoid tissues	Blood and lymphoid tissues	Blood and lymphoid tissues
Effector capability	CTLs, Cytokine release (TH1>TH2)	CTLs ($CD8^+$) Cytokine release (TH1/TH2)	Ig production
Function	Immunoregulation and immunosurveillance	Immune protection and pathogen eradication	Humoral immunity

Table 1: Human γδ T cells can be distinguished from other lymphocyte lineages CTLs. Cytotoxic T lymphocytes; iIELs, intestinal intraepithelial T lymphocytes; Ig, immunoglobulin; TH cell, T helper cell; TCR, T-cell receptor [table modified from (Carding and Egan 2002)]

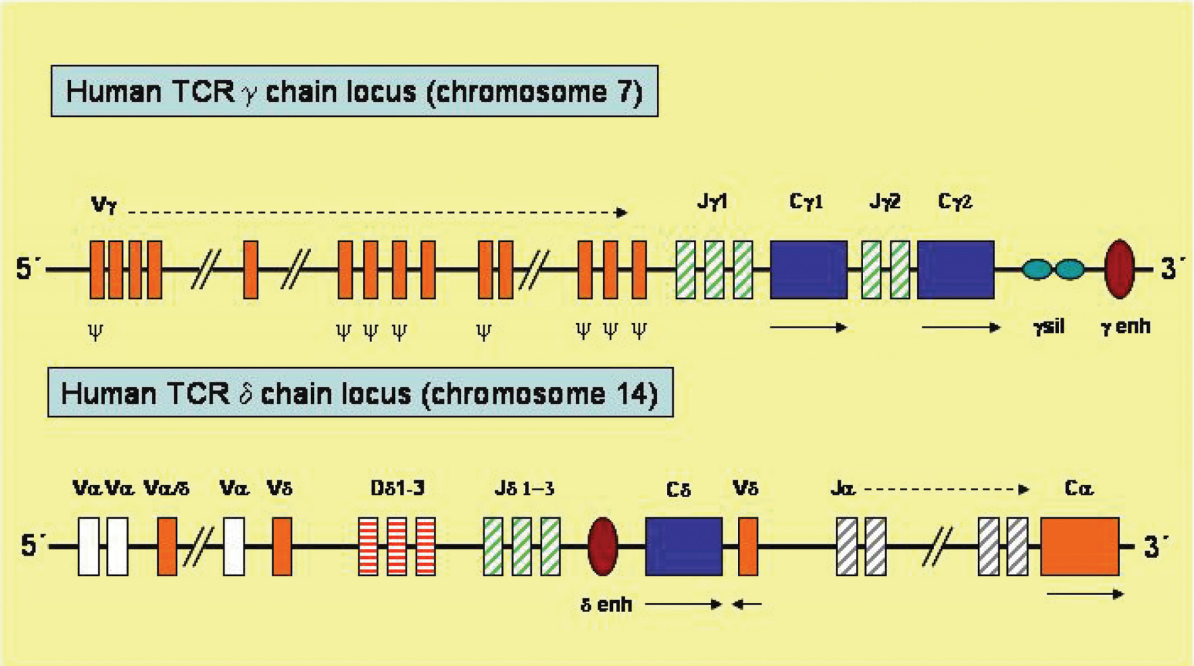
1.1 The human γδ TCR repertoire

The potency of the adaptive immune system lies in its capacity to generate billions of different antigen receptors from multiple gene segments that are assembled by somatic recombination.

This recombination joins in case of α, γ or light chains any one of several variable (*V*) gene segments with any one of several joining (*J*) gene segments and in case of β, δ or heavy chains, V, diversity (*D*) gene and J segments, to form the variable region of antigen receptors. The V–(D)–J junctional-region complexity increase by the addition or removal of nucleotides at joining sites, which further contributes to receptor-structure diversity (Fig.1) (Hayday 2000; Allison, Winter et al. 2001).

The combinations of *V*, *D* and *J* genes generate receptors with different amino-acid sequences and thus distinct molecular surfaces at their complementarity-determining regions (CDRs). While the number of available TCR Vγ and Vδ elements is small, the γδ TCR repertoire is likely to be at least as diverse as the αβ TCR or the Ig repertoire (Hayday 2000). This is a consequence of the possibility to use two (and three in case of humans) Dδ segments at the same time and in all of the possible reading frames. Insertion (respective usage of N and P nucleotides) or imprecision in V(D)J joining are mechanisms that increase the possibility to generate diversity in the γδ TCR (Hayday 2000; Kabelitz, Glatzel et al. 2000; Allison, Winter et al. 2001).

Some γδ T-cell subsets such as the Vγ5–Vδ1 γδ T cells in the skin (dendritic epidermal T cells; DETCs) and the Vγ6–Vδ1 γδ T cells of the uterine epithelia (mouse *V*γ gene nomenclature from Heilig and Tonegawa (Heilig and Tonegawa 1986)) have no junctional diversity; they have a short complementarity-determining region 3 (CDR3) and are essentially an oligoclonal population of cells (Hayday 2000; Carding and Egan 2002; Hayday and Tigelaar 2003). These cells have been described in mice and rats but not in humans (Kuhnlein, Mitnacht et al. 1996; Hayday 2000; Das, Wang et al. 2001; Carding and Egan 2002).



Graphic 1: Schematic representation of human γ and δ TCR gene families
The human TCR γ and δ are shown as indicated. TCR-V (variable) gene segments are depicted as small solid boxes; D (diversity) as striped boxed; J (join) as hatches boxes; sil, silencer (sequence that regulated TCR gene transcription). Each constant (C) gene is depicted as a single big gene box although is composed of several exons. Exon and introns are not drawn to scale and only representative Vγ and Vα are shown, TCR gene segments indicate pseudogenes. (Modified from Hayday 2000)

The mechanisms responsible for shaping the antigen-receptor repertoires of γδ T cells remain unknown. Intrinsic and genetic factors seem to be primarily responsible for generating specific subsets of γδ T cells, with extrinsic and environmental factors acting to further shape and select specific clones (Table 2) (see below). These factors probably act independently of those that influence αβ T-cell development, given that, in many animal species, γδ T cells are the first lymphocyte lineage to be generated during fetal development (Hayday 2000; Carding and Egan 2002).

In the human peripheral blood, there is a clear dominance of cells expressing Vγ9 together with Vδ2 [The human *V*γ gene nomenclature is that of Lefranc (Sturm, Braakman et al. 1989)], which can account for up to 95% of the γδ T cells (Kabelitz 1999; Das, Wang et al. 2001). Interestingly, they are only found in primates and a murine equivalent does not exits (Kabelitz 1999; Das, Wang et al. 2001). The proportion of Vγ9Vδ2 T cells gradually increases in the peripheral blood during childhood, suggesting that exposure to environmental and/or bacterial antigens (see below) might shape the γδ TCR repertoire during postnatal development (Bukowski, Morita et al. 1999; Kabelitz 1999; Morita, Lee et al. 1999; Hayday

2000; Allison, Winter et al. 2001; Das, Wang et al. 2001; Morita, Lee et al. 2001; Carding and Egan 2002; Shen, Zhou et al. 2002).

The second most frequent subset of human peripheral blood γδ T cells expresses Vδ1 in association with various Vγ elements (Hayday 2000; Kabelitz, Glatzel et al. 2000; Carding and Egan 2002; Hayday and Tigelaar 2003). However, due to the dominance of Vγ9Vδ2 cells, the Vδ1 subset usually accounts for only 10–30 % of the peripheral blood γδ T cells, and might be difficult to detect given the low percentage of γδ T cells among CD3+ peripheral blood T cells (Hayday 2000). Quantitative increases in circulating Vγ1 cells are observed in HIV infections and, even more strikingly, in cytomegalovirus (CMV) infections following renal transplantation (Dechanet, Merville et al. 1999). Under physiological conditions, however, the primary localization of Vδ1-expressing γδ T cells is not the peripheral blood but rather the intraepithelial T cell compartment of the intestine, where Vδ1 cells constitute the major T cell population (Groh, Steinle et al. 1998; Hayday 2000). The preferential expression of certain TCR Vγ and Vδ elements in different anatomical sites is usually considered to reflect the importance of a particular γδ T cell subset in the local immune surveillance (Groh, Steinle et al. 1998; Hayday 2000; Carding and Egan 2002).

Human γδ T Cell population (origin)	Functional phenotype	Antigen specificity	Biological Function
VγX–Vδ1 (iIEL lines and clones)	Cytotoxicity	MICA, MICB	Elimination of stressed/transformed cells (Groh, Steinle et al. 1998; Groh, Rhinehart et al. 1999)
VγX–Vδ1 (LA, synovial fluid clones)	?	Lipohexapeptides + DC + IL-2	Fas-mediated modulation of immune response to infection (Vincent, Roessner et al. 1996; Vincent, Roessner et al. 1998)
Vγ9–Vδ2 (PB clones)	Cytotoxicity, TH1 cytokine release	Organic phosphoesters, alkylamines, nucleotide-conjugates and cell–cell contact	Unique role in immunity to infection, homeostatic control of immune and non-immune processes? (Constant, Davodeau et al. 1994; Tanaka, Morita et al. 1995; Bukowski, Morita et al. 1999)
Vγ9–Vδ2 (TL, PB clone)	Cytotoxicity,IFN-γ Release	Pamidronate + monocytic APCs	Elimination of myeloid-cell targets? (Miyagawa, Tanaka et al. 2001)
Vγ9–Vδ2/1 (PB clones)	Cytotoxicity	SEA	Anti-microbial immunity? (Rust, Verreck et al. 1990; Loh, Wang

			et al. 1994)
Vγ9–Vδ2 (RA, synovial fluid)	Cytotoxicity	Mycobacterial $Hsp65_{180–188}$ /tetanus $toxoid_{1229–1252}$ + HLA-DRw53	Eliminate autoreactive cells? (Holoshitz, Vila et al. 1992; Holoshitz 1999) (**controversial data, which await confirmation)**
Vγ9–Vδ2 (PB lines and clones)	Cytotoxicity, cytokine release	GroEL homologue on Burkitts lymphoma cells	Tumor surveillance? (Fisch, Malkovsky et al. 1990) (**controversial data, which await confirmation)**

Table 2: Characteristics of human γδ T cells. DC, dendritic cell; GRoEL, heat-shock 60 kD protein 1; HLA, human leukocyte antigen; Hsp, heat-shock protein; iIEL, intestinal intraepithelial lymphocyte; IFN-γ, interferon-γ; IL-2, interleukin-2; MICA or MICB, MHC class I polypeptide-related sequence A or B, respectively; PB, peripheral blood; RA, rheumatoid arthritis; SEA, staphylococcal enterotoxin A; TH cell, T-helper cell; TL, tuberculoid leprosy; ?, not known. [table modified from (Carding and Egan 2002)]

1.2 Structure of the human γδ T-cell receptor: studies on the Vγ9Vδ2 TCR G115 and data obtained with mouse TCR

Sequence analysis as well as analysis of crystals of a Vδ3 domain and the Vγ9Vδ2 TCR G115, demonstrated overall similarity between γδ and αβ TCRs although γδ TCRs additionally display some characteristics structural features (Allison, Winter et al. 2001). The framework structure of Vδ more closely resembles that of the variable immunoglobulin heavy chain domain (VH) than the TCR Vα or Vß domains (Allison, Winter et al. 2001). Together with the observation that the CDR3 length distribution of TCR δ chains is similar to that of immunoglobulin heavy chains, it appears that ligand recognition by γδ T cells shares more features with antigen binding of immunoglobulins than peptide/MHC recognition by αß T cells (Hayday 2000; Allison, Winter et al. 2001; Carding and Egan 2002; Adams, Chien et al. 2005).

Sequence similarities between Vγ and Vβ and between Vδ and Vα clearly indicate their relatedness, but whether Vγ or Vδ are closer to antibody VL or VH domain is more difficult to determine (Allison, Winter et al. 2001). Amino acid alignment of the γδ TCR domains with their analogous domains in Fabs and αβ TCRs confirms the overall similarity of these immunoglobulin-like domains and also highlights their differences (Hayday 2000). The CDR loops of γδ TCRs, αβ TCRs, and antibodies compose the sites of antigen recognition. The CDR-containing surface of the G115 V domains exhibits more protrusions and clefts compared to the flatter surfaces of αβ TCRs that bind a peptide/MHC complex and of antibodies that bind protein antigens (Allison, Winter et al. 2001). The jagged surface of this γδ TCR resembles the surface of an antibody that binds a small antigen molecule. If non-

peptide antigens are recognized while bound to presentation molecules, the shape of the molecule that would complement this rather rough γδ TCR surface would likely be different from the shape of MHC molecules (Hayday 2000; Allison, Winter et al. 2001; Carding and Egan 2002).

1.3 Ligands recognized by human Vγ9Vδ2 T cells

Human Vγ9Vδ2 T cells recognize small phosphate or amine containing compounds (Pfeffer, Schoel et al. 1990; Belmant, Espinosa et al. 1999; Shen, Zhou et al. 2002), as well as alkylamines from several natural sources (Kamath, Wang et al. 2003) and aminobisphosphonates (ABP) (Kunzmann, Bauer et al. 1999; Kunzmann, Bauer et al. 2000). These non-peptide antigens do not require the classical MHC-presentation, but cell–cell contact is necessary for adequate stimulation (Kunzmann, Bauer et al. 2000; Kato, Tanaka et al. 2003) suggesting either that non-MHC molecules may present small antigens to γδ TCRs or that co-stimulation from neighboring cells is needed for the activation of those cells (Kato, Tanaka et al. 2003). Intact proteins can also serve as antigens, e.g. the murine nonclassical MHC class I molecule T10/T22 proteins (which are known generically as thymus leukemia, TL, antigens). The expression of these proteins is up-regulated in activated cells (Hayday 2000). Only a few examples of MHC-restricted human γδ T cell clones have been reported (Allison, Winter et al. 2001) and finally, recognition of viral proteins such as glycoprotein I from *Herpes simplex* has been described (Sciammas and Bluestone 1998).

Early in vitro experiments indicated that the dominant subset of peripheral blood Vγ9Vδ2 T cells, strongly reacted in a non-MHC restricted fashion towards inactivated *Mycobacterium tuberculosis (M. tuberculosis)* and a variety of other microorganisms, including *Plasmodium falciparum*, *Toxoplasma gondii*, *Yersinia enterocolitica, Francisella tularensis* (Morita, Beckman et al. 1995; Bukowski, Morita et al. 1998; Bukowski, Morita et al. 1999; Kabelitz 1999; Morita, Lee et al. 1999; Carding and Egan 2000; Hayday 2000; Das, Wang et al. 2001; Hintz, Reichenberg et al. 2001). Further investigations revealed that the γδ T cell-stimulating moiety of microbial extracts was not protein, but rather consisted of non-proteinaceous, phosphatase-sensitive, low-molecular-weight compounds (Pfeffer, Schoel et al. 1990; Morita, Beckman et al. 1995; Bukowski, Morita et al. 1998; Bukowski, Morita et al. 1999; Kabelitz 1999; Morita, Lee et al. 1999; Carding and Egan 2000; Hintz, Reichenberg et al. 2001; Morita, Lee et al. 2001).

Studies on the immune response of Vγ9Vδ2 T cells at present are difficult, due to a lack of feasible animal models. Mice do not express homologues of Vγ9Vδ2 TCR, and there is no functional equivalent of these cells in other small laboratory animals. Only non-human primates can serve as good animal models for studying the role of Vγ9Vδ2 T cells in immune biology and diseases, since macaque Vγ or Vδ elements share up to 91% similarity in amino acid sequences to their human counterparts. More importantly, the cells of macaque recognize nonpeptide phosphoantigens as efficiently as their human counterparts (Sicard, Ingoure et al. 2005).

1.3.1 Self-ligands of the Vγ9Vδ2TCR

Most of the well-defined self-ligands of the γδ TCR belong to a group of self-molecules potentially capable of indicating cellular "stress," including CD1, MIC-A or B, mitochondrial antigens like Heat shock protein-65 (HSP), phospholipid cardiolipin (CL), and F1-ATP synthase. These antigens have been found to be stimulatory in human γδ T cells (Born, Reardon et al. 2006). HSP-65 and HSP-70 were initially proposed as promising antigenic targets for γδ T cells, owing to their role as sensors during cell stress or cell transformation. Accordingly, several observations confirmed a correlation between HSP up-regulation on tumor cells and γδ T cell infiltration/activation, suggesting HSP65-dependent recognition of tumor cells by Vγ9Vδ2 T lymphocytes (Bonneville and Fournie 2005). Subsequent studies, however, failed to demonstrate the direct activation of Vγ9Vδ2 T cells by HSP (either HSP65 or HSP70), either as native proteins and crude extracts of cells that expressed these proteins. Therefore, the contribution of these chaperones to Vγ9Vδ2 T cell activation remains largely unclear. HSP may indirectly contribute to γδ T cell activation, e.g. through regulation of γδ T cell Ag processing or through the generation of TCR-independent costimuli such as interaction with innate receptors or induction of innate receptor-ligands.

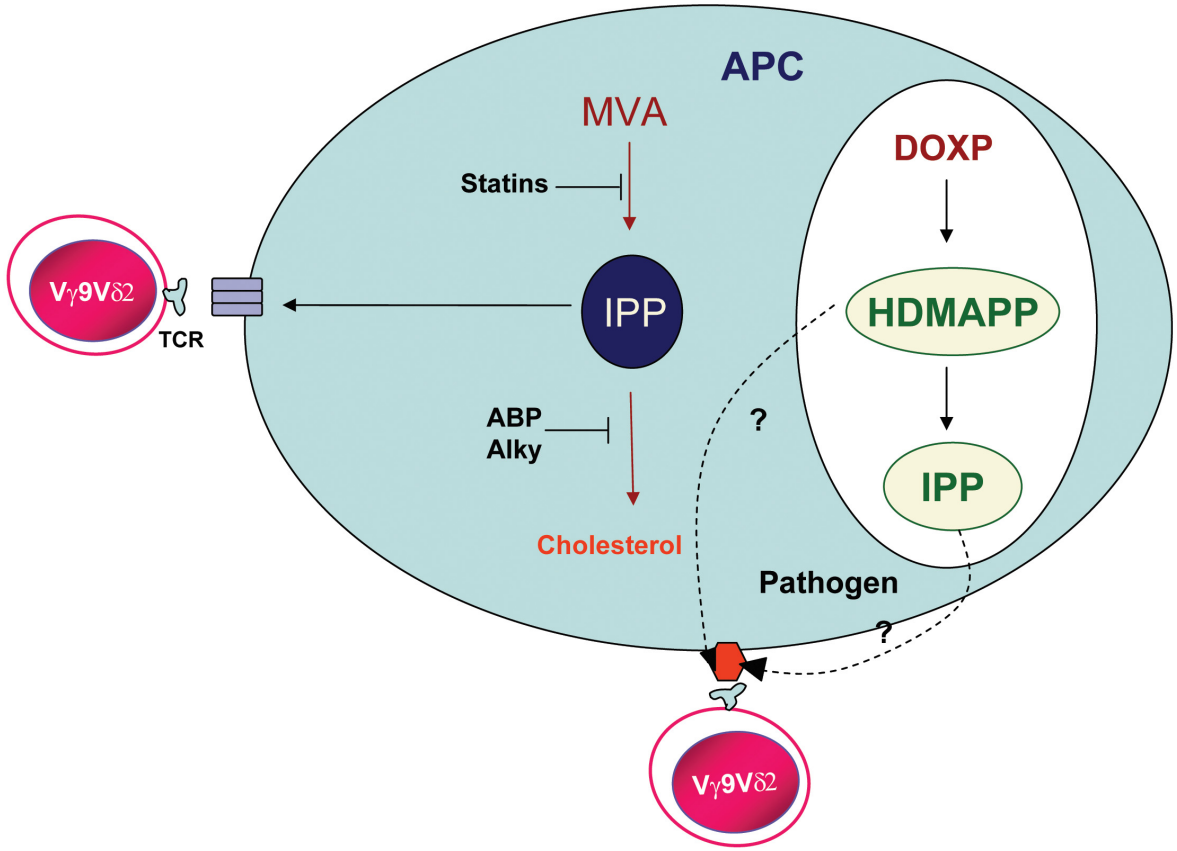
Recent evidence indicates that Vγ9Vδ2 TCR directly binds to F1-ATPase, which is normally localized in the mitochondrial membrane (Scotet, Martinez et al. 2005). F1-ATPase complexed with the serum protein apolipoprotein A1 has been identified as a target for Vγ9Vδ2 T cells through the screening of mAb able to inhibit the recognition of tumor cell line by Vγ9Vδ2 T cells in vitro (Scotet, Martinez et al. 2005).

Mitochondrial antigens could be an alerting signal that indicates the state and fate of the cell. With their inaccessible and putative microbial origin, mitochondria are intriguing as a possible source of antigens for γδ T cells, but so far, it is not known whether these molecules are involved in phosphoAg processing and/or presentation. In addition, the findings of Scotet et al. still wait for confirmation by other groups working in the field.

1.3.2 Non-peptide ligands for Vγ9Vδ2 T Cells

The biochemical structures of non-peptide antigens which specifically activate circulating human Vγ9Vδ2 T lymphocytes provide clues for the possible driving force and significance of this subset. Following the observations that human peripheral blood Vγ9Vδ2 T cells are enriched in *Mycobacterium* induced lepromatous lesions, an intensive search was undertaken to find the nature of the mycobacterial antigens. Early studies indicated that this T cell subpopulation was reactive to small non-peptide antigens (Morita, Beckman et al. 1995). Attempts to purify mycobacterial ligands were further continued in parallel with assays of synthetic non-peptidic compounds. Since then different types of phosphorylated ligands have been isolated from Mycobacteria, as a group of four structurally related phosphoesters (so-called TUBag [1-4]), comprising both thymidine and uridine nucleotide-conjugates as well as their non-nucleotide distal pyrophosphoester moieties (Constant, Davodeau et al. 1994; Poquet, Constant et al. 1996), but recently studies lead to the conclusion that none of the natural phosphoantigens (TUBags 1–4) possess the structures reported previously (Yang, Liu et al. 2001). Parallel studies in Mycobacteria identified phosphate-containing antigens as isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP); collectively, termed Phosphoantigens (Fig 2.) (Pfeffer, Schoel et al. 1990; Constant, Davodeau et al. 1994; Tanaka, Morita et al. 1995). The most potent natural phosphoantigen E-4-hydroxy-3-methylbut-2-enyl-pyrophosphate (HDMAPP) was characterized in *Escherichia coli* (Hintz, Reichenberg et al. 2001; Eberl, Altincicek et al. 2002) and also in plants. HDMAPP is a precursor of the microbial non-mevalonate pathway that ends up in isopentenyl pyrophosphate (IPP) and further in isoprenoids. In addition Vγ2Vδ2 T cells are stimulated in a cross-reactive fashion by synthetic, natural alkylamines (Bukowski, Morita et al. 1999) and therapeutic aminobisphosphonates (ABP) like pamidronate (Kunzmann, Bauer et al. 1999; Kunzmann, Bauer et al. 2000). More recently, the Vγ9Vδ2-selective antigens from human tumor cells were characterized as endogenous metabolites of the mevalonate pathway and most likely as IPP (Gober, Kistowska et al. 2003). The interaction between Vγ9Vδ2 TCR and phosphoantigens was demonstrated by inhibition assays using anti-TCRδ antibodies, and by

the transfection of Vγ9Vδ2 TCR into Jurkat T cells (TCR-) that confer reactivity against phosphoantigens.



Graphic 2: Different pathways that induce activation of Vγ9Vδ2 T cells. In mammalian cells, IPP is produced through the isoprenoid mevalonate pathway (MVA). Pharmacological agents block upstream (statins) or downstream (ABP, alkylamines) the MVA, respectively, to decrease or increase IPP levels. Accumulation of IPP is also observed in diverse tumor cells. IPP is present at the surface by not yet defined molecule. In pathogen-infected APCs (e.g. mycobacterial infection), bacterial E-4-hydroxy-3-dimethylallyl pyrophosphate (HDMAPP) or IPP are produces trough the DOXP pathway and presented by not yet defined mechanism. (Modified of Bonneville and Scotet 2006)

1.3.2.1 The isoprenoid metabolism: pathways of production of non-peptide ligands for Vγ9Vδ2 T cells

All living organisms produce isoprenoids, which are essential metabolites of their cellular and intercellular biology. These isoprenoids constitute a diverse structural family, comprising, ubiquinones, sterols, terpenes, carotenoids, gibberellins, and taxoids. Strikingly enough, the biosynthesis of such a huge structural diversity implies the same bottleneck: the repeated condensation of two branched C5 precursors, the IPP and its isomer dimethylallyl pyrophosphate (DMAPP). IPP can be synthesized via two different biosynthetic pathways.

Archaebacteria, few bacterias and most eukaryotes synthesize IPP from acetyl-CoA through the mevalonate pathway (MVA). The MVA pathway involves the initial oxidation of pyruvate into acetyl-CoA by a multimolecular complex the pyruvate dehydrogenase, initially discovered in mitochondria, (Reed 2001). Three acetyl-CoA molecules are condensed into 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA), which is reduced to mevalonate by the key enzyme HMG-CoA reductase. Then ATP-dependent steps are catalyzed by structurally related enzymes (Fu, Wang et al. 2002), wherein three successive phosphorylations of mevalonate are followed by a decarboxylation step producing IPP. Finally, IPP isomerase produces its Dimethylallyl-PP (DMAPP) isomer, and these are the C5 precursors for downstream metabolites such as sterols. On the other hand, cyanobacteria, algae, plastide and most eubacteria (including *M. tuberculosis*), make IPP in a different way, through a carbohydrate-based route referred to as 1-deoxy-d-xylulose-5-phosphate (DOXP, non-mevalonate pathway or Rohmer pathway) (Jomaa, Feurle et al. 1999). The DOXP pathway starts from pyruvate and D-glyceraldehyde-3-phosphate substrates and after several steps under oxygen-free conditions the HDMAPP synthase produce 4-hydroxy-dimethylallyl diphosphate (HDMAPP), which is than converted to IPP.

Which of these two pathways, DOXP or MVA has evolved first remains unknown, since DOXP only exists in bacteria and plastids where it provides most primary isoprenoids instead of the MVA used by *Archae* (Jomaa, Feurle et al. 1999). Some bacterial species have also kept both pathways, but for different roles, DOXP for primary metabolism and MVA for secondary metabolites (Bonneville and Fournie 2005).

In accordance with the high potency of HDMAPP as stimulator of Vγ9Vδ2 T cells, and the close correlation between the γδ T cell stimulatory activity of the bacteria exploiting the DOXP but not the mevalonate pathway (like *Mycobacterium tuberculosis* and *E. coli*) (Rohmer, Knani et al. 1993; Bukowski, Morita et al. 1999; Carding and Egan 2000; Hayday 2000; Feurle, Espinosa et al. 2002; Eberl, Hintz et al. 2003).

Since mammals make their sterols only through the MVA pathway, it is the rationale target for the lipid-lowering statin class of drugs (Poupot and Fournie 2004). In plants and yeast, regulation of the MVA pathway at the HMGR level is well documented. In response to an attack of pathogens or wounds, reduction of HMGR involves both sterol-sensor-regulated MAPK cascades which drive selected gene transcriptions and its controlled

ubiquitination/degradation (Yang, Liu et al. 2001; Hampton 2002). High levels of Farnesyl pyrophosphate (FPP), sterols or phenylalanine inhibit their HMGR activity. In mammalian cells, the HMGR activity is inhibited by statins, by phenylalanine (Shefer, Tint et al. 2000) or by a feedback inhibition with aminobisphosphonate-induced FPP accumulation (Houten, Schneiders et al. 2003). The HMGR activity, and thus, the whole MVA pathway, were found increased in cancer cell lines such as leukemia, non-Hodgkin lymphoma (Harwood, Alvarez et al. 1991), mammary and lung adenocarcinoma (Gueddari, Favre et al. 1993; Asslan, Pradines et al. 1999), in which HMGR activity is controlled by epidermal growth factor-PI3-Kinase mediated signaling. Accordingly, Vγ9Vδ2 T cells recognize metabolites of isoprenoid synthesis that are generated by the nonmevalonate pathway in certain pathogenic microorganisms but not by the mevalonate pathway in other bacteria and mammalian cells (Chen and Letvin 2003). IPP as the final product of both biosynthesis pathways, activates Vγ9Vδ2 T cells only in the μM range (medium activity), which, however, are not achieved in normal mammalian cells. Therefore, Vγ9Vδ2 T cells sense proliferating pathogens that secrete very low amounts of intermediates of the nonmevalonate isoprenoid biosynthesis pathway (Belmant, Espinosa et al. 1999). In addition, they may sense accumulation of IPP in malignant mammalian cells (Gober et al 2003) or cells, which, as explained later, accumulate IPP under treatment with inhibitors of the IPP consuming enzymes of isoprenoid biosynthesis. Together, these results indicate that IPP is a sensor of cell stress, and help to understand the role of Vγ9Vδ2 T cells in the immune defense against pathogenic microorganisms and tumors.

1.3.2.2 Aminobisphosphonates: The metabolical link to phosphoantigen -production

Aminobisphosphonates have potent and selective capacity of activate Vγ9Vδ2 T cells. This property of the aminobisphophonates was initially based on the finding that several patients with multiple myeloma (MM) treated with the well-established osteoporosis inhibitor pamidronic acid presented significantly high numbers of blood-borne γδ T cells (Kunzmann, Bauer et al. 1999). Pamidronate activates γδ T cells *in vitro* to secrete cytokines (IFNγ), proliferate and exhibit strong cytotoxicity against various cancer cell lines (Kunzmann, Bauer et al. 2000). Nevertheless, the bioactivity of aminobisphosphonates required the presence of accessory "Antigen Presenting Cells" (APC) treated with this drug prior to the assay with the γδ T cells (Miyagawa, Tanaka et al. 2001). Recently, it was established that for bioactivity on γδ T lymphocytes, the aminobisphosphonates require to be internalized and exert a statin-sensitive effect, namely inhibiting the endogenous MVA pathway (Gober, Kistowska et al.

2003). Thus, the aminobisphosphonates cause a pharmacological inhibition of the mevalonate pathway in the treated cells, leading to IPP bioaccumulation.

In agreement with the implication of the pharmacological action of amino-bisphosphonates as the rationale for their action on γδ T cell activation, non-amino bisphosphonates inhibitors for osteoporosis such as etidronate or clodronate neither inhibit the MVA pathway nor enable γδ T cell activation by etidronate- or clodronate-treated cells, respectively. On the contrary, more potent aminobisphosphonates analogues for both MVA inhibition and γδ T cell activation are now available, such as zoledronate or ibandronate (Das, Wang et al. 2001).

1.3.2.3 Alkylamines: Vγ9Vδ2 T cell specific activators different from phosphoantigens

In addition to phosphoantigens, primary alkylamines have been demonstrated to activate in the mM range (weak activity) the same subset of Vγ9Vδ2 T cells. Such alkylamines are structurally composed of non-phosphate short alkyl chains bearing a terminal amino group; prototypic bioactive alkylamines are ethylamine and sec-butylamine. This alkylamines are produced by certain plants, present in wine and in green tea or secreted by several bacteria. *Listeria monocytogenes, Bacterioides fragilis, Proteus morganii, Clostridium perfringes, and Salmonella typhimurium* produce alkylamines in concentrations able to activate the γδ T cell responses. (Kamath, Wang et al. 2003). Altogether, the alkylamines constitute a separate class of Vγ9Vδ2 T cell activators that upon ingestion of food or beverages may stimulate γδ T cells *in vivo*. The activated γδ T cells then release, mainly, Th1 cytokines. For this reason it is thought that alkylamines-rich diets may contribute to counter food allergies (Poupot and Fournie 2004).

1.3.3 Modulation of Vγ9Vδ2 T cell activation by natural killer receptors (NKR) and Toll-like receptors

Scavenger receptors, Toll-like receptors (TLR) and NK cell receptors (activating and inhibitory) are expressed by γδ T cells. The role played by TLRs expressed by γδ T cells is not completely understood, however, TLR2 is expressed by freshly isolated γδ T cells and functional assays confirmed that human Vγ9Vδ2 T cells respond to the TLR2 agonist Pam3Cys. Nevertheless, the observed response requires simultaneous stimulation of the γδ T-cell receptor and the TLR2. Dually stimulated cells produced higher levels of IFN-γ and

showed increased expression of the lysosome-associated membrane protein CD107a on the cell surface (Deetz, Hebbeler et al. 2006). Furthermore, recently, TLR3 has been reported in human γδ T cells and less extend in αβ T lymphocytes (Wesch, Beetz et al. 2006). TLR3 recognizes viral dsRNA and its synthetic mimetic polyinosinic-polycytidylic acid (poly (I:C). TLR3 expression is commonly considered restricted to dendritic cells, NK cells, and fibroblasts. Although T cells did not respond directly to poly (I:C), a dramatic increase in IFN-γ secretion and an up-regulation of CD69 was observed when freshly isolated γδ T cells were stimulated via TCR in the presence of poly(I:C) without APC. IFN-γ secretion was partially inhibited by anti-TLR3 Abs. In contrast, poly (I:C) did not co-stimulate IFN-γ secretion by αβ T cells. These results indicate that TLR3 signaling is differentially regulated in TCR-stimulated γδ and αβ T cells, suggesting an early activation of γδ T cells in antiviral immunity. In addition, it has been described that γδ T cells might indirectly depend on TLRs, expressed by myeloid cell partners (Kunzmann, Kretzschmar et al. 2004).

It is likely that the integration of signals via the TCR and other receptors produces unique responses and distinctive functions. The activation pattern of γδ T cells is modulated by signals delivered through MHC class I-specific inhibitory receptors, as with other T cells and NK cells. A large fraction of Vγ9Vδ2 T cells expresses in a non-exclusive fashion a wide array of inhibitory and activating receptors shared with NK cells. These receptors presumably ensure efficient discrimination by Vγ9Vδ2 T cells of “stress” vs. “normal” cell target through MHC, MHC like and non-MHC ligands that are under or over expressed in transformed or infected cells. Several inhibitory natural killer receptors (iNKR) are found frequently on Vγ9Vδ2 T cells from peripherical blood lymphocytes (PBL), including killer Ig-like receptors (KIR), leukocyte Ig-like receptors (LIR) and lectin-like receptors (such as NKG2A/CD94). Most of these iNKR decrease killing by Vγ9Vδ2 CTL of target cells expressing high levels of either classical and/or non-classical MHC molecules. Due to the broad cellular distribution of some Vγ9Vδ2 TCR agonists such as IPP, which are up-regulated on both activated and transformed cells, and the frequent down-modulation of MHC class I expression on tumoral cells, MHC class I-specific iNKR may selectively down-regulate recognition of “healthy” activated cells by the activity of Vγ9Vδ2 CTL. This mechanism ensures self-tolerance. Importantly, iNKR or iNKR-ligand masking may also increase Vγ9Vδ2 T cell killing of several hematopoietic and non-hematopoietic tumors (Trichet, Benezech et al. 2006), and thus may unveil broad anti-tumor activities of this γδ T cell subset.

Vγ9Vδ2 T lymphocytes also express several activating NKR (aNKR), which could play a major role in strengthening responses against infected or tumor cells. In particular, NKG2D, a C-type lectin receptor shared by NK, γδ and conventional CD8 T lymphocytes, has been shown to co-stimulate NK and T cell activation upon engagement with various stress ligands such as MICA/B and ULBP in humans (Bahram, Inoko et al. 2005). Contribution of NKG2D to antitumor responses will be described in detail later. Several other aNKR, such as KLRG1, 2B4, NKPR44 or CD94/NKG2C receptors, may also contribute to co-stimulation of antitumor T cell responses (Poccia, Cipriani et al. 1997; Eberl, Engel et al. 2005; von Lilienfeld-Toal, Nattermann et al. 2006), although this possibility remains to be formally documented in the Vγ9Vδ2 T cell system and may be of lesser importance given that expression of many aNKR is rather NK cell specific. In conclusion, both TCR and NKR act in a coordinated fashion to direct Vγ9Vδ2 T cell responses against stressed, infected and/or transformed cells. Each of these receptor/ligand interactions triggers effector functions of Vγ9Vδ2 T cells in *in vitro* systems. It remains to be determined how Vγ9Vδ2 T cells integrate activating and inhibitory signals delivered by TCR and NKR *in vivo* and how such knowledge could be used to manipulate the antitumor or antibacterial properties of this subset.

1.4 Antigen presentation by activated Vγ9Vδ2 T cells: an alternative type of professional APC

A large fraction of tonsillar γδ T cells are activated and express high levels of MHC II and the CD28 ligands CD80 and CD86, a fact that prompted a detailed analysis of APC functions in peripheral blood Vγ9Vδ2 T cells (Brandes, Willimann et al. 2005). These studies were greatly facilitated by the broad responses *in vitro* of Vγ9Vδ2 T cells to IPP. The APC features depicted in these cells include (a) the expression of antigen presentation, co-stimulation and adhesion molecules, (b) antigen uptake, processing and presentation to αβ T cells, and (c) induction of primary αβ T cell responses. Freshly isolated Vγ9Vδ2 T cells lacked most APC molecules and had reduced levels of adhesion molecules.

Dendritic Cells (DC) derived from monocytes, express several of the characteristics mentioned before, but still do not have potent APC function. By contrast, 24 h treatment of Vγ9Vδ2 T cells with IPP induce high levels of APC molecules, similar to those seen in 12 h lipopolysaccharide (LPS) treated (mature) monocyte-derived DCs. Except for the DC maturation molecules CD40 and CD83, these APC molecules remain expressed in activated

Vγ9Vδ2 T cells during prolonged *in vitro* proliferation. APC–responder-cell interactions are observed by rapid and extensive cell clustering between Vγ9Vδ2 T cells stimulated for 24–48 h with IPP (Vγ9Vδ2 T-APCs) with autologous, naïve αβ T cells. Autocrine and/or paracrine factors, such as cytokines produced in response to IPP-induced Vγ9Vδ2 TCR triggering, might also contribute to the induction of APC characteristics in this γδ T cells.

Vγ9Vδ2 T-APCs also have an efficient antigen uptake and processing machinery. This was demonstrated by the induction of antigen-specific $CD4^+$ αβ T cell responses during stimulation with Vγ9Vδ2 T-APCs presenting peptides derived from the simple and highly complex protein antigens tetanus toxoid (TT) and *M. tuberculosis* purified protein derivative (PPD), respectively (Brandes, Willimann et al. 2005). Antigen presentation was sensitive to chloroquine, an inhibitor of endosomal acidification, and neutralizing anti-HLA-DR antibodies, indicating that the route of MHC II processing does not differ fundamentally from that of DCs. The ultimate test for 'professionalism' in Vγ9Vδ2 T-APCs involved the induction of primary αβ T cell responses. A variety of APCs trigger memory responses, because of the lower threshold of activation in memory T cells. By contrast, DCs present peptide–MHC complexes in the context of sufficient co-stimulation for induction of naïve αβ T cells (which have a high threshold of activation) and are therefore called 'professional' APCs. Vγ9Vδ2 T cells also qualify as professional APCs, as shown by the responses of naïve $CD4^+$ and $CD8^+$ αβ T cells to Vγ9Vδ2 T-APCs presenting alloantigens and/or superantigens (Brandes, Willimann et al. 2005). Primary responses included αβ T cell proliferation, T helper cells, and cytotoxic T cell generation. Collectively, activated (but not resting) Vγ9Vδ2 T cells can have potent APC functions and thus clearly differ from monocytes and αβ T cells, which completely lack 'professionalism'.

1.5 Expression of CD28 and NKG2D on Vγ9Vδ2 Ţcells

CD28, the ligand for B7 (CD80/86), which is the primary co-stimulatory receptor of αβ T cells, is expressed on some γδ cells. Although it was shown that CD28 regulate the response of mouse γδ cells to TCR cross-linking, and of human γδ cells to allogeneic dendritic cells, its expression is variable, and it is not clear that as a general rule γδ cells utilize CD28 for interaction with B7 (+) professional APCs (Hayday 2000; Carding and Egan 2002; Diefenbach and Raulet 2003; Jameson, Witherden et al. 2003; Kato, Tanaka et al. 2003). Nevertheless, with recent work showing that even B cell antigens are modified so as to co-

engage both the B cell receptor (BCR) and CR2/CD21, it seems certain that γδ cell activation requires more than stimulation through the antigen receptor alone (Kato, Tanaka et al. 2003). Newer evidence also shows that the NKG2D receptor, which is expressed on γδ T cells and NK cells of mouse and human respectively, mediates such a co-stimulatory signal (Bauer, Groh et al. 1999; Groh, Rhinehart et al. 1999; Das, Groh et al. 2001; Diefenbach and Raulet 2003; Kato, Tanaka et al. 2003; Wilhelm, Kunzmann et al. 2003). NKG2D recognizes the MHC class I chain-related A (MICA/MICB) and ULBPs (UL16 binding proteins) (expressed in humans) and Rae-1 (retinoic acid early inducible 1 proteins) and H-60 (dominant minor histocompatibility antigen) (expressed in mouse) in the absence of CD4 or CD8. (Das, Groh et al. 2001; Girardi, Oppenheim et al. 2001; Carding and Egan 2002; Vivier, Tomasello et al. 2002; Kato, Tanaka et al. 2003; Raulet 2003).

Interestingly MICA/B molecules are not expressed by most normal tissues but are up-regulated in many epithelial tumor cells, in cells infected with human cytomegalovirus, in cells infected by bacteria and in "stressed" cells (Groh, Steinle et al. 1998; Bauer, Groh et al. 1999; Groh, Rhinehart et al. 1999; Das, Groh et al. 2001; Groh, Bruhl et al. 2003; Raulet 2003). A low level of MICA/B expression is maintained on the epithelial cells lining the gastrointestinal surfaces, which may be due to interactions of these cells with various environmental "stressors" (Groh, Steinle et al. 1998). Finally, the fact that expression of ULBPs, MICA/B in human and Rae1 proteins and H60 is modulated in response to stress or as a result of transformation provides the means by which γδ T cells might function in anti-tumor immunity, which is recognition and elimination of damaged or malignant (epithelial) cells as a result of signals derived through both the γδ TCR and these co-receptors (Bukowski, Morita et al. 1999; Diefenbach and Raulet 2003; Raulet 2003; Watzl 2003).

1.6 NKG2D receptor signaling: data from mouse to human

The expression of NKG2D ligands on target cells potently induces NK cell cytotoxicity. Depending on the levels of NKG2D ligands, the stimulatory signal can override coexisting inhibitory signals provided by the same target cells (Vivier, Tomasello et al. 2002). However, the stimulatory signal provided by NKG2D is not entirely refractory to inhibitory signals (Vivier, Tomasello et al. 2002; Watzl 2003). Crosslinking of the NKG2D receptor on murine NK cells triggers several effector mechanisms from NK cells (e.g. mobilization of intracellular Ca^{2+}, production of cytokines including IFN-γ, GM-CSF, TNF-α and β, and production of chemokines such as macrophage inflammatory protein (MIP-1β) (Bauer, Groh

et al. 1999; Groh, Wu et al. 2002; Jamieson, Diefenbach et al. 2002; Vivier, Tomasello et al. 2002; Raulet 2003; Watzl 2003).

Interestingly, triggering of the NKG2D receptor alone is sufficient to stimulate NK cells activation (Raulet 2003). By contrast, CD8+ T cells that express NKG2D cannot be directly stimulated via NKG2D. In CD8+ T, including CD28- CD8+ T cells, however NKG2D provides a co-stimulatory signal that synergizes with T cell receptor signal (Fisch, Malkovsky et al. 1990; Bauer, Groh et al. 1999; Groh, Rhinehart et al. 2001; Groh, Wu et al. 2002; Jamieson, Diefenbach et al. 2002; Vivier, Tomasello et al. 2002; Raulet 2003; Watzl 2003). Hence, interactions of NKG2D with induced ligands provide functions appropriate to the cells in which it is expressed; direct activation of innate immune cells and enhancement of the specific response of antigen-specific CD8+ T cells.

Expression of NKG2D on the cell surface requires its association with DAP10, a transmembrane-anchored adaptor protein expressed as a disulfide-linked homodimer (Diefenbach and Raulet 2003; Raulet 2003). The short cytoplasmic domain of DAP10 contains a YxxM motif (in amino acid one-letter code, where x denotes any amino acid) that binds to the p85 subunit of phosphatidylinositol-3 kinase (PI3 kinase) and Grb2 upon phosphorylation (Wu, Song et al. 1999; Diefenbach, Tomasello et al. 2002; Gilfillan, Ho et al. 2002; Jamieson, Diefenbach et al. 2002; Raulet 2003; Watzl 2003). In mice, in addition to the ‘long’ NKG2D (NKG2D-L) glycoprotein, which associates exclusively with DAP10, a ‘short’ (NKG2D-S) alternatively spliced isoform of mouse NKG2D exists that is able to associate with both DAP10 and DAP12. NKG2D-S was also observed in NK cells obtained from DAP10-deficient mice (Gilfillan, Ho et al. 2002; Raulet 2003; Watzl 2003).

Human NK cells express DAP10 and DAP12. Alternatively spliced and truncated NKG2D transcripts are generated in activated human lymphocytes, but these isoforms exclusively bind DAP10 with NKG2D (Raulet 2003; Andre, Castriconi et al. 2004). The stimulation via NKG2D-DAP10 in human NK cells induces cytotoxicity, cytokine production, and phosphorylation of DAP10 but not DAP12 (Gilfillan, Ho et al. 2002; Billadeau, Upshaw et al. 2003), therefore it appears likely that mechanisms different from those found in mice regulate NKG2D function. The phosphorylated hexameric NKG2D-DAP10 complex, once activated, couples two adaptors that propagate separate branches of the signaling pathway. Grb2 directly binds Vav1 and is responsible for PLCγ2 and SLP-76 phosphorylation, while the p85a

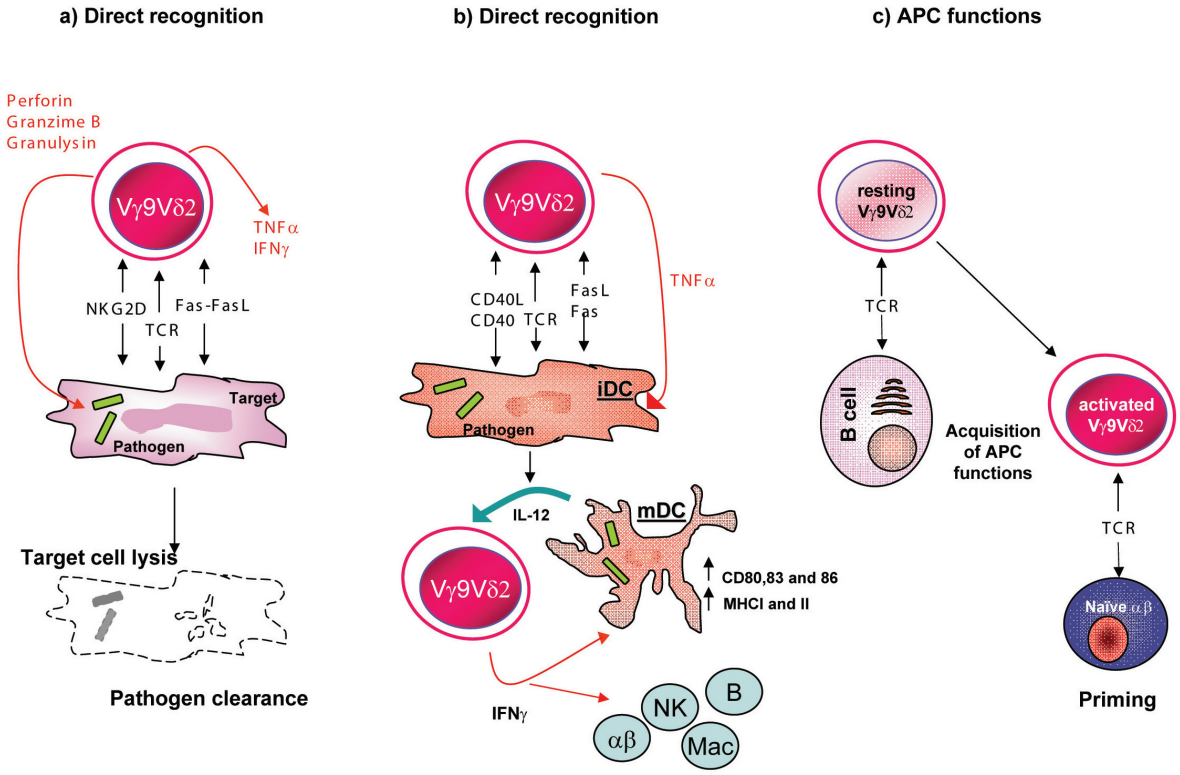
regulatory subunit of PI3K activation leads to ERK phosphorylation (Gilfillan, Ho et al. 2002; Billadeau, Upshaw et al. 2003). Both are required for optimal calcium flux and cytotoxicity. In resting human NK cells, NKG2D may only provide co-stimulation, but in activated human NK cells and in certain types of activated CTLs exclusive activation of NKG2D can trigger cytotoxicity.

The downstream events accompanying NKG2D stimulation have not been extensively characterized. Interestingly, Sutherland et al. observed that stimulation of human NK cells with soluble NKG2D ligands resulted in the activation of Janus kinase 2, STAT5, MAPK and PI3 kinase/Akt signal transduction pathways (Sutherland, Chalupny et al. 2002; Billadeau, Upshaw et al. 2003). Although activation of PI3 kinase/Akt is explained by the presence of the YxxM motif in DAP10 (Billadeau, Upshaw et al. 2003), the mechanism by which the other signaling pathways are recruited requires further investigation.

1.7 Possible functions of Vγ9Vδ2 T Cells: implication on infections and tumor control

Clearly, Vγ9Vδ2 T cells are a unique population of T cells that evolution has preserved to perform some important protective roles for the host. Although the nature and mechanisms of these roles are not yet fully understood, the ability of Vγ9Vδ2 T cells to interact with a various other immune cells can help in the course and outcome of a variety of inflammatory immune responses. Activated γδ T lymphocytes release growth factors, produce cytokines/chemokines and lysed infected or malignant cells (Fig 3.) (Sturm, Braakman et al. 1991; Bauer, Groh et al. 1999; Kabelitz 1999; Carding and Egan 2000; Hayday 2000; Girardi, Oppenheim et al. 2001; Carding and Egan 2002). Recently these functions have been translated into evidence for biological applications such as tissue repair, tumor regression and regulation of inflammation (Sturm, Braakman et al. 1991; Carding and Egan 2000; Hayday 2000; Girardi, Oppenheim et al. 2001; Carding and Egan 2002).

Only two important functions of γδ T cells will be discussed in the following part: the response to infections and the contribution to tumor control, since these two aspects are the focus of the current study.



Graphic 3: Role of Vγ9Vδ2 T cells against tumors and infections. a) Direct recognition of tumor or target cell. Following TCR engagement, Vγ9Vδ2 T cells release inflammatory cytokines (TNF-α, IFN-γ) and lytic mediators (perforin, granzyme B and granulolysin), leading to the destruction of the target cells. b) Priming of other immune effector cells, promotes DC maturation through strong release of TNF-α and CD40 engagement. Maturing DC cells releases IL-12, which amplifies IFN-γ secretion by activated T lymphocytes. Release of cytokines contributes to the priming of innate and adaptive immune effectors, involved in the clearance of pathogens. c) APC functions. Upon short-term antigenic stimulation, Vγ9Vδ2 T cells acquire attribute of APCs (CD40, MHC I and II, and co-stimulatory molecules) and promote priming of naïve conventional αβ T cells. The B cells are required for get optimal induction of those APC features. (Modified from Bonneville and Scotet 2006).

1.8 Vγ9Vδ2T cell response to infection

γδ T cells have been implicated in several models of infection as effectors and regulators of the immune response. In table 3 bacterial and parasitic infections in which activation and expansion of Vγ9Vδ2 T cells has been observed are listed. The role of Vγ9Vδ2 T cells in *M. tuberculosis* (Mtb) infection will be described in greater detail (Kaufmann, Schoel et al. 1991; Munk, Gatrill et al. 1991; Munk and Kaufmann 1991; Boom, Chervenak et al. 1992; Carding and Egan 2000; Chen and Letvin 2003).

INFECTION	Mean (max) % of Vγ9Vδ2 T cells in normal individuals	Mean (max) % of Vγ9Vδ2 T cells in patients	References
Bacterial			
Tuberculosis	6	14 (35)	(Balbi, Valle et al. 1993)
Leprosy, reversal reaction	5	29*	(Modlin, Pirmez et al. 1989)
Tularemia	3	33 (50)	(Sumida, Maeda et al. 1992; Poquet, Kroca et al. 1998)
Salmonellosis	5	18 (48)	(Hara, Mizuno et al. 1992)
Legionellosis	5	15 (42)	(Kroca, Johansson et al. 2001)
Brucellosis	4	29 (48)	(Bertotto, Gerli et al. 1993)
Ehrlichiosis	5	57 (97)	(Caldwell, Everett et al. 1995)
Listeriosis	2	12 (33)	(Jouen-Beades, Paris et al. 1997)
Meningitis (*H. influenzae*)	7	27 (37)	(Raziuddin, Mir et al. 1994)
Meningitis (*N. Meningitidis*)	7	25 (42)	(Raziuddin, Mir et al. 1994)
Meningitis (*S. pneumoniae*)	7	35 (46)	(Raziuddin, Mir et al. 1994)
Protozoal Parasites			
Acute malaria (non-endemic)	4	16 (26)	(Ho, Webster et al. 1990)
Toxoplasmosis	4	9 (15)	(Scalise, Gerli et al. 1992)
Visceral leishmaniasis	8	44	(Raziuddin, Telmasani et al. 1992)

Table 3: Expansion of human Vγ9Vδ2 T cells in response to infection. * % of γδ T cells among CD3+ T cells in skin lesion (table modified from Morita 2007)

1.8.1 Immune regulation of Vγ9Vδ2 T cells: responses in vitro and in vivo in mycobacterial infections

Tuberculosis is probably the infectious disease, which, with respect to the Vγ9Vδ2 T cell response, has been most intensively studied. Nevertheless, the pathophysiological role of these cells is not yet understood.

Vγ9Vδ2 T cells present cytolytic capacity *in vitro* against a variety of targets, including extracellular and intracellular *M. tuberculosis* (Mtb-infected macrophages), through the

granule exocytosis pathway and release of effector molecules such as perforin and granulysin (Dieli, Troye-Blomberg et al. 2000; Dieli, Troye-Blomberg et al. 2001). *In vivo* in early infections the immune responses of mycobacterial phosphoantigen-specific Vγ9Vδ2 T cells may contribute to control of bacterial burdens and resolution of active BCG infection in monkeys (Shen, Zhou et al. 2002; Lai, Shen et al. 2003). In addition, a rapid recall expansion of Vγ9Vδ2 T cells in the pulmonary compartment is associated with low levels of bacteria in acutely fatal tuberculosis after *M. tuberculosis* challenge of BCG-vaccinated monkeys by aerosol (Shen, Zhou et al. 2002). Pulmonary infection with *M. tuberculosis* leads to an increase in numbers of macaque activated Vγ9Vδ2 T cells, that express high levels of chemokine receptors (Cipriani, Borsellino et al. 2000; Glatzel, Wesch et al. 2002). This migration chemokine/receptor-mediated may facilitate the pulmonary infiltration of Vγ9Vδ2 T cells in the lung and is associated with a decrease in circulating of Vγ9Vδ2 T cells.

These data suggest that adult pulmonary tuberculosis usually occurs as result of a loss of immunity against latent infection or re-infection, due to a fundamental immune suppression or defect of immune cells including Vγ9Vδ2 T cells in patients with tuberculosis. Such immunosuppression helps to explain the reduced level of Vγ9Vδ2 T cell proliferation in response to *in vitro* antigen stimulation (Gioia, Agrati et al. 2002; Gioia, Agrati et al. 2003).

Nevertheless, until now, it is inconclusive how human Vγ9Vδ2 T cell responses during early *M. tuberculosis* infection. Studies done at a chronic phase of tuberculosis have reported a decrease in percentage of Vγ9Vδ2 T cells in both the Bronchial Alveolar Lavage (BAL) fluid and blood of patients with pulmonary tuberculosis when compared with normal individuals (Li, Rossman et al. 1996; Carvalho, Matteelli et al. 2002). Additionally, Vγ9Vδ2 T cells of patients with active pulmonary tuberculosis exhibit an impaired ability to produce cytokines or proliferate in response to *in vitro* stimulation with phosphoantigens (Li, Rossman et al. 1996; Dieli, Sireci et al. 2002; Gioia, Agrati et al. 2002). This down-regulation or impaired function of Vγ9Vδ2 T cells seems to be regulated through complex immune mechanism and appears to be consistent with an absence or anergy of CD4+ T cell responses and PPD skin test reactivity identified in patients with active tuberculosis (Delgado, Tsai et al. 2002).

Finally, tuberculous patients present a defect in the effector function of Vγ9Vδ2 T cells, which is probably due to the exhaustion of these cells by chronic activation. A recent finding demonstrates a decrease numbers of effector memory CD45RA- CD27- Vγ9Vδ2 T cells,

supporting this possibility. Moreover, the loss of *in vitro* effector function in responses to phosphoantigens in patients with active tuberculosis suggests that their Vγ9Vδ2 T cells are pre-terminally activated. Further studies are necessary to elucidate the precise mechanisms by which tuberculosis induces down-regulation or defects of Vγ9Vδ2 T cells (Gioia, Agrati et al. 2002; Gioia, Agrati et al. 2003).

1.9 Antiviral mechanisms of Vγ9Vδ2T cells

Adaptive and innate immune reactions cooperate to protect the host during viral infections. The early synthesis of soluble factors (cytokines, chemokines) influences the immune response and consequently may affect the course of infection. One of the most important effectors of natural immunity are γδ T lymphocytes, which exhibit a broad antiviral activity against different viruses such as retroviruses, flaviviruses, herpesviruses, hepadnaviruses, and orthopoxviruses (table 4) (Poccia, Agrati et al. 2005). Both, cytolytic (i.e., cytotoxicity against virus- infected cells) and non-cytolytic activities of Vγ9Vδ2 T cells play a crucial defensive role comprise rapid immune responses against viral infections.

Clearly, γδ T cells play an important role in innate and adaptive immune responses to viral infections. The molecules recognized by Vγ9Vδ2 T cells during viral infections are probably of cellular rather than viral origin and appear to be metabolites of altered cellular pathways, in particular the products of the mevalonate pathway or stress induced NKG2D ligand molecules like ULBPs. Moreover, virus-exposed γδ T cells can be rapidly activated by type I interferons (IFN-α, IFN-β), a phenomenon that contribute to the effective antiviral response (Kunzmann, Kretzschmar et al. 2004).

In humans, Vγ9Vδ2 T cells are activating early during the acute phase of most viral infections and induce potent antiviral responses. Moreover, a plethora of soluble factors with antiviral characteristics induced by Vγ9Vδ2 TCR-stimulatory molecules influence the outcome of viral infections. In addition to their direct antiviral properties, many of these molecules play crucial immunoregulatory roles and are decisive in controlling the complex antiviral immunosurveillance function, as well as in establishing the correct immunological memory environment *in vivo*.

Viruses	Functional phenotype of Vγ9Vδ2 T cells and biological function *	References
RNA infection virus		
Coronavirus	Selective expansion of Vγ9Vδ2 T cells after the onset of the infection. In vitro experiments indicated IFN-γ dependent anti SARS-CoV (severe acute respiratory syndrome) activity and killing of SARS-CoV-infected target cells	(Poccia, Agrati et al. 2006)
Flavivirus	Vγ9Vδ2 T cells induces a non-cytolytic inhibition of sub-genomic Hepatitis C Virus replication, mediated by IFN-γ	(Agrati, Alonzi et al. 2006)
Paramyxovirus	In measles paramyxovirus infection, the expansion of human Vγ9Vδ2 T cells *in vitro* is negativalety regulated by the measles virus glycoproteins	(Bieback, Breer et al. 2003)
Retrovirus	Transient increase in the percentage of activated Vγ9Vδ2 T cells in *Macaca mulatta* infected with simian immunodeficiency virus (SIV)	(Gan, Pauza et al. 1995)
DNA infection virus		
Herpesvirus	PBMC from HSV-seropositive individuals stimulated with autologous HSV-infected PHA blast show expansion of Vγ9Vδ2 T cells with cytolytic capacity against HSV-infected cells. γδ T cells are expanded during acute phase of Epstein-Barr virus (EBV)-induced mononucleosis infections	(Bukowski, Morita et al. 1994) (De Paoli, Gennari et al. 1990)
Hepadnavirus	Increase in γδ T cells during hepatitis B virus (HBV) seroconversion	(Sing, Butterworth et al. 1998)
Orthopoxvirus	In individuals who received live recombinant canarypox virus expressing HIV proteins or soluble MN rgp120, is observed an increase of γδ T-cells responses detectable after secondary in vitro expansions. These augmented of γδ T-cell responses are specific for canarypox virus, but not for HIV antigens, and are mediated primarily by IFN-γ-producing Vγ9 T cells	(Worku, Gorse et al. 2001)
	Control of Vaccinia Virus by *in vitro*-stimulated Vγ9Vδ2 T cells mediated through IFN-γ production	(Agrati, Castilletti et al. 2006)

Table 4: Human γδ T cells in RNA and DNA virus infections. * Natural infections or experimental datas.

1.9.1 Non-cytolytic antiviral immunity mediated by Vγ9Vδ2 T cells

The innate non-cytolytic activity encompasses the production and release of several soluble molecules. Peripheral blood mononuclear cells (PBMC) stimulated with isopentenyl pyrophosphate release an array of cytokines (e.g., IFN-γ, TNF-α, IL-1α, IL-6, GM-CSF, TPO, and OSM) and chemokines (e.g., MIP-1α/β, RANTES, SDF-1, MCP-2 MDC, ENA-78 and GRO) with known antiviral and/or immunomodulatory properties. After stimulation, and in sharp contrast to many non-γδ TCR-expressing lymphoid cells, single Vγ9Vδ2 T lymphocytes produce more than one of these soluble antiviral/immunoregulatory factors (Poccia, Agrati et al. 2005). Many of these soluble molecules influence the humoral and cellular adaptive immune responses, which follow the innate response. In addition, the non-cytolytic antiviral molecules continue to suppress the infectious process in vital organs without destroying important cells, also can boost the antiviral potency of classical αβ cytotoxic T lymphocytes (CTLs) (DeVico and Gallo 2004). The main factor with known

antiviral activities released by Vγ9Vδ2 T cells is IFN-γ that suppresses viral replication without direct cytolytic effects on host cells (Patterson, Thomis et al. 1995; Bovolenta, Lorini et al. 1999) and may recruit and activate killer T cells, NK cells and macrophages. *In vitro*, antiviral activity of interferons against infections with HCV, hepatitis B virus (HBV), herpesviruses, orthopoxviruses, picornaviruses, retroviruses, influenza and other type of viruses has been reported (Capobianchi, Abbate et al. 2003; DeVico and Gallo 2004). The induction of IFN-γ production by Vγ9Vδ2 T lymphocytes could represent a strategy to inhibit viral replication and to support the Th1-type immune response.

In HIV infection, Interleukin-1α (IL-1) produced by activated γδ T cells, can have either stimulatory or inhibitory effects (Osborn, Kunkel et al. 1989). In addition, it has been shown that the cytomegalovirus (CMV) infection is reduced in marrow stromal cells that either secrete IL-1 or are treated with exogenous IL-1 (Randolph-Habecker, Iwata et al. 2002). Stimulated γδ T cells also produce interleukin-6 (IL-6), a potent lymphoid cell growth factor, which affects B lymphocytes, T-lymphocytes or hybridoma cells, and may influence cytotoxic T cells in combination with other factors such as IL-2 and IFN-γ (Kurane, Janus et al. 1992). The granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by activated γδ T cells is a potent stimulator of precursors of granulocytes, macrophages and eosinophils. Antiviral effects of GM-CSF have been reported in dengue infections (Kurane, Janus et al. 1992). GM-CSF boosts antiviral humoral immunity to influenza and simian immunodeficiency virus (SIV) (Lena, Villinger et al. 2002), increases humoral and cellular immune responses against herpes simplex virus-2 (HSV-2) (Sin, Kim et al. 1998) and improves protection against Epstein–Barr virus (EBV)-induced lymphoproliferative disorders (Baiocchi, Ward et al. 2001).

Another proinflammatory factor released by activated γδ T cells is TNF-α, a potent lymphoid factor with cytotoxic effects on a wide range of target cells. Beneficial effects of TNF-α in cellular immunity to vesicular stomatitis virus (VSV) (Wong and Goeddel 1986), cytomegalovirus (CMV) (Pavic, Polic et al. 1993), Herpes simplex virus (HSV)-1 (Rossol-Voth, Rossol et al. 1991), and vaccinia virus (VV) (Sambhi, Kohonen-Corish et al. 1991) have been noted.

The role of TNF-α in HIV infections is somewhat intricate, since in HIV-1 infection TNF-α was shown to enhance replication in chronically infected pro-monocytic and T-lymphoid cell

lines by activation of the nuclear factor NF-κB, which stimulates the long terminal repeat (LTR) of the provirus (Osborn, Kunkel et al. 1989), while IFN-α (an important enhancer of TNF-α production by macrophages) inhibits HIV-1 growth in primary macrophages (Meylan, Spina et al. 1993).

Chemokines, a superfamily of proinflammatory cytokines, act primarily as chemoattractants and activators of specific types of leukocytes. Importantly, antigen-stimulated Vγ9Vδ2 T cells rapidly establish chemotactic gradients that may shape the host inflammatory response against various pathogens including HIV-inhibitory α-chemokines MIP-1α, MIP-1β, lymphotactin and RANTES (within 4-12 h) (Poccia, Battistini et al. 1999; Cipriani, Borsellino et al. 2000). A large number of studies indicate that Vγ9Vδ2 T cells participate in immune responses against the human immunodeficiency virus (HIV) (Poccia, Gougeon et al. 2002), and the human T-cell leukemia virus (HTLV) type 1 (Furukawa, Sasai et al. 1992). These cells exert both cytolytic and non-cytolytic (through the induction of β-chemokines such as MIP-1α/β and RANTES) antiviral activity (Wallace, Bartz et al. 1996; Poccia, Cipriani et al. 1997). The efficacy of inhibition is comparable to that of CD8+ T lymphocytes (Wallace, Bartz et al. 1996).

Vγ9Vδ2 T cells also express many β-chemokine receptors including CCR1, CCR5 (Tikhonov, Deetz et al. 2006) and CCR8, which can be down-regulated by activation stimuli. Activating signals for γδ T cells also induce the production of macrophage chemoattractant protein-2 that has an important role in the inflammatory response of blood monocytes and tissue macrophages, and inhibits the replication of HIV-1 via CCR5 (Yang, Garcia-Zepeda et al. 2002). Furthermore, Vγ9Vδ2 T cells produce MDC that is chemotactic for monocytes, dendritic cells, activated lymphocytes, and NK cells and exhibits anti-HIV-1 activity (Struyf, Proost et al. 1998). Cumulatively, the high levels of proinflammatory cytokines and chemokines with antiviral properties produced by activated Vγ9Vδ2 T cells may influence the progression of HIV disease. Peptide-78 (ENA-78), which is a chemotactic and activating factor for neutrophils is an IPP-induced molecule, and GRO (MGSA), which promotes neutrophil chemotaxis and degranulation (Poccia, Agrati et al. 2005). Through these molecules, Vγ9Vδ2 T cells may influence the behavior of granulocytes, that is, the cells that are likely to release of α-defensins and other non-cytolytic antiviral factors.

Antimicrobial peptides such as defensins are produced in animals (including human and non-human primates) and plants. They play an important role in infections, inflammation, wound repair, and acquired immunity. Based on the disulfide pairing of their characteristic six-cysteine residues, they are divided into α-defensins and β-defensins. Granulocytes release α-defensins and inhibit HIV infection in vitro (Nakashima, Yamamoto et al. 1993). In addition to their anti-HIV activities, α-defensins interfere with the replication of HSV-1 and HSV-2, CMV, vesicular stomatitis virus (VSV), and influenza virus (Daher, Selsted et al. 1986). The expression of α-defensins in γδ T cells has been described (Agerberth, Charo et al. 2000), but further studies are required to explore the contribution of γδ T cells to the production and action of defensins during viral infections.

1.10 Role of Vγ9Vδ2 T cells in tumor regression

Soon after their discovery, Vγ9Vδ2 T cells were shown to display strong natural MHC-unrestricted cytotoxic effector activity against tumor cells, especially of hematological origin such as lymphoma (e.g. Burkitt lymphoma cell line Daudi) or myeloma cell lines (e.g. RPMI 8226) (Sturm, Braakman et al. 1990; Selin, Stewart et al. 1992). This potent cytotoxic potential against tumor cells was reminiscent of NK cell activity, although the tumor targets recognized by Vγ9Vδ2 T cells are only partly overlapping with NK cell targets (Fisch, Meuer et al. 1997; Sicard, Al Saati et al. 2001). However, the ligands interacting with the Vγ9Vδ2 TCR on tumor cells have not been identified in detail. Besides the TCR, the cytotoxic activity of Vγ9Vδ2 T cells is influenced by the expression of the typical NK cell receptors (KIRs and KARs), explaining the stronger cytolysis of targets with reduced or missing MHC class I expression (Fisch, Meuer et al. 1997; Poccia, Cipriani et al. 1997; Rothenfusser, Buchwald et al. 2002). Moreover, under appropiate culture conditions, Vγ9Vδ2T cells display diverse characteristics in the production of cytokines, i.e.; the proinflamatory cytokines (TNF-α and IFN-γ) or chemokines (MIP 1α/β and lymphotactin). In addition it has been reported the production of fibroblast growth factor-9 (FGF-9) and connective tissue growth factor (CTGF), indicating that γδ T cells have a distinct role in local immunosurveillance and tissue repair (Cipriani, Borsellino et al. 2000; Workalemahu, Foerster et al. 2003; Workalemahu, Foerster et al. 2004).

1.10.1 Role of Vγ9Vδ2 T cells in the immunosurveillance of lymphoid malignancies

The powerful anti-tumor potential of human γδ T cells has been described in severe combined immune deficiency (SCID) mouse model and *in vitro* experiments by their anti-lymphoma activity (Ensslin and Formby 1991). However, conventional mouse models cannot be used to explore the possible anti-lymphoma activity of human γδ T cells *in vivo*, since mouse γδ T cells lack the human Vγ9Vδ2 homologous TCR and thus the reactivity to phosphoantigens. Therefore, the potential usage of human Vγ9Vδ2 T cells for immunotherapy of lymphoid malignancies can only be studied by using mouse models where human Vγ9Vδ2 T cells and human lymphoma cells are adoptively transferred into SCID or nude mice. These *in vivo* chimeric models have been successfully used for the evaluation of anti-lymphoma efficacy of human Vγ9Vδ2 T cells. Activated human γδ T cells as well as Vγ9Vδ2 T cell clones significantly reduced the *in vivo* growth of the Burkitt lymphoma Daudi (Groh, Bruhl et al. 2003). Similarly, a high survival rate is obtained when Vγ9Vδ2 TCR+ tumor infiltrating lymphocyte (TILs) (expanded from human colorectal tumors in vitro) are transferred into Daudi cell-bearing BALB/c nude mice compared with the transfer of αβ TCR+ TILs or mice without treatment (Chen, Niu et al. 2001). Several other recently published human–SCID mouse studies confirmed the anti-tumor effect of adoptively transferred γδ T cells against other malignancies (nasopharyngeal carcinoma, melanoma, pancreatic carcinoma) (Kabelitz, Wesch et al. 2004; Lozupone, Pende et al. 2004). Notably, the recognition of lymphoid malignancies by human γδ T cells has been exclusively assigned to the Vγ9Vδ2 subset, whereas γδ T cells expressing the Vδ1 TCR have been implied in the defense against epithelial cancers (Kabelitz, Wesch et al. 2004).

1.10.2 Anti-lymphoma activity of human γδ T cells in vivo

Selective stimulation of Vγ9Vδ2 T cells in humans is feasible by application of phosphoantigens *in vivo*. This strategy is used to explore the anti-tumor activity in the clinical setting, and is supported by observations with aminobiphosphonate treatment, that exert stimulatory activity on Vγ9Vδ2 T cells at pharmacologically achievable concentrations (Kunzmann, Bauer et al. 2000). ABPs such as pamidronate or zoledronate stimulate human Vγ9Vδ2 T cells in a TCR-dependent manner by inducing the cell mediated cytotoxicity against tumor cells *in vitro* and in the secretion of pro-inflammatory cytokines (i.e. TNF-α, IFN-γ) (Pfeffer, Schoel et al. 1990; Das, Wang et al. 2001; Kabelitz, Wesch et al. 2004). ABP induces γδ T cell stimulation indirectly by inhibition of the enzyme farnesyl pyrophosphate

synthase in the antigen presenting cells, which results in an accumulation of stimulating mevalonate metabolites (i.e. IPP) (Gober, Kistowska et al. 2003).

The administration of ABP with low doses of IL-2 induces a selective activation and proliferation of Vγ9Vδ2 T cells *in vivo* in patients with multiple myeloma. In addition, this clinical trial provided the first evidence that the selective stimulation of γδ T cells can be accompanied by anti-lymphoma activity *in vivo*. In this study, the expansion of γδ T cells *in vivo* was a prerequisite for tumor regression, as only patients with significant *in vivo* proliferation of γδ T cells responded to the treatment with pamidronate and IL-2, indicating that γδ T cells contributed to this anti-lymphoma effect. Nevertheless, the precise mechanisms involved in tumor cell recognition by γδ T cells will require supplementary *in vitro* and *in vivo* studies.

However, further evidences suggest at least two different anti-tumor effects of pamidronate/IL-2-activated γδ T cells. The first one indicates a rapid and direct (i.e. perforin/granzyme-mediated) cytotoxic effect, which might contribute to the anti-lymphoma activity of activated γδ T cells. This observation is supported by *in vitro* studies showing significant enhanced direct cytotoxicity of γδ T cells against various lymphoma targets after stimulation with phosphoantigens (Wilhelm, Kunzmann et al. 2003), and is observed in the fast responding profile of the patients described on the previous study.

This clinical trial also revealed a second much slower response profile, which strongly argues for an indirect influence of γδ T cells on lymphoma cells. One possible mechanism for this kind of anti-tumor effect might be the induction of IFN-γ-producing Vγ9Vδ2 T cells *in vivo*. Multiple anti-tumor effects have been attributed to IFN-γ, including direct inhibition of tumor growth or more indirect effects such as the blocking of angiogenesis (Qin, Schwartzkopff et al. 2003). Interestingly, a significant negative correlation between the serum levels of the angiogenic factors VEGF (vascular endothelial growth factor) and IFN-γ was found in cancer patients treated with ABP (Santini, Vincenzi et al. 2002).

A general disadvantage of autologous γδ T cell mediated tumor immunotherapy is the frequent impaired function of γδ T cells in cancer patients. The anergy of γδ T cell has impact in the anti-lymphoma effect. These phenomenon has been described in certain chronic

infectious diseases such as HIV infection or tuberculosis, although the cause of this γδ T cell anergy is not fully understood (Li, Rossman et al. 1996; Poccia, Boullier et al. 1996).

1.11 Aim of the work

Focus of the thesis was a better understanding of the role of NKG2D, and its interplay with the TCR, in the activation of Vγ9Vδ2 T cells. The follows pages dissect in detail the capacity of NKG2D ligation to trigger various effector functions in Vγ9Vδ2 T cells, especially with respect to the cytolytic machinery, and the differential activation of chemokine and cytokine production and the activation status of the Vγ9Vδ2 T cells.

Parts of the thesis have been published (Rincon-Orozco, Kunzmann et al. 2005)

2 MATERIALS

2.1 Chemical reagents

Agar-agar	Roth (Karlsruhe, Germany)
Agarose	Roth (Karlsruhe, Germany)
Ammoniumchloride	Roth (Karlsruhe, Germany)
Ampicillin	Gibco BLR (Eggenstein, Germany)
Aprotinin	Calbiochem (Darmstadt, Germany)
APS (Ammoniumpersulfat)	Roth (Karlsruhe, Germany)
β-mercaptoethanol	Gibco BLR (Eggenstein, Germany)
BSA (Bovine Serum Albumin)	Roth (Karlsruhe, Germany)
Boric acid	Roth (Karlsruhe, Germany)
Calcium Chloride ($CaCl_2$)	Roth (Karlsruhe, Germany)
5-(and-6)-carboxyfluorescein diacetate Succinimidyl ester (CFSE)	Molecular Probes (Eugene, Oregon)
Concavalin A	ICN (Meckenheim, Germany)
DEPC (Diethylpyrocarbonate)	Sigma (Deisenhofen, Germany)
Diatomaceous earth	Sigma (Deisenhofen, Germany)
DMSO (Dimethyl sulfoxide)	Sigma (Taufkirchen, Germany)
5,5´dithio-bis-2-nitrobenzoic acid	Pierce (Bonn, Germany)
dNTP Set	Peqlab Biotechnologie (Erlangen, Germany)
EDTA	AppliChem GmbH (Darmstadt, Germany)
Ethanol	AppliChem GmbH (Darmstadt, Germany)
Ethidium bromide	Roth (Karlsruhe, Germany)
Ficoll-Paque	Amersham (Piscataway, N.J. USA)
Formaldehyde	Roth (Karlsruhe, Germany)
Formamide	Roth (Karlsruhe, Germany)
G418 (Geneticin solution)	Biochrom AG (Berlin, Germany)
Glycerol	Sigma (Schnelldorf, Germany)
Glycine	Roth (Karlsruhe, Germany)
HEPES	AppliChem GmbH (Darmstadt, Germany)
hrIL-2 (Proleukin Chiron)	Chiron (Novartis, USA)
hrIL-15 (recombinant human interleukin-15)	Strathmann Biotec AG (Hamburg, Germany)
Ionomycin	Sigma (Deisenhofen, Germany)
IPTG (Isopropyl- D-Thiogalactopyrasoide)	Biomol Feinchemikalien (Ilvesheim, Germany)
Isopropanol	AppliChem GmbH (Darmstadt, Germany)
JNK2 inhibitor (SP600125)	Calbiochem (Darmstadt, Germany)
LB (Broth Base medium)	Gibco BLR (Eggenstein, Germany)
Leupeptin	Calbiochem (Darmstadt, Germany)
Magnesium Chloride	Roth (Karlsruhe, Germany)
MEK 1/2 inhibitor (PD98059)	Calbiochem (Darmstadt, Germany)
Methanol	AppliChem GmbH (Germany)
Na-Butyrate	Sigma (Deisenhofen, Germany)
NaF (Sodium fluoride)	Calbiochem (Darmstadt, Germany)
Na_3VO_4 (Sodium vanadate)	Calbiochem (Darmstadt, Germany)
N-benzyloxycarbonyl lysine thiobenzyl	

Ester (BLT)	Calbiochem (Darmstadt, Germany)
NaN_3 (Sodium Azide)	Merck (Darmstadt, Germany)
Sodium Chloride	Roth (Karlsruhe, Germany)
Sodium Butyrate	Sigma (Deisenhofen, Germany)
PEG (Polyethylenglycol)	Boehringer Mannheim (Mannheim, Germany)
PKH-26	Sigma (St Louis, Mo, USA)
Penicillin	Gibco BLR (Eggenstein, Germany
Pepstatin	Calbiochem (Darmstadt, Germany)
PI3K inhibitor (Ly294002)	Calbiochem (Darmstadt, Germany)
p38 inhibitor (SB203580)	Calbiochem (Darmstadt, Germany)
PMA (Phorbol myristate acetate)	Sigma (Deisenhofen, Germany)
PMSF (Phenylmethylsulfonylfluoride)	Calbiochem (Darmstadt, Germany)
Polybrene (Hexadimethrinbromide)	Sigma (Deisenhofen, Germany)
SDS (Sodium dodecyl sulfate)	Sigma (Deisenhofen, Germany)
Tris (Tris(hydroxymethyl)-aminomethan)	AppliChem GmbH (Darmstadt, Germany)
Trypan blue	Sigma (Deisenhofen, Germany)
Tween 20	Sigma (Deisenhofen, Germany)
Zeozin	Cayla (Toulouse Cedex, France)

2.2 Reagents (Media, Buffers and Solutions):

Media used for cell cultures were obtained from Gibco-BRL (Eggenstein, Germany):

DMEM (Dulbecco's Modified Eagle´s Medium) with Pyruvate, without HEPES (#41966-029)

DMEM (Dulbecco's Modified Eagle´s Medium) without Pyruvate, with HEPES (#41966-027)

RPMI- RPMI 1640 + L-Glutamine (#21875-034)

RPMI+ RPMI- with 10% of SC (supplement complete)

- SC-supplement (50 ml of SC per 500 ml of RPMI-):

500 ml	heat-deactivated FCS
100 ml	Na pyruvate 100 mM
100 ml	non-essential amino acids
100 ml	Penicillin-Streptomycin (10000U/ml)
5 ml	β-Mercaptoethanol 50 mM
58.4 ml	L-Glutamine solution 5%

- ATV:

  0.05% Trypsin
  0.02% EDTA in PBS

- 10X Trypan Blue Buffer:

  0.5% Trypan Blue
  0.5% Xylencyanol
  50% Glycine
  10 mM EDTA

- Trypan blue solution:

  0.04% Trypan blue in PBS/BSA/Azide

- BSS (Hanks balanced salt solution):

For 1 L BSS, 125 ml of BSS I was mixed with 125 ml of BSS II and volume adjusted with water to 1 L

BSS I (8X):
50 g Glucose
3 g KH_2PO_4
11.9 g NaH_2PO_4
0.4 g Phenol red
Water to 5 L

BSS II (8X):
9.25 g $CaCl_2$
20 g KCl
320 g NaCl
10 g $MgCl_2$
10 g $MgSO_4$
Water to 5 L

- BSS/BSA

  0.2 % BSA in BSS

- DEPC water

  0.1 g DEPC
  Water to 1 L, sterilised in autoclave

- 2X HBS

  50 mM HEPES pH 7.05
  10 mM KCl
  12 mM Glucose
  280 mM NaCl
  1.5 mM NaH_2PO_4

Three different pH (pH 7.0, 7.05, 7.1) were prepared and tested. Aliquots were stored at -20°C.

- LB- medium

  20 g LB
  In 1 L dH_2O, autoclaved and stored at 4°C

- PBS (Phosphate buffered saline)

  4 mM KH_2PO_4
  16 mM Na_2HPO_4

115mM NaCl
pH 7.3

- PBS/BSA/Azide

  0.2% BSA
  0.02% Na-Azide in PBS

- TAC (Tris-Ammoniumchlorid):

  20 mM Tris-HCl pH 7.2
  0.82% NH_4Cl

- 10X TBE

  890 mM Tris pH 8.0
  890 mM Boric acid
  20 mM EDTA

- 30% Acrylamide stock Roth (Karlsruhe, Germany)

- Carbonate Buffer (pH 9.6)

  1.59g Na_2CO_3
  2.93g $NaHCO_3$
  Dissolved in 900 ml dH2O
  Volume adjusted to 1 liter with dH2O

- FACS buffer:

  0.1% BSA
  0.05% NaN_3 in PBS

- Modified RIPA buffer:

  Tris-HCl: 50 mM, pH 7.4
  NP-40: 1%
  Na-deoxycholate: 0.25%
  NaCl: 150 mM
  EDTA: 1 mM
  PMSF: 1 Mm
  Aprotinin, leupeptin, pepstatin: 1 microgram/ml each
  Na_3VO_4: 1 mM
  NaF: 1 mM

- Cytofix/Cytoperm BD Biosciences (Heidelberg Germany)
- Perm/Wash buffer BD Biosciences (Heidelberg Germany)

- Tris-glycine-SDS protein electrophoresis buffer (1X):

  25 mM Tris,
  250 mM glycin,
  0.1% SDS
  pH 8.3

- 6X SDS Protein electrophoresis loading buffer:

  300 mM Tris.Cl (pH 6.8)
  600 mM DTT
  12% SDS
  0.6% Trypanblau
  60% Glycerol

- Protein transfer buffer (1X):

  39 mM Glycine
  48 mM Tris
  0.037% SDS
  20% Methanol

- 0.1% Phosphate Buffered Saline tween-20:

  The followed component were dissolved in 800 ml of dH2O

  8 g NaCl
  0.2 g KCl
  1.44 g Na_2HPO_4
  0.24 g KH_2PO_4
  2 ml of Tween-20
  pH adjusted to 7.2 with HCl
  Volume adjusted to 1 L with additional distilled H_2O
  Sterilized by autoclaving

- Ponceau S solution (Stock solution)

  2% Ponceau S diluted in 30% trichloroacetic acid
  30% sulfosalicylic acid
  Working dilution 1:10

- HRP substrate for western-blot ECL (Amersham. Freiburg, Germany)

2.3 Cell lines:

C1R EBV-transformed B cells that have lost most HLA class I alleles and express only Cw0401 and trace amounts of B3503 (kindly provided by Dr. Steinle, Tübingen University).

C1R-Neo C1R cells that have been transfected with empty vector RSV.5-Neo (kindly provided by Dr. Steinle, Tübingen University).

C1R-MICA C1R cells that have been transfected with cDNA for MICA*01 in RSV.5-Neo (kindly provided by Dr. Steinle, Tübingen University).

C1R-ULBP-2 C1R cells that have been transfected with cDNA for ULBP-2 in RSV.5-Neo (kindly provided by Dr. Steinle, Tübingen University).

C1R-ULBP-3 C1R cells that have been transfected with cDNA for ULBP-3 in RSV.5-Neo (kindly provided by Dr. Steinle, Tübingen University).

Daudi β2-microglobulin-deficient B cell lymphoma, MHC class I negative

Mewo Human melanoma cell line (kindly provided by Dr. Wesch, Kiel University).

P815 mouse (DBA/2) cell mastocytoma FcγR+ (ATCC#TIB-64)

RAJI EBV-transformed human B cell lymphoma Burkit (ATCC#CCL 86)

RMA mouse T cell lymphoma cells, which have been transfected with MICA*07 cDNA in RSV.5-Neo or with the vector alone (kindly provided by Dr. Steinle, Tübingen University).

THP1 Human monocytic cell line (kindly provided by Prof. Dr. J. Schneider-Schaulies, Würzburg University).

Vγ9δ2 T cells line HO and EP3 (kindly provided by Dr. Wesch, Kiel University).

2.4 Antibodies

The following antibodies were purchased from BD-Pharmingen (Heidelberg Germany)

- anti human CD3 (clone HIT3a)
- anti human CD4 (clone RPA-T4)
- anti-human CD8 (clone RPA-T8)
- anti-human CD19 (clone HIB19)
- anti-human CD25 (clone M-A251)
- anti-human CD28 (clone CD28.2)
- anti-human CD69 (clone FN50)
- anti-human Vδ2 TCR (clone B6)
- anti-Vγ9 TCR (clone B3.1)
- anti-HLA-DR (clone L243)
- anti-human CD107a (clone H4A3)
- anti-human Perforin (clone: δG9)
- anti-human $CD45^{RO}$ (clone: UCHL-1)
- anti-human CD27 (clone:340425)
- anti-human IL-6 (clone:340427)
- anti-human CD16 (clone 3G8)
- anti-human CD83 (clone HB15e)
- anti-human CD86 (clone IT2.2)
- anti-human CD14 (clone M5E2)

- anti-human CD80 (clone L307.4)
- anti-human CD1a (clone HI149)
- anti-human CD40 (clone 5C3)
- anti-human CD95 (clone DX2)
- anti-human CD56 (clone B159)
- anti-human CD54 (clone HA58)
- anti-human IL-10 (clone JES3-19F1)
- anti-human TNFα (clone Mab11)
- anti-human MCP-1 (clone 5D3-F7)
- anti-human IFNγ (clone 4S.B3)
- anti-human GM-CSF (clone BVD2-21C11)
- anti-human MIP-1α (clone 11A3)

The following antibodies were purchased from different companies

o anti-human RANTES (clone VL1)	Caltag (Burlingame, USA)
o anti-human NKG2D (clone 149810)	(R&D Systems Minneapolis,USA)
o anti DAP 10 (sc-20782)	(Santa Cruz Biotechnology, Santa Cruz, CA, USA)

The following antibodies were kindly provided by Dr. Steinle, Tübingen University

- anti MICA (clone AMO-1)
- anti MICB (clone BMO-1)
- anti MICA-B (clone BAMO-1)
- anti ULBP-1 (clone AUMO-01)
- anti ULBP-2 (clone BUMO-3)
- anti ULBP-2 (clone CUMO-3)

The following antibodies were kindly provided by Dr. D. Cosman (Amgen, Seattle, WA)

- NKG2D-specific mAb M585

2.5 Isotypes controls, secondary antibodies and recombinant proteins

PE-conjugated Mouse IgG2b, k	(clone 27-35) Pharmingen (Heidelberg Germany)
PE-conjugated Mouse IgG1, k	(clone MOPC-21) Pharmingen (Heidelberg Germany)
Cy-Chrome-conjugated Mouse IgG1, k	(clone MOPC-21) Pharmingen (Heidelberg Germany)
FITC-conjugated Mouse IgG1, k	(clone MOPC-21) Pharmingen (Heidelberg Germany)

D MIg -PE, FITC or Cy5.5 conjugated	
(Fab')2 fragment of donkey anti-mouse IgG	Serotec (Düsseldorf, Germany)
Goat anti-mouse magnetic microparticles	(MACS system; Miltenyi Biotec)
Rabbit polyclonal horseradish peroxidase	(Santa Cruz Biotechnology, Santa Cruz, CA, USA)
MICA-Fc recombinant protein	(R&D Systems Minneapolis, USA)

2.6 Kits

Rneasy(r) MiniKit	QIAGEN (Hilden, Germany)
First Strand cDNA Synthesis Kit	MBI Fermentas (St.Leon-Rot, Germany)

2.7 PCR Primers

PCR Primers were obtained from MWG- Biotech AG (Ebersberg, Germany)

- Control primers:

β-actin-For	5'-CTA TCG GCA ATG AGC GGT TC-3'
β-actin-Rev	5'-CTT AGG AGT TGG GGG TGG CT-3'

- Primers sequences for human DAP 10:

DAP-10-For	5´-TCT GGG TCA CAT CCT CTT CC-3´
DAP-10-Rev	5´-GAT GTA GTT GTA CGG TCC GT-3´

2.8 Enzymes and Inhibitors

Pfu-DNA-Polymerase	Stratagene (Heidelberg, Germany)
MBI Taq-DNA Polymerase	MBI Fermentas (St.Leon-Rot, Germany)
dNTPs mixture	MBI Fermentas (St.Leon-Rot, Germany)
10X PCR Buffer, Minus Mg	MBI Fermentas (St.Leon-Rot, Germany)
$MgCl_2$	MBI Fermentas (St.Leon-Rot, Germany)

2.9 Consumables

12 well flat bottom culture plates	Greiner-Bio-One (Germany)
24 well flat bottom culture plates	Greiner-Bio-One (Germany)
48 well flat bottom culture plates	Greiner-Bio-One (Germany)
96 well flat bottom culture plates	Greiner-Bio-One (Germany)

3 cm tissue culture dish	Greiner-Bio-One (Germany)
50 ml cell culture flask	Greiner-Bio-One (Germany)
1.5 ml Eppendorf centrifuge tube	Eppendorf GmbH (Germany)
15 ml centrifuge tube	Greiner-Bio-One (Germany)
50 ml centrifuge tube	Greiner-Bio-One (Germany)
Cuvette	Bio-Rad (USA)
10 µL tips	Molecular Bioproducts (USA)
200 µL yellow tips	Roth (Karlsruhe, Germany)
1000 µL blue tips	Roth (Karlsruhe, Germany)
Syringe: 2ml and 5ml single-use syringe	Braun (Melsungen, Germany)
96 well flat bottom microplate	Nunc (Denmark)
Absorbent paper for Western-Blot	Whatman (USA)
PVDF membrane for Western-Blot	Roth (Karlsruhe, Germany)

2.10 Apparatus

Magnetic station	Milteny (BergischGladbach, Germany)
*auto*MACS	Milteny (BergischGladbach, Germany)
FACS-Calibur	Becton Dickinson (USA)
Power supply unit (protein electrophoresis and transfer)	Pharmacia Biotech (USA)
UV/Visible Spectrophotometer:Ultrospe 2000	Pharmacia Biotech (USA)
Transmenbrane apparatus	Hofer Scientific Instrument (USA)
Eppendorf centrifuge 5417R	Eppendorf AG (Hamburg, Germany)
Hettich centrifuge Rotixa 50 RS	Hettich (Tuttlingen, Germany)
Microcentrifuge Biofuge pico	Hereaus (Osterode, Germany)
Cell incubator	Forma Scientific (USA)
Heating block	VWR (Germany)
Water bath	Lauda (Lauda, Germany)
Liquid Scintillation Counter Microbeta Trilux	Perkin Elmer (Turku, Finland)

3 METHODS

3.1 Cellular Biology

3.1.1 Isolation of peripheral blood mononuclear cells (PBLs)

PBLs were isolated from fresh blood samples collected from healthy donors by the following procedure: 10 ml of Heparin treated blood were collected with the vacutainer system, and diluted with an equal volume of phosphate buffered saline (PBS). Blood samples were carefully layered over 10 ml of Ficoll-Hypaque in a 50 ml tube and centrifuged at 400 x g for 30 minutes at 20 °C. The upper layer containing the platelets was drawn off with a pipette, taking care not to disturb the PBMC layer at the interface. The PBLs layer was subsequently transferred to a clean centrifuge tube using a sterile Pasteur pipette, washed twice with 3 volumes of PBS and then centrifuged at 400 x g for 10 minutes at 20 °C. The PBLs were finally resuspended in 1 ml of RPMI medium (10% FCS).

3.1.2 Establishment of Vγ9Vδ2 T cell lines by stimulation of fresh Vγ9Vδ2 T cells from peripheral blood

PBLs from blood of adult healthy donors were isolated by Ficoll-Hypaque density gradient centrifugation. The PBLs (1 x 10^6/ml) in RPMI medium (10% FCS) were stimulated with Bromohydrin pyrophosphate (BrHPP3) (200 nM) or IPP (1µg/ml) in the presence of 100 U/ml rhIL-2 and incubated in a 24 well plate for 15 days at 37°C and 5% CO_2. Half of the medium was replaced by fresh medium on days 3, 7, 10 and 14. Afterward, the cells were analyzed by flow cytometry at day 15. At this time point approximately 70-80% of all the CD3+ cells comprised Vγ9Vδ2 T cells. Subsequently, the Vγ9Vδ2 T cells were enriched by negative isolation; these cells were used for further studies.

To establish Long Term Vγ9Vδ2 T cell lines (Vγ9Vδ2 T cell lines), phosphoantigen- or IPP-activated PBLs were stimulated twice at 14-days interval with irradiated PBLs (4000 Rad) and EBV-transformed B cells lines (8000 Rad) as feeder cells, PHA and rhIL-2; after the irradiation the cells were washed at 200 x g for 5 min, and the cell suspension was adjusted to 1 x 10^6 PBLs and 1 X 10^5 EBV-transformed B cells lines per ml in RPMI (10% SC) containing 0.5µg/ml of PHA. Subsequently 100 µl of feeder mix cells (irradiated PBLs, EBV, and PHA) were added to 1 x 10^6 cells in a 1 ml of expanded Vγ9Vδ2 T cells and plated every 2 or 3 day with RPMI (10% SC) + 100 UI rhIL-2. Dead feeder cells were removed 3–5 days

after re-stimulation by Ficoll-Hypaque density gradient. This protocol induced the selective and dramatic expansion of Vγ9Vδ2 T cells. After a second stimulation, Vγ9Vδ2 T cells were separated from the remaining non-γδ T cells by magnetic isolation using the MACS system to deplete CD4+, CD8+, TCR αβ, and CD16+ cells. Finally, the cells were maintained and stimulated at 2-3 weeks interval with the protocol mentioned before.

3.1.3 Magnetic isolation of Vγ9Vδ2 T cells

The PBLs isolated by the standard Ficoll-Paque method described above, were suspended at a concentration of 1 x 10^7 cells per 100 µl in cold buffer (degassed PBS pH 7.2, supplemented with 0.5% BSA and 2 mM EDTA). Staining was performed by mixing 1x107 cells with 2 µg of anti-CD4, CD8, CD19 and CD16 antibodies (Becton Dickinson) and incubated for 20 minutes in the dark at 4 °C. Subsequently the cells were washed twice to remove unbound primary antibody by adding 6 ml of buffer, and centrifuged at 400 x g for 10 minutes at 4°C. The supernatant was completely removed and the cell pellet was resuspended in 80 µl of buffer per 1 x 10^7 cells. Then the cells were mixed with 20 µl of anti-IgG mouse MicroBeads and incubated for 15 minutes at 4 °C. Later the cells were washed twice by adding 3 ml of buffer, and centrifuged at 400 x g for 10 minutes. The supernatant was completely removed and the cells were resuspended in 500 µl of buffer for magnetic separation.

The magnetic separation was performed by using an a) LS Column in the magnetic field of a MACS device or b) by using the auto-MACS sorter. For the LS column method, the column was equilibrated by rinsing it 3 times with 3 ml of buffer. Then the column was loaded with the cell suspension, the total effluent (unlabeled Vγ9Vδ2 T cell fraction) was collected and centrifuged at 400 x g for 10 minutes at 20 °C. The cells were finally resuspended in 1 ml of RPMI (10% SC + 100 UI rhIL-2). For the auto-MACS sorter method the program negative selection was used.

Cells used for RNA preparation and immunoblot were positively sorted. Briefly, *in vitro*-stimulated PBLs were preincubated with primary Abs specific for Vγ9 TCR, followed by goat anti-mouse magnetic microparticles. After washing, cells were passed through a strong magnetic field, to attach the Vγ9Vδ2 T cells. The column was separated from the magnet and washed the column for obtain the desired cells. Purity was determined by staining with PE-labeled anti-Vγ9 TCR mAb.

3.1.4 In vitro stimulation with immobilized Abs or NKG2D ligand

Ninety-six well U-bottom plates were coated overnight at 4°C with 12 µg/ml mAb of NKG2D (clone 149810) or 0.5 µg/ml for CD3 or 10 µg/ml mouse IgG1 as isotype control in 0.05 M carbonate buffer, pH 9.6, 100 µl per well. Coating with MICA-Fc recombinant protein was done with 10 µg/ml for 2 days. Subsequently, the plates were thoroughly washed with PBS three times, and 5 x 10^5 PBLs per well were cultured in medium plus 50 UI/ml IL-2 and maintained in culture for 24 or 96 h at 37°C, 5% CO2. Cells cultured for 24 h were stained for CD69, and the cells cultured for 96 h were stained for CD25. The expression of activation markers was analyzed by two-color flow cytometry in the population that was positive for Vγ9 TCR, CD16, or CD8. Cell stimulation to evaluate cytokine production by intracellular staining or granule release was performed on the same types of plates and in similar way as described above.

3.1.5 Staining of cell surface molecules of Vγ9Vδ2 T cells

Vγ9Vδ2 T cells from PBLs were suspended at a concentration of 1 x 10^5 cells per 100 µl of staining buffer (PBS, 0.1% BSA). These cells were stained for the determination of their phenotype by adding 5 µl of fluorochrome-conjugated monoclonal antibody specific for a cell surface antigen such as CD3, CD4, CD8, CD16, CD28, CD56, NKG2D, Vγ9 TCR, and for the determination of the presence of memory markers by adding the following mAb CD45RO, CD27, Vγ9 TCR. The cells were incubated for 30 min at 4°C. Then the cells were washed twice with 3 ml of staining buffer and pelleted by centrifugation at 400 x g for 5 min at 18-20 °C. The cell pellet was resuspended in 250 µl of staining buffer and was analyzed by flow cytometry on a FACScan or FACSCalibur using Cell-Quest software.

3.1.6 Staining of cell surface antigens of Vγ9Vδ2 T cells

For the determination of cell surface molecules the cells were suspended at a concentration of 1 x 10^5 per 100 µl of staining buffer (PBS, 0.1% BSA). These cells were stained by adding of 5 µl of fluorochrome-conjugated monoclonal antibody specific for a cell surface antigen such as CD8, CD16, CD25, CD28, CD69, CD95, Vγ9 TCR and incubated 30 min, 4°C. Then the cells were washed twice with 3 ml of staining buffer and pelleted by centrifugation at 400 x g for 5 min at 18-20 °C. The cell pellet was resuspended in 250 µl of staining buffer and analyzed by flow cytometry on a FACScan or FACSCalibur using the Cell-Quest software.

3.1.7 Analysis of the production of cytokines (TNFα and IFNγ) by intracellular staining of Vγ9Vδ2 T cells

For the intracellular cytokine or chemokine staining, Vγ9Vδ2 T cells from PBLs or IPP-activated Vγ9Vδ2 T cells (see above: establishment of Vγ9Vδ2 T cell lines by stimulation of fresh Vγ9Vδ2 T cells from peripheral blood) were washed and living cells were isolated by Ficoll-Hypaque, resuspended in medium plus 50 U/ml IL-2, and transferred at 5 x 10^5 cells/200 µl of culture plates coated with mAb or ligand (see above: *in vitro* stimulation with immobilized Abs or NKG2D ligand). After 1 h of stimulation, 1 µg/ml of the protein transport inhibitor brefeldin A was added, and the cells were further incubated for 5 h; afterward the cells were resuspended in staining buffer with 1 µg/ml of conjugated mAb Vγ9 TCR-FITC. Subsequently, cells were washed once with staining buffer, and fixed for 20 min at 4°C using 100 µl of Cytofix/Cytoperm. Fixed cells were permeabilized by washing twice with 200 µl of Perm/Wash buffer and resuspended in 50 µl of Perm/Wash buffer, plus 0.5 µg of the corresponding antibody: anti-IFN-γ, anti-TNF-α (all conjugated with PE), or isotype-PE conjugated control mAb, incubated for 30 min at 4°C, and washed twice with 200 µl of Perm/Wash buffer. Finally, samples were resuspended in 200µl of staining buffer and analyzed by flow cytometry on FACSCalibur using Cell-Quest software.

3.1.8 Analysis of the production of chemokines (GM-CSF, MIP1α, MCP-1, RANTES) and cytokines (IL-6) by intracellular staining of Vγ9Vδ2 T cells

For intracellular cytokine and chemokine analysis, Vγ9Vδ2 T lines cells (see above: establishment of long term Vγ9Vδ2 T cell lines) were washed and living cells isolated by Ficoll-Hypaque. Vγ9Vδ2 T lines cells were resuspended at 1 x 10^5 cells/100 µl in RPMI (10% SC) in a ninety-six well U-bottom plate and co-cultured with RMA-Neo or RMA-MICA cells, at an effector-target ratio of 1:5, medium alone served as the negative control. Positive control cells were stimulated with phorbol-12-myristate-13-acetate (PMA) (2.5 ng/ml) and ionomycin (0.5 µg/ml). After 1 h of stimulation, 1 µg/ml of the protein transport inhibitor brefeldin A was added, and re-incubated for 5 h, afterward the cells were resuspended in staining buffer with 1 µg/ml of conjugated mAb Vγ9 TCR-FITC. Subsequently, cells were washed once with staining buffer, fixed for 20 min at 4 °C using 100 µl of Cytofix/Cytoperm. Fixed cells were permeabilized by washing twice with 200 µl of Perm/Wash buffer and resuspended in 50 µl of Perm/Wash buffer, subsequently, 0.5 µg of the corresponding antibody: anti-IL-6, anti-RANTES, anti-MCP-1, anti-MIP-1α, anti-GM-CSF (all conjugated with PE), or isotype-PE

conjugated control mAb, incubated for 30 min at 4°C, and washed twice with 200 µl of Perm/Wash buffer. Finally, samples were resuspended in 200µl of staining buffer and analyzed by flow cytometry on FACSCalibur using Cell-Quest software.

3.1.9 Analysis of the release of perforin by Vγ9Vδ2 T cell by intracellular staining

The detection of perforin was performed essentially in the same way as for intracellular cytokines (see above), but without addition of brefeldin A or IL-2. mAb used for the staining were 0.5 µg of anti-Vγ9 TCR FITC, for the extracellular staining, and 0.5 µg of anti-perforin PE, for the intracellular detection. After the intracellular staining, the samples were resuspended in 200 µl of staining buffer and analyzed by flow cytometry on a FACSCalibur instrument using Cell-Quest software.

3.1.10 CD107a up-regulation after stimulation of Vγ9Vδ2 T cells

The percentage of degranulating Vγ9Vδ2 T cells was quantified by multi-parameter FACS staining. PBLs were resuspended at 5 x 10^5 cells/100 µl in RPMI (10% SC) in a ninety-six well U-bottom plate and co-cultured with C1R-ULBPs or C1R-Neo cells, at an effector-target ratio of 1:1, medium alone served as the negative control. Positive control cells were stimulated with PMA (2.5 ng/ml) and ionomycin (0.5 µg/ml). CD107a-PeCy5 antibody was added directly to the wells at 10 µl per 200 µl. Cells were incubated for 1 h at 37 °C in 5% CO_2 after which monensin (Golgi-Stop) was added at a final concentration of 10 µg/ml and incubated for additional 4 h at 37 °C in 5% CO_2. Monensin prevents the acidification of endocytic vesicles avoiding the degradation of re-internalized CD107a proteins from the surface and allowing for the visualization of this marker following stimulation. Afterwards the PBLs were stained for surface Vγ9 TCR-FITC, for 30 min. After washing, cells were resuspended in FACS buffer and the cytometric analysis was performed on a FACSCalibur instrument using Cell-Quest software.

3.1.11 Measurements of granule release

Ninety-six well U-bottom plates were coated overnight at 4°C with 12 µg/ml mAb to NKG2D (clone 149810) or 0.5 µg/ml for CD3 (clone HIT3a) or 10 µg/ml mouse IgG1 as isotype control in 0.05 M carbonate buffer, pH 9.6, 100 µl per well. Coating with MICA-Fc recombinant protein was performed for 2 days at a concentration of 10 µg/ml. Afterwards, the plates were thoroughly washed with PBS three times, and 5 x 10^5 cells per well of the Vγ9Vδ2 T enriched cell suspension (Negative Isolation of γδ T cells from PBLs) was added

to each well in 200 µl of RPMI (10% SC) media and incubated at 37°C in 5% CO_2 for 4 h (for granule release). As the positive control cells were treated with (50 ng/ml PMA + 1 µg/ml Ionomycin final concentration) and incubated at 37°C in 5% CO2 for 4 h.

Subsequently, the supernatants of the culture were collected and evaluated for secretion of cytoplasmic granules using a standard *N*-benzyloxycarbonyl lysine thiobenzyl ester (BLT) esterase assay. Briefly, 50 µl of each culture supernatant were mixed with 950 µl of BLT substrate (0.20 mM BLT, 0.22 mM of DTNB 5,5´dithio-bis-2-nitrobenzoic acid and 0.01 Triton X-100 in PBS pH 7.2) and incubated for 40 min at 37 °C. Later the reaction was stopped by the addition of 10 µl of 0.1 M PMSF in ice. Next, 1 ml of PBS was added to achieve a final volume of 2 ml. Samples were measured in a spectrophotometer at 412 nm. The percentages of antibody-induced secretion of esterase was calculated according to the following formula: % secretion= 100 X (E-B)/(T-B), where E is the mean absorbance for antibody-stimulated cell supernatants, B is the background from γδ T cells in the untreated wells, and T is the total esterase content (for determination of total esterase release, 5 x 10^5 cells were cultured at 37°C in 5% CO2 for 4 h in 190 µl of RPMI (10% SC) plus 10 µl of 1% Triton X-100 (Takayama, Trenn et al. 1987).

3.1.12 Fluorometric assessment of T lymphocyte antigen specific lysis (FATAL)

The target cells (RMA-MICA, C1R-MICA, C1R-ULBPs and Daudi) and the control cells (RMA-Neo and C1R-Neo) were labeled with PKH-26, according to the manufacturer's instructions. Target cells (1 x 10^6) were stained with PKH-26 (final concentration of 2.5^{-6} M) at room temperature for 3 min. To stop the reaction a volume of heat inactivated fetal calf serum (FCS) was added to the cells dye mixture (1:1, v:v), incubated for 1 min at room temperature, and centrifuged for 5 min at 400 x g. After a single wash with 10 ml PBS, the target cells were centrifuged for 5 min at 400 x g and the supernatant was discarded.

PKH-26 labeled target cells were subsequently stained with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE). CFSE was diluted to a final concentration of 2.5 µM and added to the target cell suspension. Immediately after addition of the CFSE, the reaction was stopped by addition of an equal volume of FCS and the cells were centrifuged at 300 g for 5 min at room temperature (RT). Finally, the target cells were washed twice with PBS, resuspended in RPMI medium, and dispensed in duplicates at 5 x 10^4 cells per well into 96-well U-bottom plates. Effector cells were added at various effector: target ratios, and mixed

with the target cells. The FATAL assay was performed for 5 h at 37°C, 5% CO_2. Following incubation, the total content of the U bottom plate was centrifuged for 5 min at 400 x g room temperature. The supernatant was discarded and the cell pellet was resuspended in 150µl 1% paraformaldehyde (Sheehy, McDermott et al. 2001). The analysis was performed on a FACSCalibur using Cell-Quest software.

3.1.13 Determination of T lymphocyte specific lysis by ^{51}Chromium release assay

The target cells (RMA-MICA, MEWO) and control cells (RMA-Neo) were labelled using ^{51}Cr. Briefly, 1 x 10^6 target cells were resuspended in 100 µl of RPMI (10% SC) media and ^{51}Cr (100 µCi) and incubated for 2 h with occasional mixing; after the incubation the cells were washed three times (10 ml per wash) with media and resuspended to 1 ml. Cell viability and enumeration were performed using trypan blue and the cell concentration was adjusted to 1 x 10^6 per ml.

Subsequently 100 µl containing 1 x 10^4 labeled target cells were distributed in triplicate in 100 µl per well of a ninety-six well U-bottom plates, for each effector: target (E:T) ratio, and for spontaneous and maximum release groups. A volume of 100 µl of effector cells (described previously in establishment of Vγ9Vδ2 T cell lines by stimulation of fresh Vγ9Vδ2 T cells from peripheral blood) were plated at different effector : target ratios in a final volume of 200 µl. The mixtures of cells were cultured for 4 h in 5% CO_2, at 37 °C. Ten minutes before harvesting, 100 µl of bleach (PBS 2% Triton-100X) was added to the maximum release wells. Finally the supernatant was harvested and counted in a Liquid Scintillation Counter Microbeta Trilux. Specific lysis was calculated as follows: % specific lysis = (cpm_{test} - $cpm_{spontaneous}$/cpm_{max} – $cpm_{spontaneous}$) x 100, where spontaneous release was determined in medium alone and maximal release was determined in Triton-lysed target cells. The result was considered as successful when an increasing percentage of the specific lysis correlated with the increasing E: T ratios and the spontaneous release did not exceed 30% of the maximal release.

Finally the inhibition assays of cellular cytotoxicity were performed by adding the mAbs to effector cells 30 min before addition of target cells, with a final concentration of 10 µg/ml of NKG2D-specific mAb M585 (Sutherland, Chalupny et al. 2002) or Vγ9-specific mAb 7A5 (Janssen, Wesselborg et al. 1991) and isotype specific controls.

3.1.14 Determination of the modulation of co-stimulator molecules (CD40, CD80, CD86) antigen presenting molecules (CD1a, HLA-DR), adhesion molecules (CD54), and activation molecules (CD69, CD95) after the stimulation with NKG2D ligands.

Fresh PBLs were suspended at a concentration of 5 x 10^5 per 100 µl in RPMI (10% SC) + rhIL-2 and co-cultured with 5 x 10^5 per 100 µl of C1R-ULBPs or C1R-Neo (negative control) mitomycin treated cells (1 x 10^6 cells incubated with 1 µg/ml of mitomycin in BSS 1h at 37 °C in 5% CO2, and then washed twice with BSS/BSA). Afterwards cells were stained by the addition of 10 µl of fluorochrome-conjugated monoclonal antibody specific for cell surface antigens such as HLA-DR, CD1a, CD69, CD54, CD40, CD80, CD86, CD95 vs. Vγ9 TCR and incubating 30 min at 4°C. Then the cells were washed twice with 3 ml of staining buffer and pelleted by centrifugation at 400 x g for 5 min at 18-20 °C. The cell pellet was resuspended in 500 µl of staining buffer and the analysis was performed on a FACSCalibur instrument on selected cells that fell within the lymphocyte population. Then, Vγ9+ cells population was selected. Within this population of cells, we noted the expression of the different co-stimulatory, adhesion, APC molecules for each sample following stimulation with NKG2D ligand. The analysis was performed on a FACSCalibur using Cell-Quest software.

3.1.15 Cytotoxicity inhibition assays using specific inhibitors of PLCγ (U-73122), PI3K (Ly294002), MEK1/2 (PD98059), p38 (SB203580), Src Kinases (PP2) and JNK2 (SP600125)

For this purpose ^{51}Cr-labeled RMA-MICA cells were used as a target cells. For the direct specific lysis assay ^{51}Cr-labeled RMA-MICA cells were used as described (determination of T lymphocyte specific lysis by ^{51}Chromiun release assay). The Vγ9Vδ 2 T cell lines (effector cells) were treated for 30 min at 37 °C prior to the cytotoxic assay respectively, with 100 µM of the PLCγ inhibitor (U-73122), 100 µM of the PLCγ inhibitor analog (U-73343), 50 µM of Src Kinase inhibitor (PP2), 10 µM PI3K inhibitor (Ly294002), 50 µM of the MEK1/2 inhibitor (PD98059), 5 µM of p38 inhibitor (SB203580), 25 mM of JNK2 inhibitor (SP600125), or equivalent concentrations of the DMSO as control. Subsequently the cells were incubated for 4 h at 50:1 E: T ratio. The mean of triplicates of lysis for each cell line was expressed as the percentage of specific killing.

3.1.16 IFNγ ELISpot

Precoated ninety-six nitrocellulose plates ELISpot^{pro} with anti-human IFNγ mAb (anti IFNγ mAb 1-D1K) were blocked 2 h at 37°C with RPMI (10% SC). After the blocking, 1000 Vγ9Vδ2 T cells were co-cultered with 25000 Raji (plus 30 µg/ml of Zolendronate) or P815 cells (cultured previously for 30 min with the following concentration of mAb: 5µg/ml of anti-NKG2D (clone 149810), 0.5 µg/ml of anti-CD3 or 5 µg mouse IgG1 as isotype control) in 200 µl of media for 24 h in 5% CO_2 at 37 °C.

After the incubation, the cells were removed and the wells were washed 5 times with PBS. Subsequently the one-step detection reagent (alkaline phosphatase-conjugated detection anti IFNγ mAb 7-B6-1) was added in a dilution 1:200 in filtered PBS-FSC (0.5 % fetal calf serum), and incubated for 2 h at RT. After incubation the wells were washed 5 times with PBS, and 100 µl of filter ready-to-use substrate solution (BCIP/NBT-plus) was added to the wells, and developed for 10-15 min until spot emerge. Color development was stopped by washing extensively in tap water. After the washes, the plate was dried and the spots counted in a dissection microscope (40X).

3.1.17 Phosphospecific intracellular proteins by flow cytometry analysis

The Vγ9Vδ2 T cell lines (1 x 10^6 cells/ml) were incubated with anti-NKG2D mAb (5 µg/ml, clone 149810) or anti-CD3 mAb (0.5 µg/ml) for 1 h at 4 °C; after the incubation the cells were washed twice with BSS-BSA and prepared for the differents time points of stimulation (0, 1, 3, 8, 15 min), the stimulation was performed as follow: 1 x 10^5 cells (preincubated with the corresponded antibody) were suspended in 100 µl of RPMI and added to 100 µl of prewarmed RPMI containing 10 µg/ml of goat anti mouse polyclonal Ab and incubated at 37°C in the different time points; the stimulation was stopped immediately after every time point by adding 400 µl of PhosFlow Fix Buffer I solution to the cell suspension, incubated at 37°C for 10 min. After the incubation, the cells were washed twice by adding 400 µl of PhosphoFlow perm/wash buffer I and centrifuged for 5 min at 250 x g RT. Subsequently, the cells were resuspended in 100 µl of PhosphoFlow perm/wash buffer I, and 5 µl of PhosphoFlow specific mAb (p-ERK 1/2, p-PLC-γ 1) was added and incubated 1 h at 4°C. After the incubation the cells were washed twice with PhosphoFlow perm/wash buffer I, and resuspended in PhosphoFlow perm/wash buffer I. Finally the analysis was performed on a FACSCalibur using Cell-Quest software.

3.2 Molecular Biology and Biochemistry Methods

3.2.1 RNA extraction and quantification

Pure γδ T cells or PBLs (1 x 10^6) were placed into 1.5 ml conical tubes, centrifuged for 5 min at 300 x g, and the supernatant removed by aspiration. After addition of 350 µl of buffer RLT, the cells were disrupted and the sample was homogenized by vortex, and subsequently 1 volume (350 µl) of 100% ethanol was added into the conical tube, the sample was mixed and 700 µl of this sample was applied into RNeasy mini columns. The sample was centrifuged for 15 s at ≥8000 x *g* (≥10,000 rpm) RT. Next, 700 µl buffer RW1 were added into of the RNeasy column and the sample was centrifuged again for 15 s at >8000 x *g* (≥10,000 rpm) to wash the column.

The washed RNeasy column was transferred into a new 2 ml tube and 500 µl of buffer RPE were pipetted onto the RNeasy column. Then the column was centrifuged for 15 s at 8000 x *g* (>10,000 rpm) RT, to wash the column, afterwards 500 µl of buffer RPE was added into the RNeasy column and centrifuged this time for 2 min at ≥8000 x *g* (≥10,000 rpm) RT, to dry the RNeasy silica-gel membrane. Finally 50 µl of RNAse free water was added to the RNasy column and centrifuged for 1 min at ≥8000 x *g* (≥10,000 rpm) RT, for elution of the RNA.

The concentration of eluted RNA was determined by measuring the absorbance at 260 nm (*A*260) in a spectrophotometer. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml (*A*260 =1= 40 µg/ml). The estimation RNA purity was made by the ratio between the absorbance values at 260 and 280 nm.

3.2.2 RT-PCR amplification of human DAP 10 and β actin mRNA.

The cDNA of human DAP10, DAP 12 and β actin were amplified by the following procedure: 1 µg of template total RNA, 0.5 µg primer oligo$(dT)_{18}$ and deionized water (nuclease free) up to 11 µl were added to 0.5 ml tube for PCR and incubated at 70°C for 5 minutes. Then the samples were chilled on ice and the following reagents were added in the order indicated: 5X reaction buffer 4µl, 2 µl of 10 mM dNTP mix (1 mM - final concentration), ribonuclease inhibitor 20 U and deionised water (nuclease free) a volume of 19 µl. The reagents were mixed and incubated at 37°C for 5 min and immediately after 40 units of M-MuLV Reverse Transcriptase were added into the mix and incubated again at 37°C for 60 minutes. The amplification of cDNA was stopped by heating at 70°C for 10 min and

chilling on ice. Subsequently serial dilution of cDNA (0.01, 0.1, and 1 µg/ml) were used in the amplification of the genes of human DAP 10, DAP 12 and β actin by Polymerase Chain Reaction (PCR) as follow:

Components	Volume	Final Concentration
10X PCR Buffer, Minus Mg	5 µl	1X
10 mM dNTP mixture	1 µl	0.2 mM each
50 mM MgCl2	1.5 µl	1.5 mM
primer Forward*	1 µl	0.2 µM
primer Reverse*	1 µl	0.2µ µM
Template DNA (0.01, 0.1, and 1 µg/ml)	1 µl	100 ng
Taq DNA Polymerase	0.2 µl	1.0 unit
Autoclaved, deionised water	40.3 µl	

* Pair of primers for specific amplification of DAP 10 or β actin

The tubes were closed and centrifuged briefly. Afterwards the tubes were incubated in a thermal cycler at 94°C for 2 min and 35 cycles of PCR amplification were performed as follows: Denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 1 min, the reaction was maintained at 4°C after cycling. The products of the reaction were analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining, using appropriate molecular weight standards 100 bp leader.

3.2.3 Immunoblot of DAP 10

The cells were washed in ice-cold PBS and centrifuged at 250 g for 5 min RT, ice-cold modified RIPA buffer (Tris-HCl: 50 mM, pH 7.4; NP-40: 1%; Na-deoxycholate: 0.25%; NaCl: 150 mM; EDTA: 1 mM, PMSF: 1 mM, Aprotinin, leupeptin, pepstatin: 1 microgram/ml each, Na_3VO_4: 1 mM, NaF: 1 mM) was added to the pelleted cells (100 µl per 1 x 10^7 cells) and the suspension was gently rocked on an orbital shaker at 4 °C for 30 min. The lysed cells were centrifuged at 14,000 x g in a precooled centrifuge for 30 min, immediately after the supernatant was transfered to a new centrifuge tube. The sample was mixed with 6 X electrophoresis sample buffer and boiled for 2-3 minutes 96 °C. The unused samples were stored at -70 C.

The samples were analyzed by 15 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE), according to standard protocols for 1 h at 110 volt. The proteins were transfered from the gel

to a PVDF membrane using an electroblotting semi-dry apparatus with transfer buffer (25 mM Tris, 40 mM Glycine and 10% methanol, final pH 9.4) and applying 1.2 mA per cm^2 of membrane for 1 h. After the transfer, the PDVF membrane was washed twice with distilled H_2O and stained with Ponceau S solution for 5 min to visualize the protein bands (Stock solution: 2% Ponceau S in 30% trichloroacetic acid and 30% sulfosalicylic acid; diluted 1:10 for use). The membrane was rinsed with H_2O until protein bands appeared, the positions of the molecular weight markers were then labeled.

For blocking of the unspecific binding, the PDVF membrane was incubated in freshly prepared PBS containing 3% nonfat dry milk for 1 h at RT under constant agitation. Then the PDVF membrane was washed five times for 5 min each with TBS that contained 0.1 % Tween 20 and subsequently incubated for 1 h at RT with slow agitation with the primary antibody DAP 10, diluted 1/500 in fresh PBS/3% nonfat dry milk. Then the PDVF membrane was washed 3 times and incubated with the secondary antibody rabbit polyclonal horseradish peroxidase 1/20000 for 1 h at RT with slow agitation. After incubation, the PDVF membrane was washed and the proteins were detected using chemiluminescence system (ECL).

3.3 Statistical analysis

Data were analyzed using the two-way ANOVA test and the Bonferroni post-test to compare replicate means by row for paired samples.

4 RESULTS

4.1 Expression of the activation markers CD95, CD69, CD25 and the memory marker CD45+RO expression on Vγ9Vδ2 T cells.

PBLs were isolated from blood samples from five voluntary young/adult donors by using the Ficoll separation method, subsequently, the expression of the memory markers CD45RO, CD27 and the activation markers CD25, CD69, CD95 on Vγ9Vδ2 T cells were analyzed by two-color (Vγ9 vs. CD95) or three-color (Vγ9 vs. CD25, CD69 or Vγ9 vs. CD45RO, CD27) FACS staining (Fig. 1a). The basal levels of expression of these molecules were studied immediately after the PBLs isolation (i.e., 0 h) without any stimulation. As depicted in Fig. 1b, most of the circulating Vγ9Vδ2 T cells derived from the donors were CD27+CD45RO+ (mean 75.7% SD ± 4.2) (central memory T cell phenotype); moreover a small percentage of Vγ9Vδ2 T cells were CD27- CD45RO+ (22.23% SD ± 0.3) (terminally differentiated phenotype). Furthermore, we observed that Vγ9Vδ2+ T cells express high levels of CD95+ (mean 94.1% SD ± 3.7) (Fig. 1c), and a high proportion of Vγ9Vδ2 T cells also express the activation markers CD69 (mean 35.91 SD ± 5.6%), and CD25 (mean 41.5 SD ± 13.15 %). (Fig. 1c) These Vγ9Vδ2+ T cells from PBLs were used for: a) Expression analysis of NK associated receptors and co-stimulatory molecules; b) expression analysis of activation markers induced after NKG2D ligation, and c) modulation of the expression of CD107a on the cell surface of Vγ9Vδ2+ T cells after NKG2D ligation.

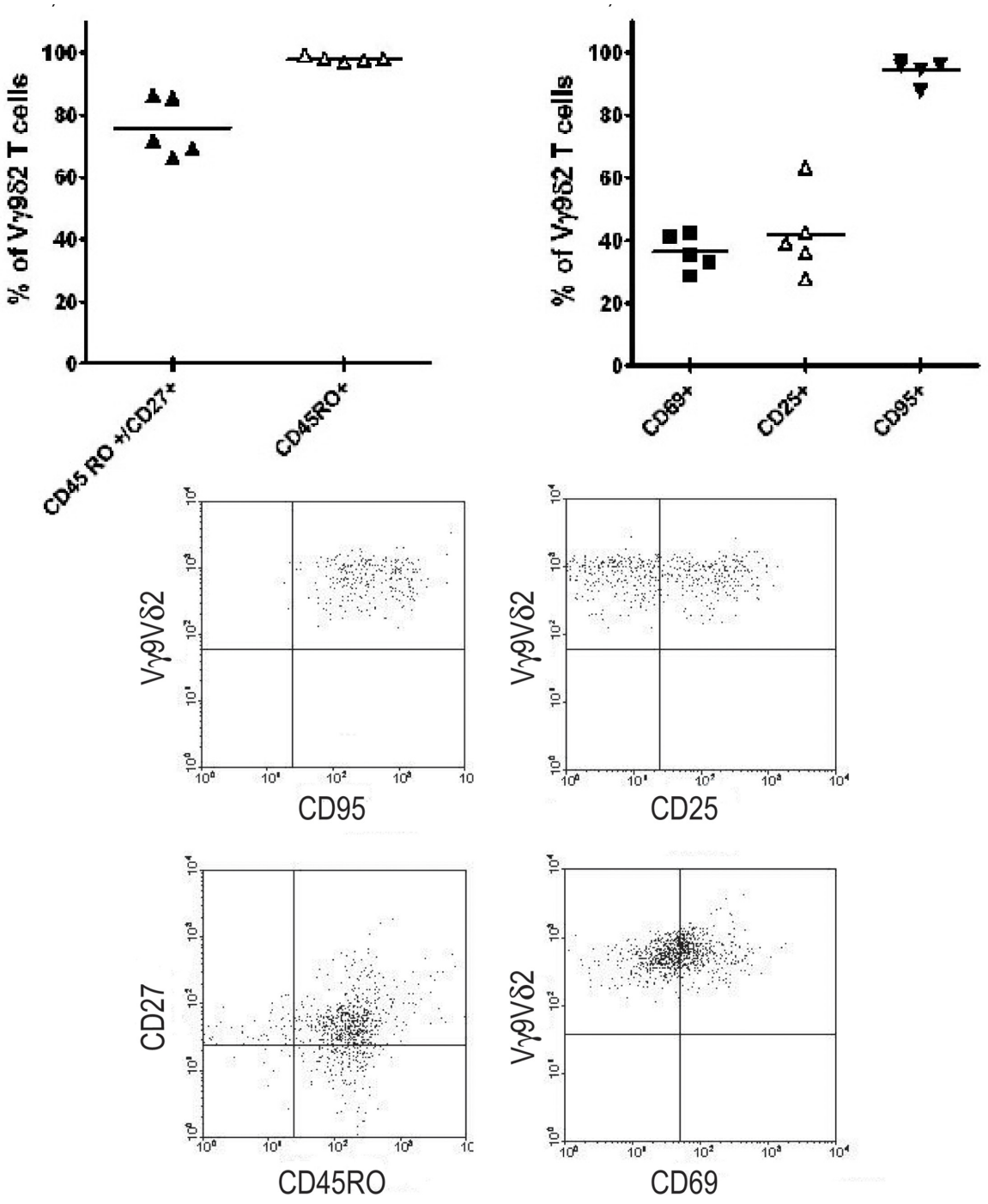
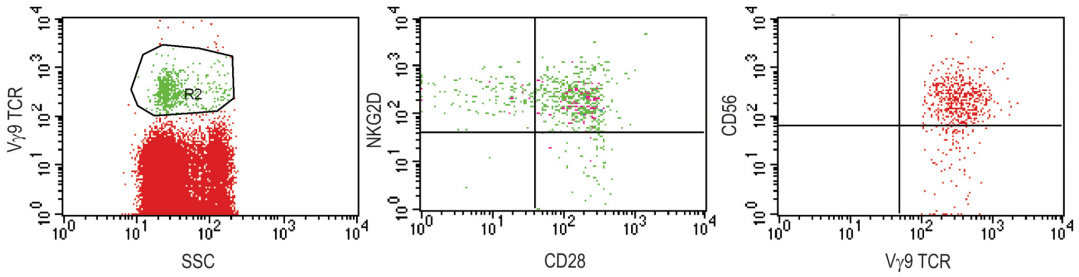


Figure 1: Characterization of Vγ9Vδ2 T cells derived from PBLs. Fig. 1a) Level of expression of memory markers (CD27 and CD45RO) on fresh Vγ9Vδ2 T cells from PBLs of different donors. Fig. 1b) Level of expression of activation markers (CD25, CD69 and CD95) on fresh Vγ9Vδ2 T cells from PBLs of different donors. Fig. 1c) depicts a representative examples of FACS two or three-color staining of CD95, CD25, CD27/CD45RO and CD69 expression on Vγ9Vδ2 T cells. The percentages of expression of different markers were determined by FACS analysis. Lines indicate the mean values of stained samples of five different donors.

4.2 Analysis of NK associated receptors (CD16, CD56 and NKG2D) and co-stimulatory molecule (CD28) expression on Vγ9Vδ2 T cells from freshly isolated PBLs.

The Vγ9Vδ2 T cells from PBLs previously described were analyzed for the expression of NK associated receptors such as CD16, CD56 and NKG2D and the co-stimulatory molecule CD28 by two or three-color FACS staining, one representative example is shown in Fig. 2a. The NK associated markers CD56 (mean 83% SD ± 1.15) and NKG2D (mean 90.12% SD ± 0.9) were highly expressed in Vγ9Vδ2 T cells; however, the level of CD16 (mean 41.9% SD ± 18.8) expression varied between the donors. Subsequently, the co-receptor CD28 (mean 80.67% SD ± 4.5) expression was determined in Vγ9Vδ2 T cells (Fig. 2b). A high proportion of Vγ9Vδ2 T cells co-express CD28 and NKG2D (mean 83.5 SD ± 2.6%) (Fig. 2b). Additionally a small proportion of Vγ9Vδ2 T cells expresses the phenotype of CD28+ NKG2D- (mean 7.8 SD ± 1.8%) or CD28-NKG2D+ (mean 8 SD ± 1.5%). The modulation of NK associated receptors and co-stimulatory molecules in Vγ9Vδ2 T cells stimulated *in vitro* with IPP/IL-2 was analyzed later.

2 a)



2 b)

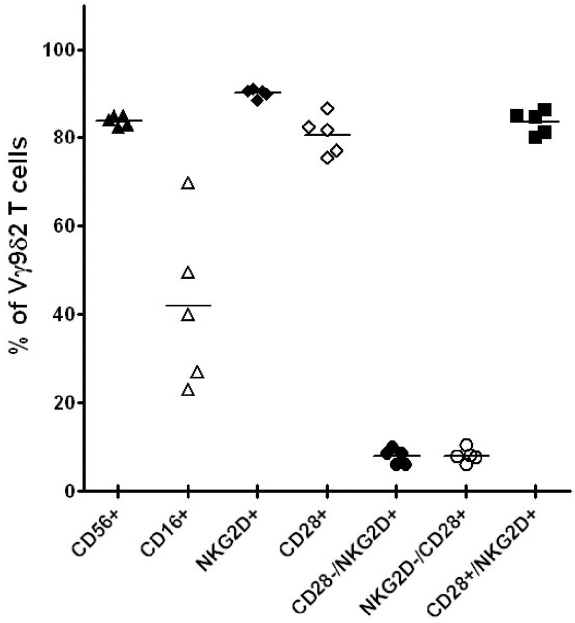


Figure 2: Expression levels of NK associated receptors (CD16, CD56 and NKG2D) and co-stimulatory molecule (CD28) on Vγ9Vδ2 T cells derived from PBLs. Fig. 2a) depicts a representative example of FACS three-color staining of CD28, CD56 and NKG2D expression on Vγ9Vδ2 T cells. Fresh isolated Vγ9Vδ2 T cells from PBLs were stained with anti-CD28 mAb, anti-CD56 mAb and anti-NKG2D mAb vs. anti-Vγ9 TCR mAb. Fig. 2b) Represent the percentages of expression of the different markers were determined by FACS analysis. Lines indicate the mean values of stained samples of five different donors.

4.3 NKG2D ligation induces expression of activation markers on Vγ9Vδ2 T cells from PBLs

PBLs were suspended at a concentration of 5 x 10^5 cells/200µl and cultured in the presence of 50 UI/ml IL-2 in 96 well culture plates coated with mAb specific for CD3 or NKG2D, with a MICA-Fc construct or isotype-specific control mAb. The activation markers CD69 and CD25 were analyzed after 24 h and 96 h of stimulation, respectively in Vγ9Vδ2 T cells (Vγ9 TCR mAb), NK cells (CD16 mAb), and CD8 cells (CD8 mAb) by two-color FACS staining. A representative example is depicted (Fig. 3a, dot plot) and the frequencies of CD69-positive and CD25-positive cells were determined from three independent experiments with PBLs from three different donors (Fig. 3b, histogram). No significant changes were found for CD8 cells after stimulation with anti-NKG2D (mean 8% SD ± 1.5), as compared with the positive control anti-CD3 (mean 21% SD ± 1.9). The proportion of CD69-positive Vγ9Vδ2 T cells increased following NKG2D ligation (mean 51% SD ± 2.5). The activation of these cells is comparable with anti-CD3 stimulation (mean 80% SD ± 2.9) (Fig. 3b). This effect was also demonstrated by a shift in the mean fluorescence after NKG2D stimulation (Fig. 3A, dot plot). For NK cells, un-stimulated cells exhibited high expression of CD69 (mean 27% ± 1.8) probably due to the presence of IL-2 in the culture media. The effects of NKG2D ligation were smaller (mean 35% SD ± 2.9) compared to Vγ9Vδ2 T cells. Determination of CD25 expression after 96 h of culture confirmed the capacity of NKG2D-mediated activation of Vγ9Vδ2 T (mean 57% SD ± 2.9) cells compared to the anti-CD3 stimulation (mean 85% SD ± 0.5) (Fig. 3b. lower panel). Similar results were also observed in un-stimulated (mean 17% SD ± 0.7) and NKG2D stimulated NK cells (mean 35 SD ± 1.5%). In contrast weak effect on the modulation of CD25 was observed in CD8 cells after NKG2D stimulation (Fig. 3b, histogram).

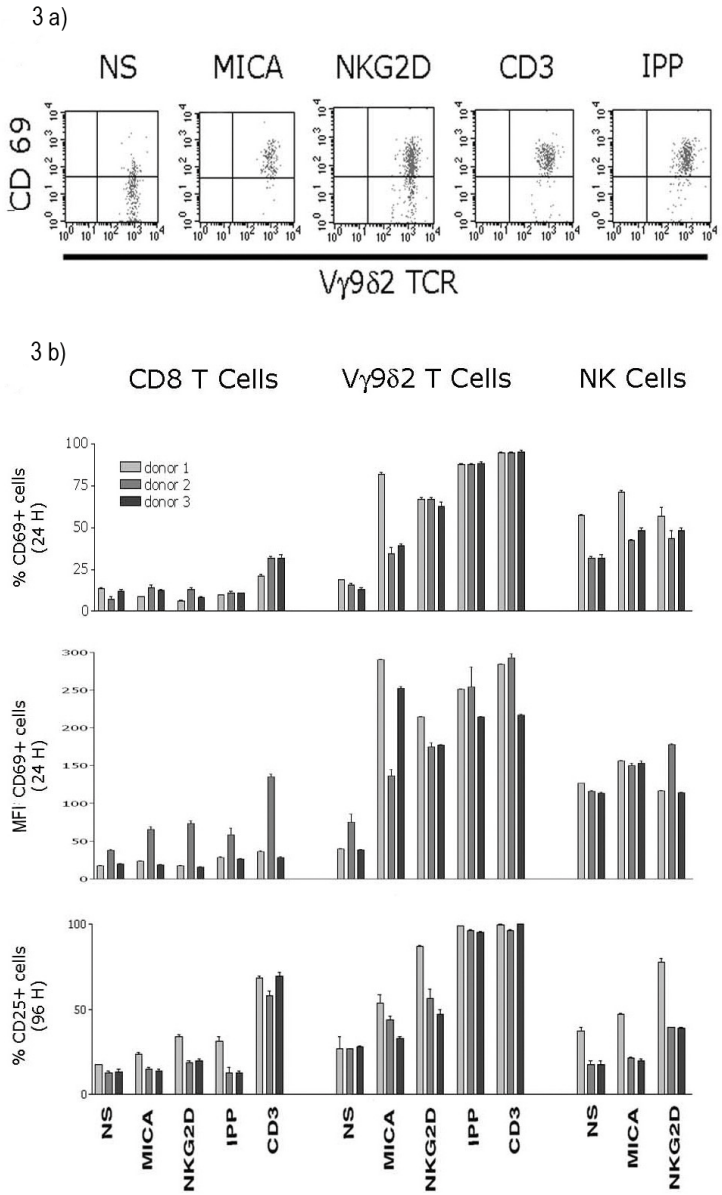


Figure 3: Differential induction of CD69 and CD25 expression on CD8 cells, Vγ9Vδ2 T cells, and NK cell after ligation of NKG2D. Fig. 3a) Depicts a representative example analysis of CD69 expression. Vγ9Vδ2 T cells from PBMC were stained with anti-CD69 vs. anti-Vγ9 TCR after 24 h of stimulation in medium plus 50 UI/ml IL-2 with indicated stimuli: NS (non-stimulated; immobilized isotype control), MICA (immobilized recombinant MICA-Fc), NKG2D (immobilized anti-NKG2D), IPP and CD3 (immobilized anti-CD3). Fig. 3b) PBMC were cultured for 24 h (upper panel) or 96 h (lower panel) in medium plus 50 UI/ml IL-2 with indicated stimuli. Proportion of CD69-positive cells (upper panel), mean fluorescence intensity (middle panel), and percentage of CD25-positive cells (lower panel) were determined by two-color FACS analysis. Error bars indicate SEM of duplicates stained for each sample

4.4 Analysis of activation markers (CD95, CD69 and CD25), memory markers (CD45RO, CD27), NK associated receptors (CD16, CD56 and NKG2D) and co-stimulatory molecule (CD28) expression on Vγ9Vδ2 T cells after IPP/IL-2 induced in vitro proliferation

Vγ9Vδ2 T cells from PBLs were stimulated *in vitro* for 10 days with IPP (1μg/ml) and IL-2 (100 UI/ml). This stimulation led to a massive and specific expansion of Vγ9δ2 T cells (Vγ9Vδ2 T-STL) (Fig. 4a middle dot plot). Importantly, similar levels of expression of NKG2D were observed in Vγ9Vδ2 T cells from PBLs and Vγ9Vδ2 T-STL. Vγ9Vδ2 T cells were purified from Vγ9Vδ2 T- STL by negative selection (purity 93%, Fig. 4a, right panel) and were further analyzed for various surface markers expression. The analysis of Vγ9Vδ2 T-STL by FACS demonstrated a decreased in the percentage of Vγ9Vδ2 T cells CD27+CD45RO+ T cells (57.6% SD ± 10.9) (central memory cells), and an increase in Vγ9δ2 T cells CD27- CD45RO+ (40.4% SD ± 0.37) (terminally differentiated) (Fig. 4c) compared with the freshly isolated Vγ9Vδ2 T cells from PBLs (Fig. 1a). Furthermore the analysis of the activation markers on Vγ9Vδ2 T cells exhibited a decrease in CD69 (5.6% SD ± 0.4), and increase in CD25 (63.38% SD ± 2.7) expression, although the level of CD95 (99.2% SD ± 0.3) expression was not altered (Fig. 4d). Strong down-regulation of the expression of NK associated markers CD16 (9% SD ± 1.9) and CD56 (28.1% SD ± 2.9) were observed in Vγ9Vδ2 T cells, although the level of NKG2D (91.2% SD ± 0.9) was unaffected (Fig. 4c). In addition the expression of the co-stimulatory molecule CD28 (61.65% SD ± 2.7) was slightly decreased. A small proportion of Vγ9Vδ2+ T cells remained CD28+ NKG2D- (4.8% SD ± 1.08) but significant increase in CD28-NKG2D+ (33.73% SD ± 1.5) cells was observed. These *in vitro* expanded Vγ9Vδ2 T-STL cells were used for further experiments.

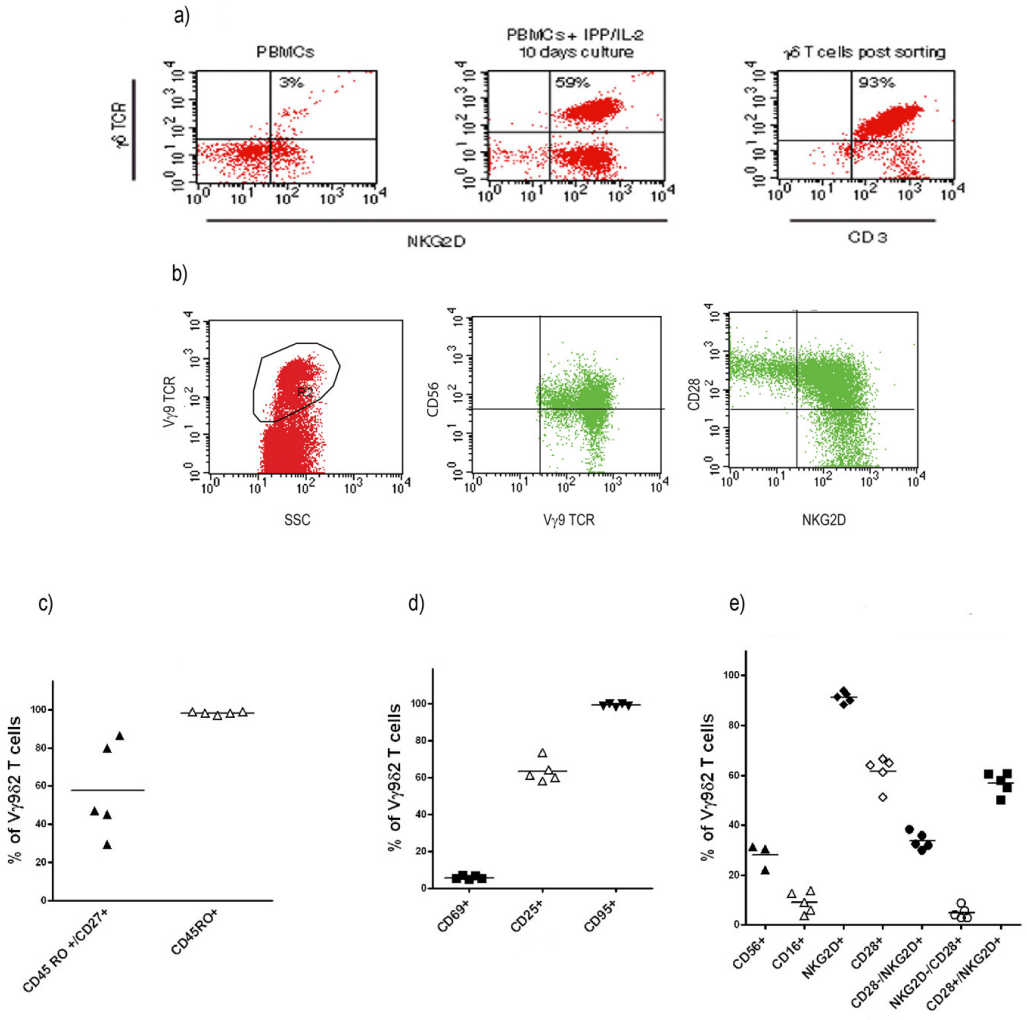


Figure 4: Characterization of Vγ9Vδ2 T-effector cells. Fig. 4a) Generation and isolation of Vγ9Vδ2 T-effector cells. Fresh Vγ9Vδ2 T cells from PBMC (left dot plot) were stimulated in vitro in the presence of TCR-Ag and IL-2 for 10 days (middle dot plot) and isolated by negative selection (right dot plot). Phenotype was determined by two colour-flow cytometry. Proportion of Vγ9Vδ2 T cells is indicated in the upper right quadrant of the dot plots. Fig. 4b) depicts a representative example of FACS three-color staining of CD56, CD28 and NKG2D expression on Vγ9Vδ2 T cells. Vγ9Vδ2 T-STL cells were stained with anti-CD56 mAb, anti-NKG2D mAb and anti-CD28 mAb vs. anti-Vγ9 TCR mAb. Fig. 4c) Level of expression of memory markers (CD27 and CD45RO) on Vγ9Vδ2 T-STL cells. Fig. 4d) Level of expression of activation markers (CD25, CD69 and CD95) on Vγ9Vδ2 T-STL cells. Fig. 4e) Level of expression of NK associated receptors (CD16, CD56 and NKG2D) and co-stimulatory molecule (CD28) on Vγ9Vδ2 T-STL cells. The percentages of expression of the different markers on Vγ9Vδ2 T-STL cells were determined by FACS analysis. Lines indicate the means values of stained samples of five different established Vγ9Vδ2 T-STL.

4.5 IFN-γ and TNF-α production of Vγ9Vδ2 T-STL after NKG2D ligation

Vγ9Vδ2 T-STL cells were suspended at a concentration of 5 x 10^5 cells per 200 µl of RPMI (10% SC) with 50 UI/ml IL-2 and stimulated for 5 h with immobilized NKG2D Ab or MICA-Fc construct as NKG2D ligand, or anti-CD3 mAb or 5 x 10^5 Daudi cells plus 1 µg/ml of IPP (as positive controls) and analyzed by intracellular FACS analysis for production of TNF-α or IFN-γ (Fig. 5 left dot plots). The data from three donors are represented on Fig. 5 (right panel). It indicated an increased frequency of TNF-α-producing Vγ9Vδ2 T cells after re-stimulation with NKG2D-specific mAb (3.4–6.2%), respectively, recombinant MICA-Fc (2–3.6%), and after co-culture with Daudi cells plus IPP (2.2–5.3%). These stimulation conditions induced in Vγ9Vδ2+ T cells a weaker activation than the positive control CD3 (47–67%). Induction of IFN-γ by Vγ9Vδ2 T-STL was not observed after NKG2D stimulation, only in one of the donors NKG2D stimulation induced weak IFN-γ production (2.6% producing cells versus 1.0% in the non-stimulated culture), although Daudi plus IPP demonstrated consistent production (3.3-6.38%)

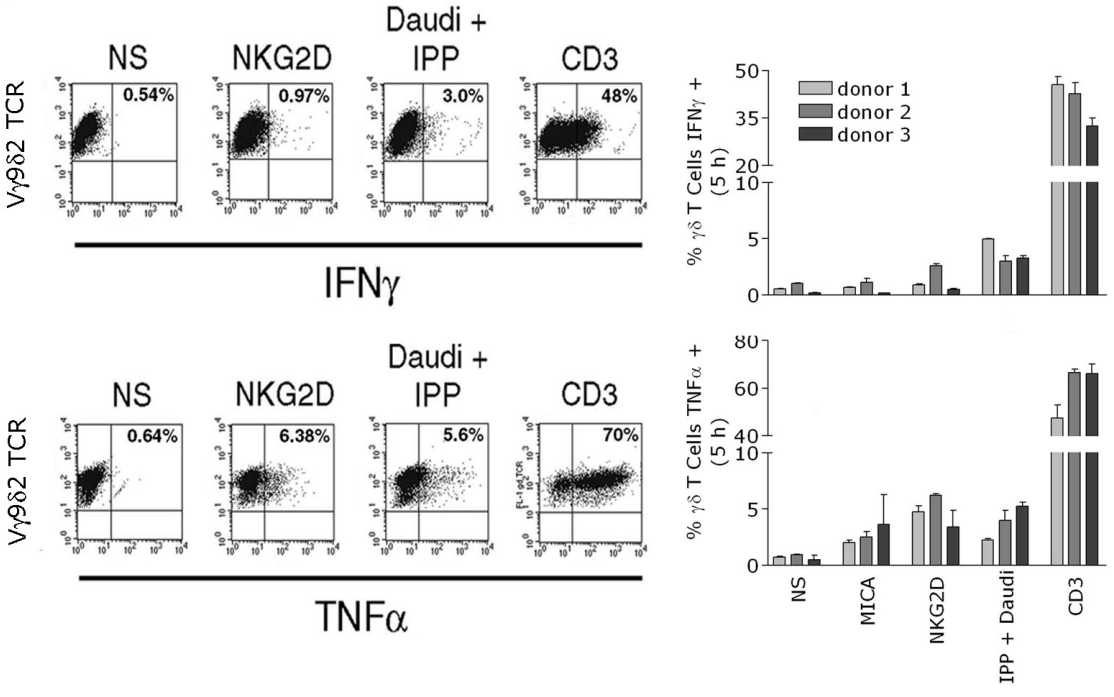


Figure 5: Analysis of IFN-γ and TNF-α production in Vγ9Vδ2 T cells by intracellular FACS staining after 5 h of stimulation. Vγ9Vδ2 T cells were stimulated with the indicated stimuli: NS (non-stimulated; immobilized isotype control), MICA (immobilized recombinant MICA-Fc), NKG2D (immobilized anti-NKG2D), IPP (Daudi IPP), CD3 (immobilized anti-CD3). The left graph provides a representative example for intracellular staining with anti-IFN-γ or anti-TNF-α vs. anti-Vγ9 TCR after 5 h of stimulation. The right graph summarizes data from three donors. Error bars indicate SEM of duplicates stained for each sample.

4.6 Analysis of chemokine (RANTES, MIP-1a, MCP-1 and GM-CSF) and (IL-6) production by Vγ9Vδ2 T cells after stimulation of NKG 2D. cytokine

Vγ9Vδ2 T cell lines were suspended at a concentration of 1 x 10^5 cells per 200 µl of RPMI (10% SC) with 50 UI/ml IL-2 and stimulated for 5 h by co-culturing with RMA cells transfected with MICA or the control vector RMA-Neomycin in the 1:5 effector: target ratio at 37°C for 5 h in RPMI media. As positive control the cells were stimulated with PMA-ionomycine. The cells were analyzed by intracellular FACS analysis for production of cytokine (IL-6) and chemokines (RANTES, MIP-1a, MCP-1 and GM-CSF) (Fig. 6a histograms). The data from two independent experiments are represented on Fig. 6b. It indicated significant increase ($P< 0.05$) on the production of MIP-1a, after NKG2D stimulation using RMA-MICA cells (media MFI: 47,78) compared with the stimulation using RMA-Neo cells (media MFI: 36.6), and non stimulated Vγ9Vδ2 T cells (media MFI: 30.39). In contrast, not significant production of IL-6 (mean MFI: RMA-MICA: 22.79 vs. RMA-Neo: 24.89, NS: 20.25), and chemokines such as RANTES (mean MFI: RMA-MICA: 44.79 vs. RMA-Neo: 38.21, NS: 33.43), MCP-1 (mean MFI: RMA-MICA: 42.49 vs. RMA-Neo: 44.62, NS: 32.22) and GM-CSF (mean MFI: RMA-MICA: 46.52 vs. RMA-Neo: 42.39, NS: 30.20) were observed in Vγ9Vδ2 T cell lines after NKG2D stimulation compared with the non stimulated cells or RMA-Neo cells (Fig. 6).

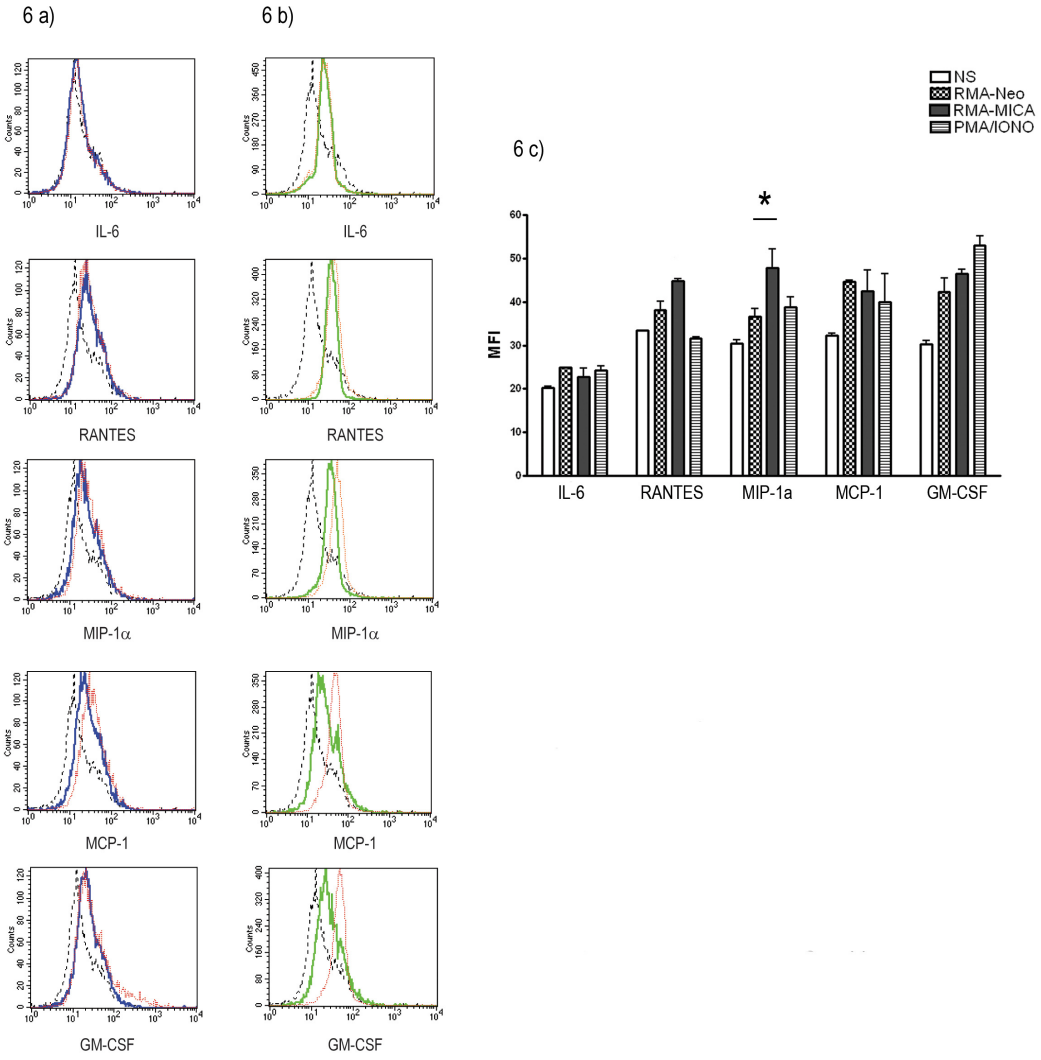


Figure 6: ICS FACS analysis of the cytokine (IL-6) and chemokines (RANTES, MIP-1α, MCP-1, GM-CSF) production after 5 hours of NKG2D stimulation. Fig. 6a) Histograms depict a representative example of the cytokine/chemokine production by pure Vγ9Vδ2 T cells line analyzed by intracellular FACS staining after stimulation. Left column of histograms depicted un-stimulated cells (blue histogram), positive control PMA/Ionomycine (red histogram), and isotype control (dotted white histogram). Fig. 6b) Right column of histograms depicted RMA-MICA (orange histogram), RMA-Neo (green histogram), and isotype control (dotted white histogram). Fig. 6c) Graphic representation [median fluorescence intensity (MFI)] of cytokine/ chemokine production by pure Vγ9Vδ2 T cells line after NKG2D stimulation. Bars indicate the SD and mean values of the MFI upon stimulation of two independent experiments. Two- way ANOVA analysis was performed to compare RMA-Neo cells with RMA-MICA stimulated cells, with significant values defined as $P < 0.05$ (*).

4.7 Analysis of IFN-γ-secreting Vγ9Vδ2 T cells after TCR or NKG2D ligation.

IFN-γ-secreting Vγ9Vδ2 T cells were detected by ELISpot assay (Fig. 7a). P815 cells were used as stimulator cells. They were pulse-labeled with anti CD3, anti-NKG2D or isotype control antibody as described in the methods section (IFNγ ELISpot). Strong spot formation was observed in all the probes where P815 cells pulse-labeled with anti-CD3 mAb (269 spots ± 11) were used as stimulators (Fig. 7b), and a substantial increment of spot formation was observed in the presence of P815 cells pulse-labeled with anti-CD3 mAb and 100 µg/ml of Poly I*C (355 spots ± 5), these proportion of IFN-γ-secreting Vγ9Vδ2 T cells after the stimulation with P815 cells pulse-labeled with anti-CD3 mAb were higher even than the positive control Raji cells cultured with 30 µg/ml of Zolendronate (69 spot ± 2). Non significant number of IFN-γ-secreting Vγ9Vδ2 T cells were detected after the stimulation with P815 cells pulse-labeled with anti-NKG2D mAb (16 spots ± 2) compared with the control P815 cells pulse-labeled with isotype mAb stimulation and only a slight increase was observed in presence of P815 cells pulse-labeled with anti-NKG2D mAb and 100 µg/ml of Poly I*C (19.5 spots ± 0.5). The number of IFN-γ-secreting Vγ9Vδ2 T cells after Poly I*C stimulation (15.5 spots ± 1.5) is similar to the non-stimulated Vγ9Vδ2 T cells (11 spots ± 2).

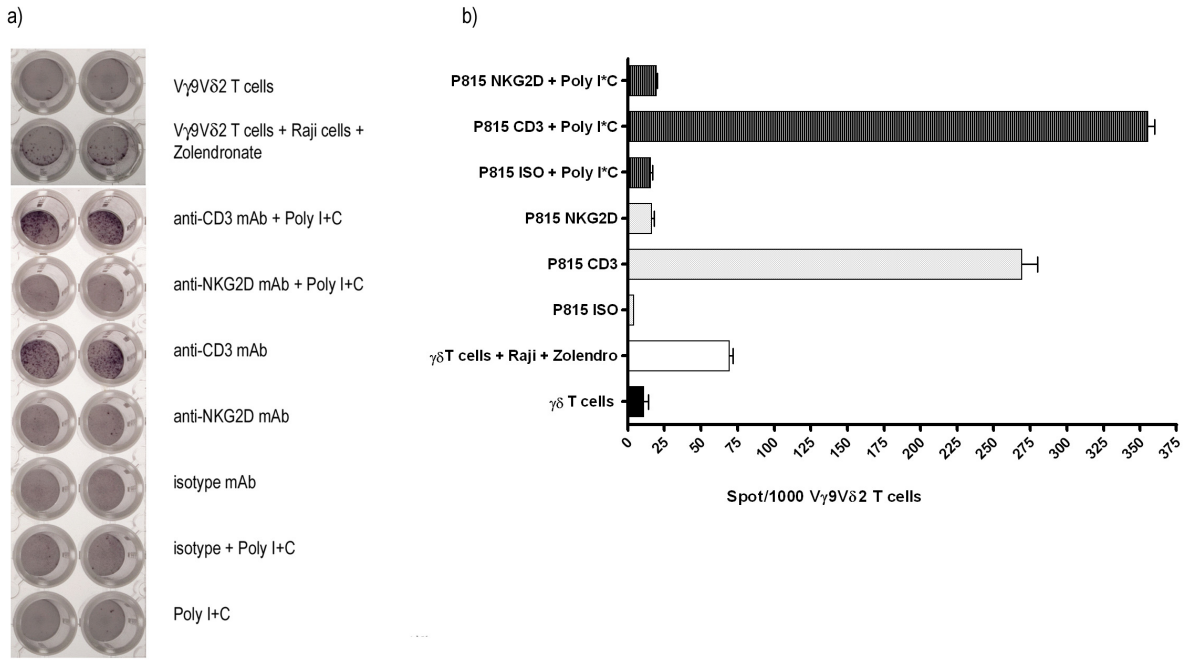


Figure 7: Analysis of Vγ9Vδ2 T cells IFN-γ-secreting by ELISpot after TCR or NKG2D ligation. Vγ9Vδ2 T cells (100 cells) were co-culture for 18 h with 25000 Raji cells (30 µg/ml of Zolendronate) or 25000 FcR-γ+ murine P815 cell line (incubated 30 min before assay with the follow mAb: 5 µg/ml of anti-NKG2D, 0.5 µg/ml of anti-CD3 or 5 µg/ml of mouse IgG1 as isotype control) as stimulator cells in presence or absence of TLR-3 ligand (Poly I+C). a) Demonstration of Vγ9Vδ2 T cell activation by direct ex-vivo IFN-γ ELISpot assay. Two

replicated were performed for each experimental condition. The numbers of spots were counted in a dissection microscope (40X). b) Number of specific spots per 1000 Vγ9Vδ2 T cells IFN-g-secreting after 18 h of specific stimulation of TCR or NKG2D detected by ELISpot IFN-γ. Bar represent spots induced by mAb stimulation using FcR-γ+ murine P815 cell line or Raji cells + Zolendronate (TCR stimulation by specific antigen). Error bars shown the SD from mean of two replicate wells.

4.8 Analysis of the regulation of Vγ9Vδ2 T cell surface molecules, which affect antigen-presentation after NKG2D stimulation.

Effects of NKG2D mediated activation on expression of molecules involved in antigen-presentation characteristics was determined in fresh PBLs co-cultured with 5 x 10^5 per 100 µl of the human B cell lymphoblastoma C1R transduced with ULBP-2 (C1R-ULBP-2) or Neomycin (C1R-Neo) (negative control) (Fig. 8b). The C1R cells were mitomycin treated at 37°C in 5 % CO_2 for 24 h in RPMI (10% SC) plus 50 UI of rhIL-2, and after the stimulation the Vγ9Vδ2 T cells were analyzed by FACS for the determine the level of expression of HLA-DR, CD1a, CD69, CD54, CD40, CD80, CD86, CD95 vs. Vγ9 TCR. The modulation of antigen-presentation molecules (HLA-DR, CD1a) and co-stimulation molecules (CD40, CD80, CD86) adhesion molecules (CD54) and activation molecules (CD69, CD95) expressed on Vγ9Vδ2 T cells after NKG2D stimulation is depicted in the figure 8. The graphic representation of four independent experiments is shown in Fig. 8a. It indicated an significant increase ($P< 0.001$) of the different molecules analyzed after NKG2D stimulation compared with RMA-Neo stimulation and non stimulated cells (NS) e.g. antigen-presentation molecules: HLA-DR (mean MFI: C1R-ULBP-2: 216,1 vs. C1R-Neo: 104.7, NS: 54,50) and CD1a (mean MFI: C1R-ULBP-2: 95,12 vs. C1R-Neo: 43.25, NS: 33.96), co-stimulation molecules: CD86 (mean MFI: C1R-ULBP-2: 179 vs. C1R-Neo: 126.5, NS: 104.5), adhesion molecules CD54 (mean MFI: C1R-ULBP-2: 96.17 vs. C1R-Neo:42.83, NS: 22.49) and activation molecules CD69 (mean MFI: C1R-ULBP-2: 577 vs. C1R-Neo: 226, NS: 32.50) and CD95 (mean MFI: C1R-ULBP-2: 723 vs. C1R-Neo: 592.5, NS: 357.5). Not significant up-regulation of the co-stimulatory molecule CD40 (mean MFI: C1R-ULBP-2: 37.25 vs. C1R-Neo:32.67, NS: 27.85) and CD80 (mean MFI: C1R-ULBP-2: 106 vs. C1R-Neo:81, NS: 14.50) were observed on Vγ9Vδ2 T cells after NKG2D stimulation.

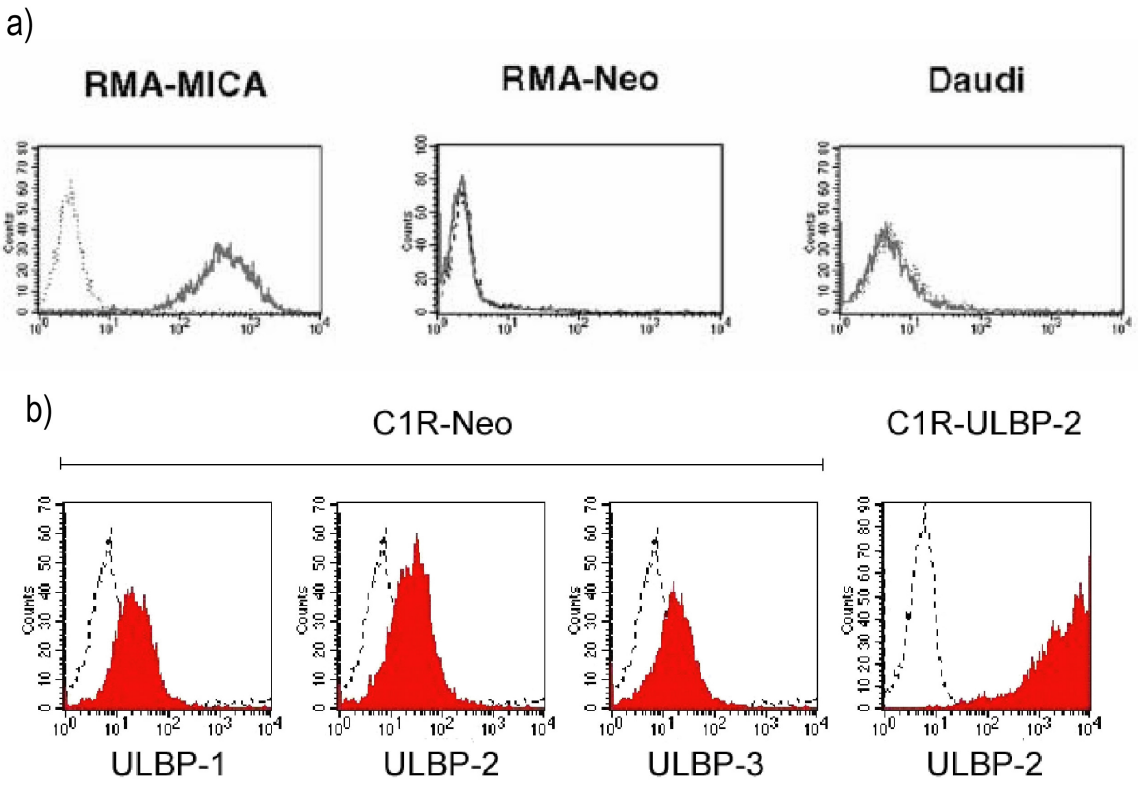


Figure 8: Analysis of NKG2D-ligands expression in target cells. a) MICA expression in target cells was determined by one-color FACS analysis. Only RMA-MICA cells express MICA. b) ULBP expression of C1R cells was determined by one-color FACS analysis. C1R-Neo (left histograms) express low level of endogenous ULBPs ligands and the expression is increased after the transfection of NKG2D ligand e.g. ULBP-2 (right histogram).

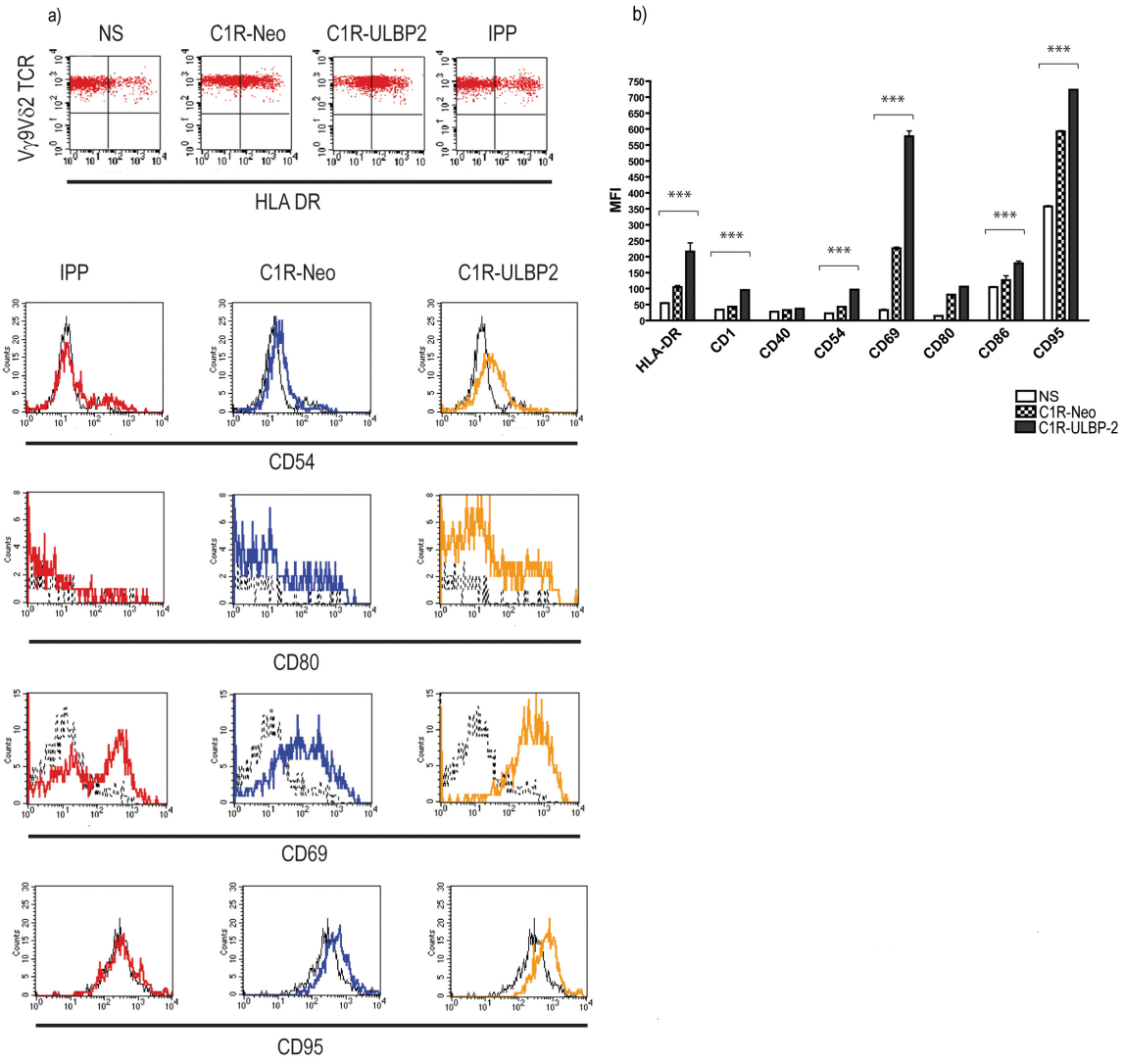


Figure 9: Stimulated Vγ9Vδ2 T cells modulated APC molecules after NKG2D stimulation. Fig. 9a) fresh Vγ9Vδ2 T cells from PBLs are activated and express antigen-presentation, co-stimulation, and adhesion molecules after NKG2D stimulation. Vγ9Vδ2 T cells were analyzed for expression of cell surface markers. Fig. 9a) Dot plot (upper part), indicated an example of the up-regulation of HLA-DR molecule on Vγ9Vδ2 T cells after NKG2D stimulation (C1R-ULBP-2) or positive control (IPP stimulation). Histograms depicted an example of the results for indicated markers on Vγ9Vδ2 T cells positive cells; red line positive control (IPP), blue line (C1R-Neo), yellow line (C1R-ULBP-2), black line (Isotype), represent stainings with specific and isotype control antibodies, respectively. The data are representative of three independent experiments. Fig. 9b) Graphic representation [median fluorescence intensity (MFI)] of the induction of antigen-presentation features upon co-culture of Vγ9Vδ2 T cells for 24 hours with the control cells C1R-Neo analyzed by flow cytometry. Bars indicate the mean values and SD of the MFI upon stimulation of three independent experiments. Two-way ANOVA analysis was performed to compare Vγ9Vδ2 T cells stimulated with C1R-Neo against C1R-ULBP-2 stimulation. The significant values $P<0.01$ were defined as (***).

4.9 Release of cytolytic granules by Vγ9Vδ2 T cells after NKG2D ligation

The release of cytolytic granules after NKG2D ligation by Vγ9Vδ2 T cells was measured by three independent methods: a) determination of CD107a in Vγ9Vδ2 T cells from PBLS by FACS analysis: freshly isolated PBLs were co-cultured with C1R cells transfected with ULBP-3 or the control vector (C1R neomycin) in the 1:1 ratio at 37°C in 5 % CO_2 for 5 h in RPMI media containing 100 UI/ml of IL-2 and CD107a-PE Ab. The basal expression of CD107a (9.27%) in the selected cells are indicated in Fig. 9a, left dot plot. Up-regulation of CD107a was evidenced after NKG2D engagement mediated by the C1R-ULBP-3 (47.6%) (Fig. 9a, middle dot plot), finally Vγ9Vδ2 T cells exhibited a weak stimulation after C1R-Neo (15.26%) (Fig. 9a, middle dot plot). b) The reduction in the content of perforin; this property was analyzed in Vγ9Vδ2 T-STL stimulated for 5 h as previously described (cytokine production of Vγ9Vδ2 T-STL after NKG2D ligation), the perforin content was then determined by intracellular FACS analysis. Fig. 9b histogram illustrates the uniform decrease in intracellular staining for perforin release after stimulation with immobilized NKG2D mAb in Vγ9Vδ2 T cells. Results for different stimulation conditions were as follows (arbitrary units of mean fluorescence intensity): non-stimulated (NS), 19.2 and 18.0; MICA-Fc, 14.3 and 14.4 (data not show); anti-NKG2D, 10.8 and 12.5; anti-CD3, 12.2 and 12.0 (data not show); for PMA plus ionomycin, 5.4 and 6.4 (data not show). Therefore, NKG2D and CD3 ligation led to an equivalent degree of perforin release, which was evident in the rapid secretion of cytolytic granules under NKG2D ligation.

c) The activation of CTL was measured by exocytosis of granules with esterase activity (BLT assay): the cytolytic capacity of Vγ9Vδ2 T cells mediated by triggering of NKG2D was tested by BLT assay. Negatively selected effector Vγ9Vδ2 T-STL cells (Fig. 4a) (90 and 93% purity respectively) were re-stimulated for 4 h with immobilized mAb. The results of two independent experiments performed with CD3 (vertical lines column) or NKG2D-specific mAbs (horizontal lines column) confirm that the cross-linking of NKG2D induce a strong release of granules with esterase activity and is efficient as CD3 stimulation Fig. 9c. The NKG2D-induced release corresponding to nearly the 80% of release found after stimulation with PMA plus ionomycin (black column). The negative control (white column) indicates that unspecific release of granule after stimulation with isotype mAb in this assay is not present.

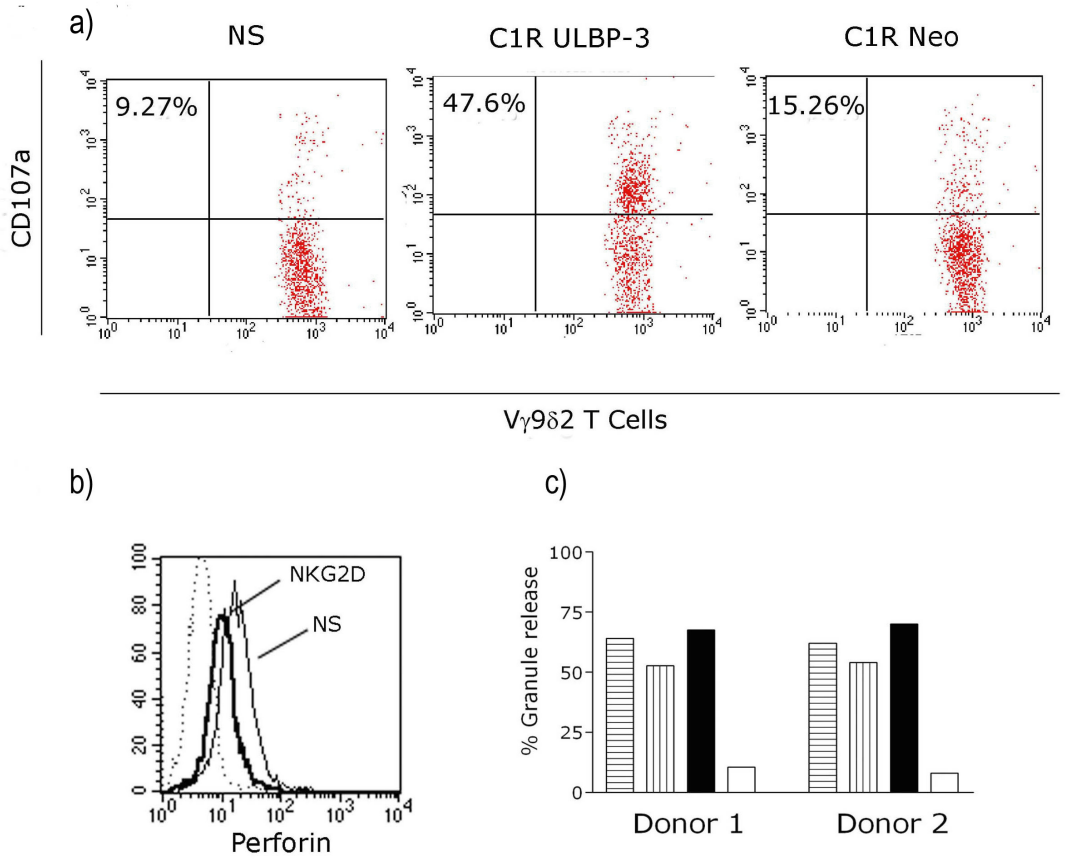


Figure 10: NKG2D dependent granule release by Vγ9Vδ2 T cells. Fig. 10 a) FACS analysis of CD107a modulation on Vγ9Vδ2 T cells from PBLs after 5 h of co-culture with C1R cells transfected with NKG2D ligand (ULBP-3) or control. Fig. 10 b) Intracellular FACS staining for perforin after incubation of Vγ9Vδ2 T cells, histogram depicts immobilized anti-NKG2D (NKG2D) or isotype-specific Ab (NS), and dotted line correspond to isotype-matched Ab. Fig. 10 c) Determination of secreted granule-associated enzymatic activity by BLT esterase after incubation of purified Vγ9Vδ2 T cell with immobilized anti-NKG2D (horizontal line column) anti-CD3 (vertical line column) and PMA plus ionomycine (black column), isotype specific control (white control). One hundred percent content of granule was estimated by analysis of cells treated with 0.2% of Triton X-100.

4.10 MICA and NKG2D-dependent lysis by in vitro-activated Vγ9Vδ2 T cells

NKG2D-triggered cytotoxicity was tested using Vγ9Vδ2 T cells purified from IPP-induced polyclonal cells (effector Vγ9Vδ2 T cells) and Vγ9Vδ2 T-STL. To exclude TCR-mediated effects by residual Ag, the STL effector Vγ9Vδ2 T cells were enriched on a Ficoll gradient and then purified by negative selection, resulting in a 85–95% pure Vγ9δ2 T cells as evidenced from FACS analysis using Vγ9 TCR-specific mAb (Fig 4a). More than 97% of the cells were CD3 positive; 1–3% of them expressed CD8 or CD16. The effector cells were tested for lysis of different target cells: a) Target mouse RMA cells transfected either with MICA expression vector (RMA-MICA) or control vector (RMA-Neo) (Fig. 8a). The RMA cells were selected because mouse cells do not present ligands for the Vγ9Vδ2 TCR. In addition, the species difference between effector and target cells makes ligation of KIRs of effector Vγ9Vδ2 T cells by target MHC class I molecules rather unlikely. b) Daudi cells express endogenous TCR mediated ligands for the Vγ9Vδ2+ T cells and are negative for surface MHC class I and MICA (Fig. 8a). c) The human B cell lymphoblastoma C1R express low levels of endogenous MICA and ULBPs, these cells were transfected with MICA (C1R-MICA) or ULBP2 (C1R-ULBP2), or with vector alone (C1R-Neo) (Fig. 8b) and d) the melanoma line Mewo which has been previously demonstrated (Kabelitz, Wesch et al. 2004) to express Vγ9Vδ2 TCR-antigens as well as NKG2D ligand (MICA).

A representative cytotoxicity assay of six independent experiments using different donors and the Fatal Assay as readout is depicted in Fig. 11. In all the experiments, the percentage of lysis between RMA-MICA cells and Daudi cells was similar, whereas no dose-dependent lysis was observed for RMA-Neo cells (Fig. 11a). Involvement of NKG2D-mediated killing was analyzed by addition of 20 μg/ml of blocking anti-NKG2D mAb (M585). In this assay only the killing of the NKG2D ligand-expressing RMA-MICA cells was inhibited (Fig. 11b), but not TCR dependent killing of Daudi cells (Fig. 11c). Finally, the lysis of C1R-Neo cells, C1R-MICA cells, and C1R-ULBP2 cells were compared (Fig. 11d). A small percentage of killing was found for C1R-Neo cells (4-5 %), which express low levels of endogenous ULBP2 and MICA. The lysis was increased for C1R cells that express either MICA or ULBP2.

NKG2D-mediated lysis was also analyzed using two previously described highly purified (98%) Vγ9Vδ2 T cell lines (denominated HO and EP3) as effectors, tested in a standard ^{51}Cr

release assay (experiment made by P. Wrobel in collaboration with Dr. Kabelitz and Dr Wesch from Kiel, Germany). As shown above with the FATAL method, only RMA-MICA (Fig. 12 c and d) but not RMA-Neo cells (Fig. 12 a and b) were lysed by the Vγ9Vδ2 T cell lines. To establish the respective contribution of NKG2D and TCR to the lysis of the target cells, Ab inhibition experiments were performed. The presence of NKG2D-specific mAb M585 blocked lysis of RMA-MICA very efficiently, while only weak effects of TCR-specific mAb were found (Fig. 12 c and d). The results contrast with those obtained with Mewo melanoma as target cells, which express ligands for Vγ9Vδ2 TCR and NKG2D. In this case, blockade of lysis by TCR-specific mAb (70–80% inhibition) was very efficient, compared with NKG2D-specific mAb (30–50% inhibition). Combining both mAb only negligibly increased the anti-TCR inhibition (Fig. 12 e and f).

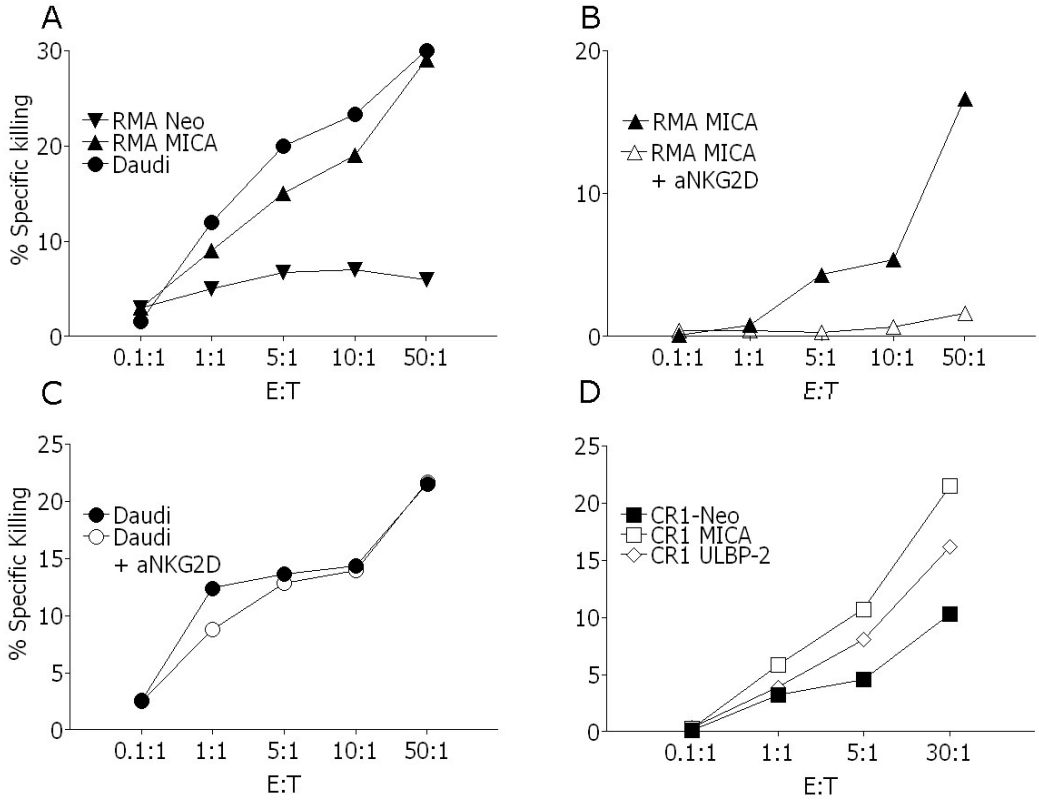


Figure 11: Analysis of NKG2D-dependent cytotoxicity by Vγ9Vδ2 T cells. Depicted lysis 5-h cytotoxicity assay at the indicated E:T ratio A–D, killing by purified polyclonal Vγ9Vδ2 T cells). A, Comparison of lysis of RMA MICA (black triangle), RMA Neo (invert black triangle), Daudi (black circle). B, Lysis of RMA-MICA in the presence (black triangle) or absence of 5 µg/ml anti-NKG2D (white triangle). C, Lysis of Daudi in the presence (white circle) or absence of 5 µg/ml anti-NKG2D (black circle). D, Lysis of C1R-Neo (black square) C1R-MICA (white square) and C1R- ULBP2 (white diamond). The effector cells were prepared as shown in Fig. 4a. NKG2D ligand expressions on target cells were exhibited in figure 8.

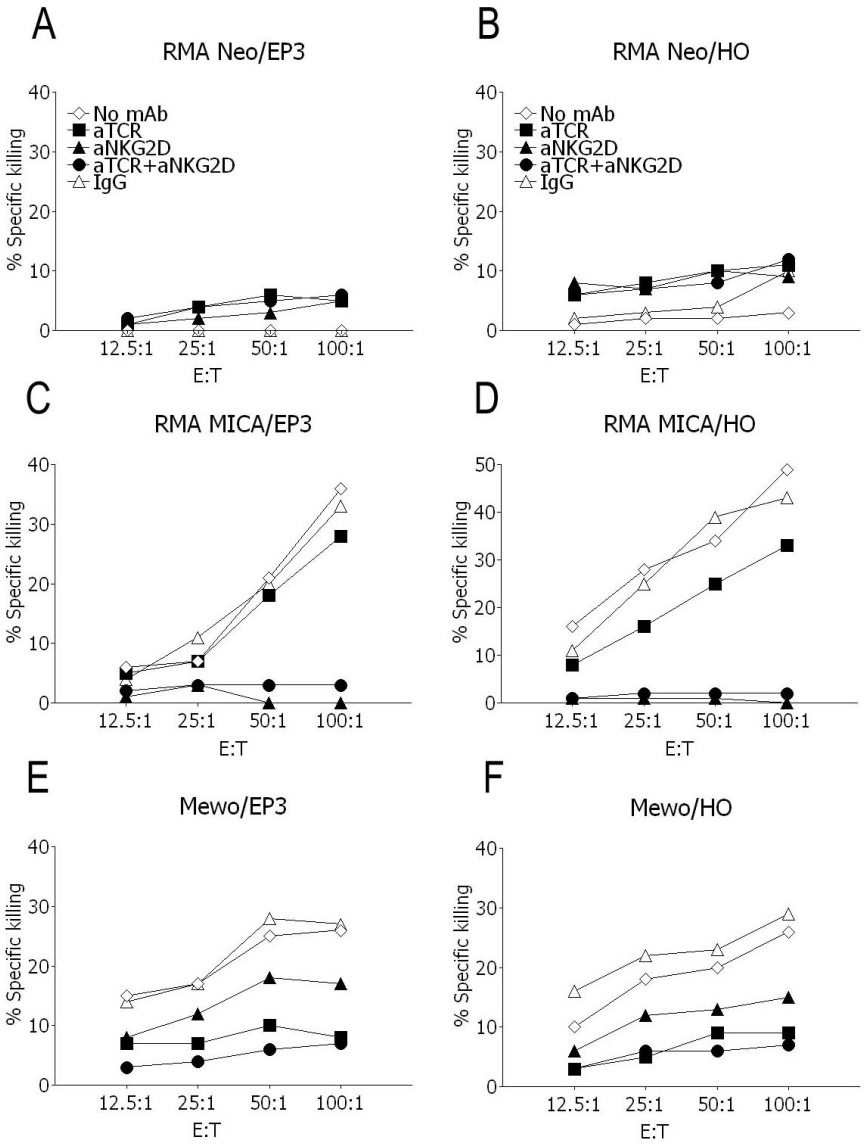


Figure 12 Analysis of TCR and NKG2D-dependent cytotoxity by Vγ9Vδ2 T cell lines. Lysis in a 4 h cytotoxity assay at the indicated E:T ratios and combinations. Vγ9Vδ2 T cell line EP3 (left column) or HO (right column). (A-B) Lysis of RMA Neo; (C-D) lysis of RMA; (E-F) lysis of Mewo cells. Abs were added as indicated. No Ab (white diamond), anti-TCR (black square), anti-NKG2D (black triangle), anti TCR plus anti-NKG2D (black circle), isotype control (with triangle).

4.11 NKG2D-dependent but not CD28-dependent cytotoxicity by in vitro-activated Vγ9Vδ2 T cells

Analysis of specific induced cytotoxicity by Vγ9Vδ2 T cells was performed by redirected killing using ^{51}Cr release assay and FcR-γ+ murine P815 cell line as a target cells. P815 cells were pulse-labeled 30 min before the assay with the following mAb: anti-NKG2D mAb (5 μg/ml), anti-CD3 mAb (0.5 μg/ml), anti-CD28 conventional mAb (5 μg/ml), anti-CD28 superagonistic mAb (5 μg/ml), or isotype control mAb (5 μg/ml), and as effectors cells were used the EP3 Vγ9Vδ2 T line. To exclude TCR-mediated effects by residual Ag, the effectors Vγ9Vδ2+ T cells (90–95% pure) were enriched on a Ficoll gradient. The result is depicted in Fig. 13 and represents three independent experiments using Vγ9Vδ2 T cell lines EP3. High percentage of redirected lysis was founded with P815 cells pulse-labeled with anti-NKG2D mAb (26%), and anti-CD3 mAb (39%), whereas no significant redirected killing was found with P815 cells pulse-labeled with anti-CD28 conventional (7%) and anti-CD28 superagonistic mAb (4%).

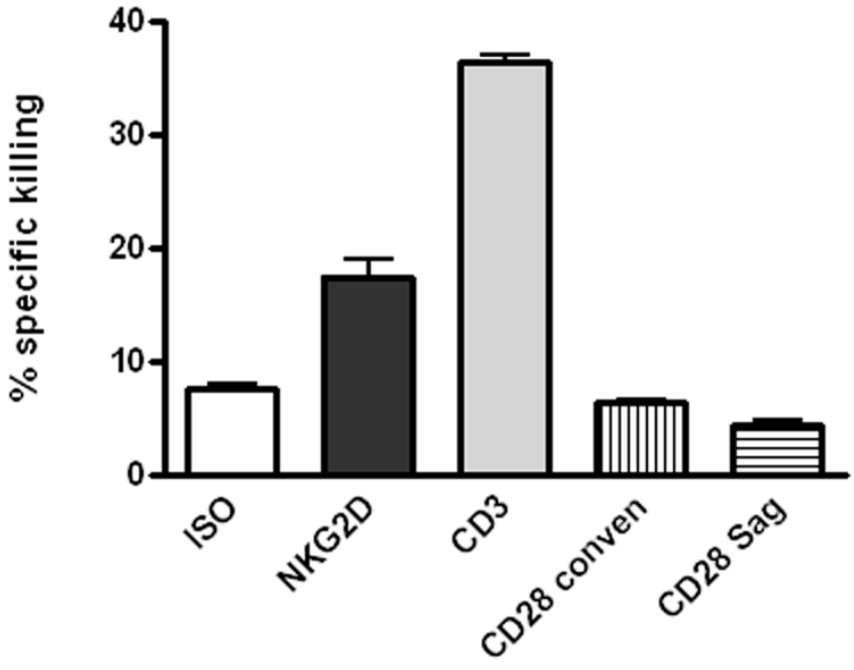


Figure 13: Percentage of specifically induces cytotoxicity by Vγ9Vδ2 T cells in a redirected killing assay using FcR-γ+ murine P815 cell line. 51Cr release assay was performed using Vγ9Vδ2 T cells lines as effectors and FcR-γ+ murine P815 cell line pulse-labeled 30 min before assay with the followed mAb: anti-NKG2D mAb (5 μg/ml), anti-CD3 mAb (0.5 μg/ml), anti-CD28 conventional mAb, anti-CD28 superagonistic mAb or isotype control mAb (5 μg/ml) as a targets. Effector-to-target cell ratio 50:1. Error bars show the standard deviation from mean values of three experiments performed with Vγ9Vδ2 T cells.

4.12 DAP10 expression on Vγ9Vδ2 T cells

DAP10 expression in Vγ9Vδ2 T cells was tested by semiquantitative RT-PCR analysis, as depicted in figure 14a. It shows DAP10 mRNA expression in resting and activated Vγ9Vδ2 T cells. cDNA for this analysis was synthesized from positively selected resting Vγ9Vδ2 T cells (95% purity, no NK cells) or Vγ9Vδ2 T cells after 7 days of culture with IPP and intermediate (100 U/ml; 96% purity, no NK cells) or high concentration of IL-2 (300 U/ml; 93% purity, no NK cells). As positive controls cDNA of the human monocytic line THP-1 and PBLs were used and as negative control cDNA from human B-cell lymphoma (Raji) was used. Expression of DAP10 at the protein level (Fig. 14b) was also tested by immunoblot analysis of lysates of Vγ9Vδ2 T effector cells, human monocytic line THP-1, and Daudi cells. DAP10 was strongly expressed in Vγ9Vδ2 T effector cells and in THP-1 cells.

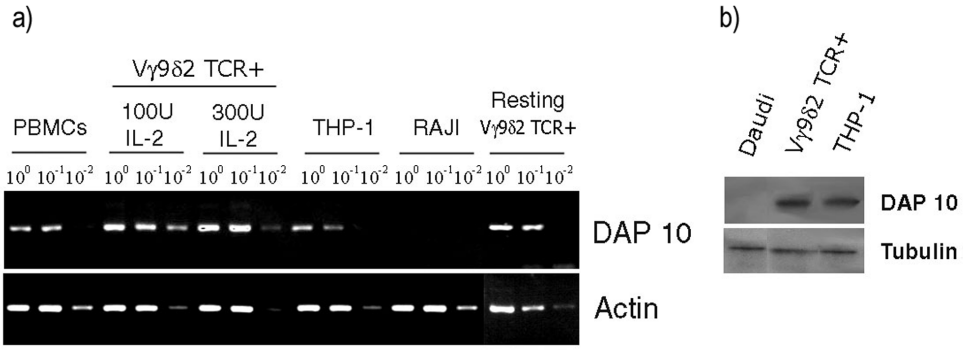


Figure 14: Determination of DAP10 expression in Vγ9Vδ2 T cells by RT-PCR and immunoblot. Fig. 14a) Depicts RT-PCR for DAP10 and beta-actin using serial dilutions of cDNA from the indicated cell populations. Fig. 14b) Immunoblot analysis of DAP10 protein in indicated cell types.

4.13 Analysis of the role of PLC-γ 1, Src kinases, P38, MEK 1/2, JNK and PI3K in NKG2D-mediated lysis by Vγ9Vδ2 T cells treated with specific pharmacological inhibitors

In order to determine the signaling pathways involved in NKG2D-mediated cytolysis in Vγ9Vδ2 T cells. The effects of a panel of kinase inhibitors were tested on Vγ9Vδ2 T cell lines. The cytolytic assays were performed using ^{51}Cr labeled RMA-MICA cells as a target. Pretreatment of Vγ9Vδ2 T cells with PLC-γ inhibitor U73122 (63% of inhibition) (but not with the inactive analog U73343, 5.66% of inhibition) and the PI3K inhibitor Ly294002 (72% of inhibition) strongly suppressed NKG2D mediated lysis. Additionally the Src kinases inhibitor (PP2) (50% of inhibition) and the P38 inhibitor SB203580 (27% of inhibition) present a middle suppression of the NKG2D mediated lysis. In contrast, the MEK 1/2 inhibitor PD98059 (1.8% of inhibition) and the JNK inhibitor SP600125 (1.6% of inhibition) not present any inhibitory effect in the NKG2D mediated lysis. In the DMSO-treated Vγ9Vδ2 T cells lines is observed a percentage of killing of 25 % (SD ± 4%)

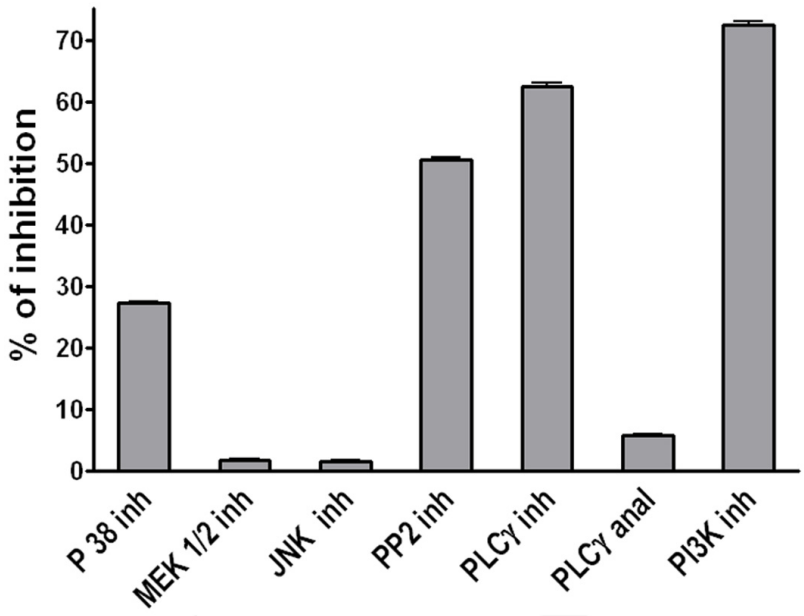


Figure 15: Suppression of NKG2D mediated lysis of RMA cells by using a panel of kinase inhibitors on Vγ9Vδ2 T cell lines. The effector cells lines were treated for 30 min with 100 μM of the PLC-γ 1 inhibitor (U-73122), 100 μM of the PLC-γ 1 inactive analog (U-73343), 50 μM of Src Kinase inhibitor (PP2), 10 μM PI3K inhibitor (Ly294002), 50 μM of the MEK1/2 inhibitor (PD98059), 5 μM of p38 inhibitor (SB203580), 25 mM of JNK2 inhibitor (SP600125) or equivalent concentrations of the DMSO as control. Subsequently the cells were incubated for 4 hr at 50:1 E: T ratio with 51Cr labeled RMA-MICA cells. Error bars show the standard deviation from mean values of two independent experiments. The percentage of inhibition was calculated by reference to DMSO-treated Vγ9Vδ2 T cell lines killing capacity.

4.14 Analysis of phosphospecific intracellular proteins (ERK 1/2 and PLC-γ 1) in Vγ9Vδ2 T cells after stimulation with anti-NKG2D mAb or anti-CD3 mAb.

The specific phosphorylation of ERK 1/2 and PLC-γ 1 after NKG2D or CD3 stimulation were analyzed in pure Vγ9Vδ2 T cells lines by three-color flow cytometry. Graphic representation of two independent experiments is depicted in Fig. 16 a. It indicated that PLC-γ 1 is rapidly but weak phosphorylated (1.49 fold induction) after 1 min of NKG2D stimulation (Fig. 16b). This state of phosphorylation is maintained after 15 min of stimulation (1.40 fold induction) (Fig. 16b). Additionally, CD3 stimulation induced a rapidly and strongly PLC-γ 1 phosphorylation (2.65 fold induction after 1 min). This activation started to decrease after 8 min of stimulation (2.5 fold induction) and the higher decreasing was observed after 15 min (1.81 fold induction) (Fig. 16b), The phosphorylation levels of PLC-γ 1 after CD3 stimulation is high comparing with NKG2D stimulation (Fig. 16b). ERK 1/2 is rapidly phosphorylated after 1 min of NKG2D stimulation (5.65 fold induction) and this level start decreasing after 3 min of stimulation (4.8 fold induction) reaching nearly half of the maximum phosphorylation level after 15 min of stimulation (3.55 fold induction) (Fig. 16c). CD3 stimulation leads to a rapid and strong phosphorylation of ERK 1/2 after 1 min of stimulation (19.6 fold induction), achieving the maximum phosphorylation after 3 min of stimulation (20.1 fold induction). A decrease of the phosphorylation of ERK 1/2 is observed after 8 min of stimulation (12 fold induction) and 15 min of stimulation (8.50 fold induction), which still is higher than the maximum phosphorylation levels reached with NKG2D stimulation (Fig. 16c).

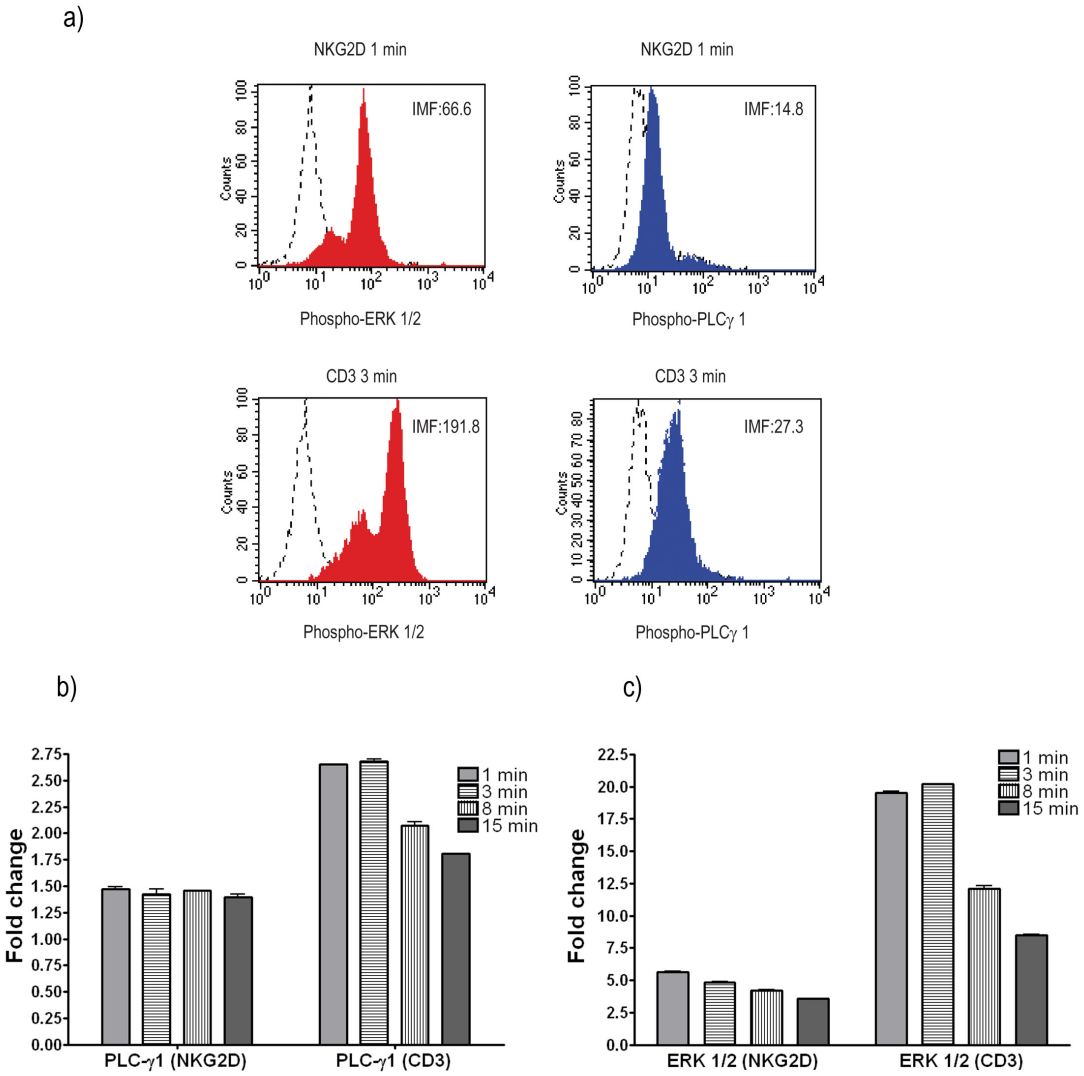


Figure 16: Analysis of ERK 1/2 and PLCγ-1 phosphorylation in Vγ9Vδ2 T cells by ICS flow cytometry (phosflow). Figure 16a) Histograms as representative example of the kinetic phosphorylation of ERK 1/2 and PLCγ-1 in pure Vγ9Vδ2 T cells analyzed by intracellular FACS staining after stimulation with anti-NKG2D mAb (red histogram) or anti-CD3 mAb (blue histogram), compared with un-stimulated cells (dotted white histogram). Figure 16b) Graphic representation (fold induction) of the kinetic stimulation of PLC-γ 1 in pure Vγ9Vδ2 T cells (0, 1, 3, 8, 15 min) with anti-NKG2D or anti-CD3 mAb. Figure 16c) Graphic representation (fold induction) of the kinetic stimulation of ERK1/2 in pure Vγ9Vδ2 T cells (0, 1, 3, 8, 15 min) with anti-NKG2D or anti-CD3 mAb. Bars indicate the mean values of three independent experiments. Fold induction=MFI_{stimu}/MFI_{unstim}

5 DISCUSSION

Vγ9Vδ2 T cells recognize ligands not seen by conventional αβ T cells, thereby providing an essential additional pathway of local immunosurveillance with immediate relevance for tumor defense. In addition, Vγ9Vδ2 T cells also play a non-redundant role in the antimicrobial immune response through the selective recognition of bacterial metabolites and some viral antigens. Vγ9Vδ2 T cells frequently express activating and/or inhibitory NK cells receptors (NKRs) that can fine-tune their activation threshold and the activating NKG2D receptor is one of the most studied until now.

NKG2D has been reported to be a powerful co-stimulus for Ag-mediated activation of CD8+ and Vγ9Vδ2 T cells. The aim of the study was determine the contribution of NKG2D to the activation of Vγ9Vδ2 T cells. The experiments presented in this work describe in detail the modulation of different parameters of activation such as antigen-presenting molecules, activation markers, cytokines and chemokines, the cytolytic capacity against target-bearing NKG2D ligands cells and finally value data about the NKG2D-intracellular signaling in Vγ9Vδ2 T cells. In this thesis [data partially published (Rincon-Orozco, Kunzmann et al. 2005)] we demonstrate that NKG2D is able to partially activate Vγ9Vδ2 T cells through the association with DAP10 adapter molecules and independently of TCR-Ag recognition, similar to the NKG2D-mediated activation of NK cells. All together, this information contributes to the better understanding on how Vγ9Vδ2 T cells recognize and help in the control tumor or infected cells.

5.1 Modulation of the memory/effector phenotype on Vγ9Vδ2 T cells after in vitro stimulation with TCR-specific antigen and regulation of activation markers (CD69 and CD25) on Vγ9Vδ2 T cells after NKG2D stimulation.

High percentages of the peripheral Vγ9Vδ2 T cells used in this study exhibit the central memory T cells phenotype CD27+ CD45RO+. Previously, it was reported that the central memory T cells phenotype is characterized by the expression of lymph nodes homing receptors such as CCR7, CCR5, CXCR3 and CD62L, and lacking the immediate effectors functions such as cytokine production (IFN-γ) and cytotoxicity (Dieli, Poccia et al. 2003). Moreover, a significant percentage of the peripherical Vγ9Vδ2 T cells display the terminal differentiated phenotype CD27- CD45RO+, that is characterized by the expression of

receptors for migration to inflamed tissues and exhibit immediate effectors functions (proliferation, BLT-esterase release, IFN-γ production) (Dieli, Poccia et al. 2003).

The peripheral Vγ9Vδ2 T cells obtained from different donors used in this work, express intermediate levels of the activation markers CD69, and CD25; this scenario is likely due to the continuous long life antigenic load as a consequence of the ubiquitous nature of exogenous and endogenous prenylpyrophosphate that could lead to the accumulation of Vγ9Vδ2 T cell effectors (Bukowski, Morita et al. 1999; Kamath, Wang et al. 2003). Subsequently stimulation with NKG2D-specific mAb 149810 of resting Vγ9Vδ2 T cells from PBLs, led to the up-regulation of the activation markers CD69 (24 h stimulation) and CD25 (96 h stimulation) in Vγ9Vδ2 T cells similar to NK cells in the presence of IL-2; moreover, this effect is not observed for CD8 cells, proposing, that NKG2D has an IL-2 dependent stimulatory role on Vγ9Vδ2 T cells.

However, we observed different levels of CD69 and CD25 expression between the fresh isolated Vγ9Vδ2 T cells and the 24 h or 96 h incubated Vγ9Vδ2 T cells without stimuli, these differences probably are due to two different reasons: first, the variability of expression between donors of the CD69 and CD25 markers (Colonna-Romano, Potestio et al. 2002); second, the Vγ9Vδ2 T cells used in the experiment of NKG2D stimulation were cultured for 24 h or 96 h in RPMI in absence of TCR-antigens, this condition can lead to a different resting state of Vγ9Vδ2 T cells.

Activated T cells, but not resting (αβ) T lymphocytes are characterized by low levels of CD95 expression and by resistance to CD95 (Fas)-mediated apoptosis. Quite differently, Vγ9Vδ2 T cells from peripheral blood showed a rather high level of constitutive CD95 expression, which correlated with the rather high level of CD69 and CD25 on these cells, and may indicate a constant stimulation by persistent antigens.

It has been reported that activated T lymphocytes become progressively more sensitive to CD95-mediated apoptosis, due to the up-regulation of both CD95 and CD95-ligand expression (Klas, Debatin et al. 1993; Latinis, Carr et al. 1997) and that such up-regulation may provide a fail safe mechanism for prevention of inappropriate T cell activation (Li, Bassiri et al. 1998; Cipriani, Fulminis et al. 2006). Therefore, it will be of interest to analyze

expression of CD95 and its ligand on resting and activated Vγ9Vδ2 T cells in detail and the sensitivity of the respective Vγ9Vδ2 T cells to CD95 mediated apoptosis.

Moreover, the peripheral Vγ9Vδ2 T cells, highly express NK cell associated markers such as CD56 and NKG2D (Battistini, Borsellino et al. 1997; Bossowski, Urban et al. 2003), however the CD16 marker exhibits high variability of expression on Vγ9Vδ2 T cells, between the donors. Previously, it has been reported that this subset of Vγ9Vδ2 T CD16+ exhibits high levels of KIRs and are potent cytotoxic effectors cells, but loss their ability to produce high amounts of cytokines (Bodman-Smith, Anand et al. 2000; Lafont, Liautard et al. 2001; Bossowski, Urban et al. 2003; Angelini, Borsellino et al. 2004). Furthermore, the level of expression of the co-receptor CD28 indicates that a high proportion of peripheral Vγ9Vδ2 T cells co-express CD28 and NKG2D molecules. These receptors can interact with their corresponding ligands; e.g.: a) NKG2D with the tumoral or infected cells that express NKG2D ligands (Groh, Rhinehart et al. 1999; Rincon-Orozco, Kunzmann et al. 2005; Smyth, Swann et al. 2005; Upshaw and Leibson 2006). b) CD28 with molecules expressed by the APC; such the case of immature dendritic cells by inducing the maturation of the dendritic cell and the correspondent fully activation of the Vγ9Vδ2 T cells (Munz, Steinman et al. 2005; Shrestha, Ida et al. 2005; Devilder, Maillet et al. 2006). Finally, a small proportion of Vγ9Vδ2 T cells express the phenotype of CD28+ NKG2D- or CD28- NKG2D+, but the specific characteristics of this subset of cells remain unknown.

Subsequently, the *in vitro* stimulation of PBLs with specific TCR-antigens IPP or HPPBr in presence of IL-2, led to a massive and specific expansion of the number of Vγ9Vδ2 T cells (Vγ9Vδ2 T-STL). A decreased percentage of these Vγ9Vδ2 T-STL cells have a central memory phenotype (Vγ9Vδ2 T cells CD27+ CD45RO+), while a corresponding increased percentage have a terminally differentiated phenotype (Vγ9Vδ2 T cells CD27- CD45RO+), compared with fresh isolated Vγ9Vδ2 T cells. In addition, the levels of the early activator marker CD69 are decreased (resting Vγ9Vδ2 T cells 35.9% - IPP activated Vγ9Vδ2 T cells 5.6%) and the level of the late activation marker CD25 is slightly increased (resting Vγ9Vδ2 T cells 41.5% - IPP activated Vγ9Vδ2 T cells 63.4%).

Additionally a strong down regulation in the NK associated markers CD16 and CD56 was observed, indicating a polarization in Vγ9Vδ2 T cells to the cytokine producing phenotype

after the *in vitro* stimulation. Moreover, the level of NKG2D was not affected; however, the percentage of expression of the co-stimulatory molecule CD28 was slightly decreased, showing a selective proliferation of CD28- NKG2D+ Vγ9Vδ2 T cells over the CD28+ NKG2D- Vγ9Vδ2 T cells or a down-modulation of the CD28 expression on Vγ9Vδ2 T cells.

Altogether, these results suggest that phenotype and function of Vγ9Vδ2 T cells can be manipulated under certain conditions of *in vitro* stimulation, such as the cytokine-environment e.g. IL-2, IL-7 or IL-15 plus specific TCR stimulation by specific phosphoantigen with or without NKG2D stimulation. In addition, human Vγ9Vδ2 memory T cells may have different patterns of proliferation and differentiation potentials that can be approached for *in vitro* and *in vivo* generation of terminally differentiated Vγ9Vδ2 T cells that can be use in therapeutic protocols against cancer or infections.

5.2 NKG2D stimulation can modulate the antigen-presenting molecules on Vγ9Vδ2 T cells.

Vγ9Vδ2 T cells stimulated with TCR specific antigen, can up-regulate essential B-cell co-stimulatory ligands such as CD40L, OX40, CD70, and ICOS. These co-receptors are functional, as evidenced by the strong B-cell help provided by the γδ T cells (Brandes, Willimann et al. 2003). Additionally to the B-cell help, it has been reported that IPP-activated Vγ9Vδ2 T cells also acquire the ability to function as professional APCs (Vγ9Vδ2 T-APC) for naive αβ T cells, by the up-regulation of MHC class II and co-stimulatory molecules, such as CD80 and CD86, to level comparables with those present in dendritic cells. These Vγ9Vδ2 T-APC were able to up-take and process soluble antigens and present and activate CD4+ and CD8+ cells (Brandes, Willimann et al. 2005).

We studied if NKG2D-ligation also modulates the expression of molecules involved in antigen-presentation (HLA-DR, CD1a), co-stimulation (CD40, CD80, and CD86), and adhesion (CD54) and used CD69, CD95 as activation markers (CD69, CD95). We observed that after co-culturing fresh PBLs with NKG2D ligands expressing cells (C1R-ULBP-2), the population of Vγ9Vδ2 T cells up-regulated HLA-DR, CD1a, CD86, CD54, CD69 and CD95, but not the co-stimulatory molecules CD40 and CD80. Nevertheless, it is necessary to confirm these preliminary results by using, for example, beads coated with anti-NKG2D mAb or recombinant fusion-proteins (Fc-MICA or ULBPs) to exclude the action of unknown molecules or TCR-ligands present in the B cell or on the Vγ9Vδ2 T cell. Indeed, it well may

be that the communication between B cells and Vγ9Vδ2 T cells is needed for the full differentiation of γδ T cells to Vγ9Vδ2 T-APC phenotype, and the isolation of Vγ9Vδ2 T-APC from human tonsils would be compatible with such notion (Brandes, Willimann et al. 2005). All together, these results suggest, that Vγ9Vδ2 T cells could have a role beyond that implied by the immediate release of cytokines and chemokines, and the killing of infected or tumoral cells after TCR or NKG2D activation. Vγ9Vδ2 T cells could also function to help or initiate adaptive αβ T cell responses by antigen presentation and B-cell responses by providing B-cell help.

Vγ9Vδ2 T-APC might complement DC in anti-microbial or tumoral immune responses, but probably are not able to substitute DCs due to the limited pinocytosis and phagocytic activity of Vγ9Vδ2 T cells and their inability to produce IL-12, which might not allow them to efficiently prime conventional Th1 cell directed against processed peptidic antigens. In addition, it remains to be analyzed whether they posses tolerogenic functions as its has been shown for some types of DC. In any case, it is necessary to design new *in vitro* experiments and describe *in vivo* animal models to confirm the role of Vγ9Vδ2 T-APC in the settings of infections or tumors, and to determine if NKG2D ligation can induce total or partial Vγ9Vδ2 T-APC phenotype.

5.3 NKG2D stimulation induces the production of cytokines/chemokines by Vγ9Vδ2 T cells.

The majority of Vγ9Vδ2 T cells are potent producers of type I cytokines, including IFN-γ, that mediates antiviral and immunoregulatory functions in the innate and adaptive phases of the immune response and TNF-α which is a potent proinflamatory cytokine, with an important role in the control of infections and tumors (Morita, Verma et al. 1991; Garcia, Sieling et al. 1997), as well as chemokines such as the macrophages inflammatory protein-1α (MIP-1α or CCL3), MIP-1β (CCL4), and RANTES (CCL-5) (Cipriani, Borsellino et al. 2000), that contribute to the direct recruitment of leukocytes with anti-microbial effectors functions, immune surveillance T cells and antigen-presenting cells precursors at the site of microbial infections or tumoral growth (Moser and Loetscher 2001; Moser and Willimann 2004; Moser, Wolf et al. 2004). Additionally, Vγ9Vδ2 T cells help to prevent the death of humanized-SCID mice from infections with Gram-negative rods (*E. coli* and *Morganella*

morganni) and Gram-positive cocci (*Staphylococcus aureus*) by rapid production of large amounts of IFN-γ (Wang, Kamath et al. 2001).

As already mentioned, NKG2D was originally described as a stimulatory receptor for NK cells, but its capacity to enhance activation of Vγ9Vδ2 T cells together with other activating receptors indicates that it also functions as a co-stimulatory molecule. The fact, that direct NKG2D-mediated activation of Vγ9Vδ2 T cells has not been described in earlier studies may be due to several reasons.

First, previous work has focused on the function of NKG2D as a co-stimulatory receptor, and measured its effect on IFN-γ production and proliferation. In our study we also did not observe IFN-γ production after ligation of NKG2D, as tested by intracellular FACS analysis and IFN-γ-ELISpot analysis; where, non significant numbers of IFN-γ-secreting Vγ9Vδ2 T cells were detected after NKG2D stimulation, however, a strong IFN-γ secretion was detected in all the probes where Vγ9Vδ2 T cells were TCR stimulated.

Previous reports suggest that the integrated signal from TLR3 and TCR together induce potent antiviral effector function in γδ T cells as evidenced by the strong IFN-γ secretion (Wesch, Beetz et al. 2006). We analyzed the possibility of IFN-γ induction by Vγ9Vδ2 T cells after the integrated stimulation of NKG2D and TLR3. No significative increase of IFN-γ production was observed, after TLR3-NKG2D stimulation, although a substantial increment of IFN-γ production was detected after TLR3-TCR stimulation, suggesting that the stimulation of TLR3 together with NKG2D do not affect the production of IFN-γ**.** Since TLR3 ligation in some cell types induces production of type I interferons, it would be of interest to study the possibility also for activated Vγ9Vδ2 T cells because it has been reported that type I interferons are important for the control of viral replication and some tumors.

One cytokine, where an increased frequency of producing cells was found after NKG2D ligation is TNF-α, which was produced after re-stimulation with NKG2D-specific mAb and recombinant MICA-Fc. Although these stimulations were weaker compared to that exerted by the positive control CD3 mAb, there were as strong as those found after co-culture with Daudi cells and IPP.

Additionally, the production of other cytokines and chemokines was analyzed on Vγ9Vδ2 T cells after 5 h stimulation by co-culturing with RMA-MICA cells. This stimulation evidenced a significant increase in the production of the chemokine MIP-1a. In contrast, a not significant increase of the production of IL-6, and chemokines such as RANTES, MCP-1 and GM-CSF was observed in Vγ9Vδ2 T cell lines after this short period of stimulation. Nonetheless, it is necessary to confirm these results by new protocols by using pure Vγ9Vδ2 T cells stimulate with beads coated with anti-NKG2D mAb or recombinant fusion-proteins (Fc-MICA or ULBPs), longer times of incubation (e.g. 24 h), together with more sensible techniques of detection, such as ELISA, ELISpot or Cytometric Bead Array (CBA), to found production/release of important cytokines or chemokines after NKG2D ligation and avoid the background induced by RMA-Neo cells in the previously experiments.

5.4 NKG2D mediated cytotoxicity

Vγ9Vδ2 T cells express an array of NK receptors, such as NKG2D, and it is likely that much of the tumor reactivity of Vγ9Vδ2 T cells is controlled by those receptors. Until very recently, NKG2D was considered exclusively as a co-stimulatory molecule for T cells. This interpretation, is now modified because some subtypes of *in vitro*-activated human CD8 cells are directly activated by NKG2D (Maccalli, Pende et al. 2003; Conejo-Garcia, Benencia et al. 2004; Verneris, Karami et al. 2004). Relevant to our work was the identification of *ex vivo* activated and expanded CD8+ T cells, which display MHC-unrestricted, TCR-independent, and NKG2D-mediated cytotoxicity against malignant targets cells (Maccalli, Pende et al. 2003). Additionally, it has been reported that in vitro-activated ovarian tumor-infiltrating CD8+ cells kill K562-FcγR+ cells loaded with ULBP4-Fc construct (Conejo-Garcia, Benencia et al. 2004). Finally, cytokine-induced killer CD8+ T cells generated by culture in the presence of 300 U/ml IL-2 have the capacity to lyse NKG2D ligand-expressing target cells in a NKG2D-dependent but TCR-independent manner. Interestingly, this lyses was not found in CD8+ T cells generated with 100 U/ml IL-2 for which DAP10 expression could not be detected (Verneris, Karami et al. 2004). In contrast, we show in our study that resting and activated Vγ9Vδ2 T cells express DAP10 and are stimulated by NKG2D ligation.

In our work, the induction of cytotoxic effector phenotype in Vγ9Vδ2 T cells after NKG2D ligation was evidenced by different methodologies. The preliminary assay consisted in the determination of cytolytic granules release by Vγ9Vδ2 T cells after NKG2D ligation, this

phenomenon was measured by three independent methods. First, modulation of CD107a on Vγ9Vδ2 T cells found in PBL; in which, the up-regulation of CD107a was observed after NKG2D engagement. Second, a uniform reduction in the content of perforin in Vγ9Vδ2 T-STL was found after stimulation with NKG2D mAb. Finally, exocytosis of granules with esterase activity (BLT assay) by Vγ9Vδ2 T cells was evidenced after stimulation with NKG2D mAb to be as efficient as CD3 stimulation. All together, these results, strongly suggest an important role of Vγ9Vδ2 T cells in the recognition and killing of target cells that express NKG2D ligands in infection or tumoral process.

The NKG2D-triggered cytotoxicity mediated by purified Vγ9Vδ2 T cells was determined using ^{51}Cr release and FATAL assays and different mouse and human cells transduced with NKG2D ligands as a target cells. This set of experiment demonstrated the specific NKG2D-mediated and TCR-independent killing by Vγ9Vδ2 T cells. Additionally, we studied the role of CD28 in the triggering of cytotoxicity by using redirected killing assay as read-out and two different class of anti-CD28 mAb (anti-CD28 conventional mAb and anti-CD28 superagonistic mAb) [previously reported superagonistic anti-CD28 mAb induced strong stimulation in T cells (Beyersdorf, Gaupp et al. 2005; Beyersdorf, Hanke et al. 2006)], whereas no significant redirected killing was found with the both CD28 mAb.

All these assays confirmed the specific and TCR-independent cytolytic mechanism mediated by NKG2D in Vγ9Vδ2 T cells. Nevertheless, there is still a discrepancy between our results on the lyses of MICA and ULBP2-expressing C1R cells and a report where such lyses was not found (Das, Groh et al. 2001). One possible explanation could be the class of effectors cells used in both studies, namely monoclonal *vs.* polyclonal γδ T cell populations. It appears quite possible that expression of KIR p58.1 by monoclonal effectors cells, which bind to HLA-Cw 0401 of C1R cells, could have inhibited lyses, although such effects would not be detected with a polyclonal effectors cell population, where only a minority of cells express p58.1 (Rothenfusser, Buchwald et al. 2002).

In addition, it has been reported that other activating natural receptors such as NKp44 that present cytotoxic activity against melanoma cells, support a functional role in cytotoxicity of these molecules (von Lilienfeld-Toal, Nattermann et al. 2006). Moreover to activating NK receptors, cytotoxicity could also be facilitated by down-regulation of MHC class I on certain tumors, such as Daudi, because this would make the tumors targets for the γδ T cells

expressing inhibitory NK receptors specific for certain alleles of MHC class I such as CD94/NKG2A/B and killer immunoglobulin-like receptors (Carena, Shamshiev et al. 1997; Fisch, Meuer et al. 1997; Poccia, Cipriani et al. 1997). These iNKRs may ensure efficient discrimination of transformed versus normal cells by Vγ9Vδ2 T lymphocytes.

Altogether, these results suggest that an alternative way to boost the activation of Vγ9Vδ2 T cell would be the co-ligation of NKG2D with other "co-receptors" like CD28. In the case of CD28, which shows some similarity to NKG2D with respect to signaling, combinations of mAb specific for CD28 and CD27 (Sunder-Plassmann, Pickl et al. 1995) or CD28 and CD2 (Van Lier, Brouwer et al. 1988) activate (αβ) T cells rather efficiently. It is now tempting to speculate that similar regimes could induce substantial NKG2D-triggered and Ag-independent activation of Vγ9Vδ2 T cells, indicating that Vγ9Vδ2 T cells recognized tumor cells through both TCR and non-TCR receptors, and that the recognition through activating and inhibitory NK receptors is probably one of the more important determinants of tumor recognition by Vγ9Vδ2 T cells. In this way γδ T cells function as a bridge cells with characteristics of both innate NK cells and adaptive αβ T cells.

5.5 Analysis of the NKG2D signaling pathway in Vγ9Vδ2 T cells

The studies about human NKG2D signaling have been performed mainly in NK cells, where NKG2D exclusively uses the adaptor molecule DAP-10 but not DAP-12 for surface expression and intracellular signaling (Wu, Song et al. 1999; Rosen, Araki et al. 2004). DAP-10 lacks the ITAM motif but instead contains a YINM motif, which closely resembles those found in co-stimulatory molecules such as CD28 (Diefenbach, Tomasello et al. 2002). The association of NKG2D with these adaptors occurs non-covalently, via oppositely charged amino-acids in the respective transmembrane domain (Wu, Song et al. 1999; Diefenbach, Tomasello et al. 2002).

The NKG2D receptor complex is an hexameric structure consisting of two NKG2D molecules, each of them associated to a DAP-10 homodimer to form a hexameric complex (Garrity, Call et al. 2005). Upon ligand binding, DAP-10 is phosphorylated by a Src family kinase and subsequently leads to at least three different reported ways, in our experiments the use of specific Src inhibitor (PP2) abrogated up to 50% of the NKG2D-mediated killing by

Vγ9Vδ2 T cells, indicating the important role of these Src family kinase in the initiation of the cytotoxic machinery mediated by NKG2D.

The first NKG2D signaling pathway reported in human NK cells is the DAP-10–PI3K pathway; the NKG2D trigger leads to the recruitment of the p85 subunit of PI3-K (Wu, Song et al. 1999), and subsequently Akt phosphorylation, which presumably activates cell survival pathways (Chang, Dietrich et al. 1999; Sutherland, Chalupny et al. 2002). PI3K is also required for optimal NKG2D-mediated calcium release (Upshaw, Arneson et al. 2006) and cytotoxicity (Billadeau, Upshaw et al. 2003; Meresse, Chen et al. 2004; Upshaw, Arneson et al. 2006). In our study, the inhibition of PI3K in Vγ9Vδ2 T cells by a specific inhibitor (Ly294002) showed a strong suppression of the NKG2D-mediated cytotoxic activity, indicating the important role of PI3K in the Vγ9Vδ2T cells-NKG2D pathway. Additionally, is important to complement this data with further analysis such as calcium flux induction after NKG2D ligation in Vγ9Vδ2 T cells, in order to corroborate possible similarities or differences between Vγ9Vδ2 T cells and NK cells.

NKG2D-initiated PI3K activation leads also to MEK and ERK phosphorylation, which are required for cytotoxicity (Sutherland, Chalupny et al. 2002; Meresse, Chen et al. 2004). Thus, it is possible that DAP10-mediated PI3K activation initiates a PI3K → Rac1 → PAK1 → MEK → ERK pathway that was shown to be necessary for non-NKG2D initiated NK cell-mediated cytotoxicity (Jiang, Zhong et al. 2000; Jiang, Zhong et al. 2002). Our results indicated a rapid and maintained phosphorylation of ERK after NKG2D stimulation in Vγ9Vδ2 T cells. Also a slight inhibition of the killing was found in Vγ9Vδ2 T cells treated with p38 inhibitors, suggesting the contribution of these two component of the MAPKs family (p38 and ERK kinases) (Johnson and Lapadat 2002) in the killing of target-bearing NKG2D ligands cells by Vγ9Vδ2 T cells. Finally, the pharmacological inhibition of MEK (PD98059) in Vγ9Vδ2 T cells does not abrogate the NKG2D mediated killing effect against RMA-MICA cells indicating some differences in the DAP-10-PI3K pathway between NK cells and Vγ9Vδ2 T cells.

The second pathway is denominated DAP-10-Grb2 pathway, where Grb2 directly bind to phosphorylated DAP-10 (Chang, Dietrich et al. 1999). Both p85 and Grb2 have to be recruited to DAP-10 for full calcium flux and cell-mediated cytotoxicity (Upshaw, Arneson et al. 2006). The recruitment of Grb2 provides a link to the phosphorylation of SLP-76, PLC-γ2

and Vav1. Furthermore, as shown in this thesis the treatment of Vγ9Vδ2 T cells with PLC-γ 1/2 (U-73122) inhibitor strongly inhibited killing; in addition a rapid but weak phosphorylation of PLCγ-1 is observed after stimulation of NKG2D in Vγ9Vδ2 T cells. Nevertheless, before drawing further parallels between NKG2D signaling with respect NK and Vγ9Vδ2 T cells mediated cytotoxicity it will be necessary to analyze the patterns of phosphorylation of PLC-γ 2, SLP76 and Vav1 that are reported preferentially activated after NKG2D trigger in NK cells.

An interesting difference with respect to NKG2D mediated cytotoxicity between Vγ9Vδ2 T cells and other cells may lay in the PI3K-independ JNK → cJunN pathway, which is required for NKG2D-mediated cytotoxicity in IL-15 activated CTLs from celiac patients (Meresse, Chen et al. 2004). Our pharmacological inhibition assays suggest that JNK is not implicated in the NKG2D mediated killing of target cells by IL-2 stimulated Vγ9Vδ2 T cells, suggesting that different *in vitro* culturing and stimulation conditions (e.g. IL-15) or the different cellular origin (Vγ9Vδ2 T cells vs. CD8 T cells) can precipitate in differential signaling pathways of NKG2D induced cytotoxicity.

All these data provide a first biochemical basis for an understanding on how NKG2D-dependent lyses occur independently from ITAM signaling and for the design of new protocols of *in vitro* activation of Vγ9Vδ2 T cells for the use in patients suffering certain classes of tumors.

5.6 Concluding remarks

There are several reasons to believe that the NKG2D recognition system represents a promising entry point to induce and/or improve immune responses in cancer. First, NKG2D ligands are generally poorly and only transiently expressed on healthy tissues, while they are constitutively expressed at significant levels on a broad variety of tumor cells of distinct tissue origins. Second, in situations where NKG2D ligands are poorly expressed, it may be possible to enhance their expression using radiation- and/or chemotherapies. Third, NKG2D is expressed on all NK cells, CD8+ T cells and Vγ9Vδ2 T cells, and our work indicates that the effects on NKG2D ligation are remarkably similar for Vγ9Vδ2 T cells and NK cells (Rincon-Orozco, Kunzmann et al. 2005), suggesting that activated Vγ9Vδ2 T cells may take over functions of NK cells in destroying NKG2D ligand-expressing tumor or infected cells. This

special quality provides the possibility to obtain a large number of potential autologous effectors Vγ9Vδ2 T cells with improved NKG2D and TCR function to be used in different protocols for treatment of cancer or chronical infections such as tuberculosis.

Finally, NKG2D-mediated adoptive immunotherapy should be applicable to all individuals as the NKG2D receptor is monomorphic. Therefore, it may be possible in the future to fully exploit the fact that diseased cells have the capacity to alert the immune system, in order to boost immune recognition of cancer.

6 REFERENCES

Adams, E. J., Y.-H. Chien, et al. (2005). "Structure of a {gamma}{delta} T Cell Receptor in Complex with the Nonclassical MHC T22." Science **308**(5719): 227-231.

Agerberth, B., J. Charo, et al. (2000). "The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations." Blood **96**(9): 3086-93.

Agrati, C., T. Alonzi, et al. (2006). "Activation of Vgamma9Vdelta2 T cells by non-peptidic antigens induces the inhibition of subgenomic HCV replication." Int Immunol **18**(1): 11-8.

Agrati, C., C. Castilletti, et al. (2006). "Interferon-gamma-mediated antiviral immunity against orthopoxvirus infection is provided by gamma delta T cells." J Infect Dis **193**(11): 1606-7; author reply 1607-8.

Allison, T. J., C. C. Winter, et al. (2001). "Structure of a human gammadelta T-cell antigen receptor." Nature **411**(6839): 820-4.

Andre, P., R. Castriconi, et al. (2004). "Comparative analysis of human NK cell activation induced by NKG2D and natural cytotoxicity receptors." Eur J Immunol **34**(4): 961-71.

Angelini, D. F., G. Borsellino, et al. (2004). "FcgammaRIII discriminates between 2 subsets of Vgamma9Vdelta2 effector cells with different responses and activation pathways." Blood **104**(6): 1801-7.

Asslan, R., A. Pradines, et al. (1999). "Epidermal growth factor stimulates 3-hydroxy-3-methylglutaryl-coenzyme A reductase expression via the ErbB-2 pathway in human breast adenocarcinoma cells." Biochem Biophys Res Commun **260**(3): 699-706.

Bahram, S., H. Inoko, et al. (2005). "MIC and other NKG2D ligands: from none to too many." Curr Opin Immunol **17**(5): 505-9.

Baiocchi, R. A., J. S. Ward, et al. (2001). "GM-CSF and IL-2 induce specific cellular immunity and provide protection against Epstein-Barr virus lymphoproliferative disorder." J Clin Invest **108**(6): 887-94.

Balbi, B., M. T. Valle, et al. (1993). "T-lymphocytes with gamma delta+ V delta 2+ antigen receptors are present in increased proportions in a fraction of patients with tuberculosis or with sarcoidosis." Am Rev Respir Dis **148**(6 Pt 1): 1685-90.

Battistini, L., G. Borsellino, et al. (1997). "Phenotypic and cytokine analysis of human peripheral blood gamma delta T cells expressing NK cell receptors." J Immunol **159**(8): 3723-30.

Bauer, S., V. Groh, et al. (1999). "Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA." Science **285**(5428): 727-9.

Belmant, C., E. Espinosa, et al. (1999). "3-Formyl-1-butyl pyrophosphate A novel mycobacterial metabolite-activating human gammadelta T cells." J Biol Chem **274**(45): 32079-84.

Bertotto, A., R. Gerli, et al. (1993). "Lymphocytes bearing the gamma delta T cell receptor in acute Brucella melitensis infection." Eur J Immunol **23**(5): 1177-80.

Beyersdorf, N., S. Gaupp, et al. (2005). "Selective targeting of regulatory T cells with CD28 superagonists allows effective therapy of experimental autoimmune encephalomyelitis." J Exp Med **202**(3): 445-55.

Beyersdorf, N., T. Hanke, et al. (2006). "CD28 superagonists put a break on autoimmunity by preferentially activating CD4+CD25+ regulatory T cells." Autoimmun Rev **5**(1): 40-5.

Bieback, K., C. Breer, et al. (2003). "Expansion of human gamma/delta T cells in vitro is differentially regulated by the measles virus glycoproteins." J Gen Virol **84**(Pt 5): 1179-88.

Billadeau, D. D., J. L. Upshaw, et al. (2003). "NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway." Nat Immunol **4**(6): 557-64.

Bodman-Smith, M. D., A. Anand, et al. (2000). "Decreased expression of FcgammaRIII (CD16) by gammadelta T cells in patients with rheumatoid arthritis." Immunology **99**(4): 498-503.

Bonneville, M. and J. J. Fournie (2005). "Sensing cell stress and transformation through Vgamma9Vdelta2 T cell-mediated recognition of the isoprenoid pathway metabolites." Microbes Infect **7**(3): 503-9.

Boom, W. H., K. A. Chervenak, et al. (1992). "Role of the mononuclear phagocyte as an antigen-presenting cell for human gamma delta T cells activated by live Mycobacterium tuberculosis." Infect Immun **60**(9): 3480-8.

Born, W. K., C. L. Reardon, et al. (2006). "The function of gammadelta T cells in innate immunity." Curr Opin Immunol **18**(1): 31-8.

Bossowski, A., M. Urban, et al. (2003). "Analysis of circulating T gamma/delta lymphocytes and CD16/56 cell populations in children and adolescents with Graves' disease." Pediatr Res **54**(3): 425-9.

Bovolenta, C., A. L. Lorini, et al. (1999). "A selective defect of IFN-gamma- but not of IFN-alpha-induced JAK/STAT pathway in a subset of U937 clones prevents the antiretroviral effect of IFN-gamma against HIV-1." J Immunol **162**(1): 323-30.

Brandes, M., K. Willimann, et al. (2003). "Flexible migration program regulates gamma delta T-cell involvement in humoral immunity." Blood **102**(10): 3693-701.

6. REFERENCES

Brandes, M., K. Willimann, et al. (2005). "Professional antigen-presentation function by human gammadelta T Cells." Science **309**(5732): 264-8.

Bukowski, J. F., C. T. Morita, et al. (1998). "Crucial role of TCR gamma chain junctional region in prenyl pyrophosphate antigen recognition by gamma delta T cells." J Immunol **161**(1): 286-93.

Bukowski, J. F., C. T. Morita, et al. (1994). "Recognition and destruction of virus-infected cells by human gamma delta CTL." J Immunol **153**(11): 5133-40.

Bukowski, J. F., C. T. Morita, et al. (1999). "Human gamma delta T cells recognize alkylamines derived from microbes, edible plants, and tea: implications for innate immunity." Immunity **11**(1): 57-65.

Caldwell, C. W., E. D. Everett, et al. (1995). "Lymphocytosis of gamma/delta T cells in human ehrlichiosis." Am J Clin Pathol **103**(6): 761-6.

Capobianchi, M. R., I. Abbate, et al. (2003). "HCV and interferon: viral strategies for evading innate defence mechanisms in the virus-host battle." Cell Death Differ **10 Suppl 1**: S22-4.

Carding, S. R. and P. J. Egan (2000). "The importance of gamma delta T cells in the resolution of pathogen-induced inflammatory immune responses." Immunol Rev **173**: 98-108.

Carding, S. R. and P. J. Egan (2002). "Gammadelta T cells: functional plasticity and heterogeneity." Nat Rev Immunol **2**(5): 336-45.

Carena, I., A. Shamshiev, et al. (1997). "Major histocompatibility complex class I molecules modulate activation threshold and early signaling of T cell antigen receptor-gamma/delta stimulated by nonpeptidic ligands." J Exp Med **186**(10): 1769-74.

Carvalho, A. C., A. Matteelli, et al. (2002). "gammadelta T lymphocytes in the peripheral blood of patients with tuberculosis with and without HIV co-infection." Thorax **57**(4): 357-60.

Chang, C., J. Dietrich, et al. (1999). "Cutting edge: KAP10, a novel transmembrane adapter protein genetically linked to DAP12 but with unique signaling properties." J Immunol **163**(9): 4651-4.

Chen, J., H. Niu, et al. (2001). "Antitumor activity of expanded human tumor-infiltrating gammadelta T lymphocytes." Int Arch Allergy Immunol **125**(3): 256-63.

Chen, Z. W. and N. L. Letvin (2003). "Vgamma2Vdelta2+ T cells and anti-microbial immune responses." Microbes Infect **5**(6): 491-8.

Cipriani, B., G. Borsellino, et al. (2000). "Activation of C-C beta-chemokines in human peripheral blood gammadelta T cells by isopentenyl pyrophosphate and regulation by cytokines." Blood **95**(1): 39-47.

Cipriani, P., A. Fulminis, et al. (2006). "Resistance to apoptosis in circulating alpha/beta and gamma/delta T lymphocytes from patients with systemic sclerosis." J Rheumatol **33**(10): 2003-14.

Colonna-Romano, G., M. Potestio, et al. (2002). "Gamma/delta T lymphocytes are affected in the elderly." Exp Gerontol **37**(2-3): 205-11.

Conejo-Garcia, J. R., F. Benencia, et al. (2004). "Ovarian carcinoma expresses the NKG2D ligand Letal and promotes the survival and expansion of CD28- antitumor T cells." Cancer Res **64**(6): 2175-82.

Constant, P., F. Davodeau, et al. (1994). "Stimulation of human gamma delta T cells by nonpeptidic mycobacterial ligands." Science **264**(5156): 267-70.

Daher, K. A., M. E. Selsted, et al. (1986). "Direct inactivation of viruses by human granulocyte defensins." J Virol **60**(3): 1068-74.

Das, H., V. Groh, et al. (2001). "MICA engagement by human Vgamma2Vdelta2 T cells enhances their antigen-dependent effector function." Immunity **15**(1): 83-93.

Das, H., L. Wang, et al. (2001). "Vgamma2Vdelta2 T-cell receptor-mediated recognition of aminobisphosphonates." Blood **98**(5): 1616-8.

De Paoli, P., D. Gennari, et al. (1990). "Gamma delta T cell receptor-bearing lymphocytes during Epstein-Barr virus infection." J Infect Dis **161**(5): 1013-6.

Dechanet, J., P. Merville, et al. (1999). "Implication of gammadelta T cells in the human immune response to cytomegalovirus." J Clin Invest **103**(10): 1437-49.

Deetz, C. O., A. M. Hebbeler, et al. (2006). "Gamma interferon secretion by human Vgamma2Vdelta2 T cells after stimulation with antibody against the T-cell receptor plus the Toll-Like receptor 2 agonist Pam3Cys." Infect Immun **74**(8): 4505-11.

Delgado, J. C., E. Y. Tsai, et al. (2002). "Antigen-specific and persistent tuberculin anergy in a cohort of pulmonary tuberculosis patients from rural Cambodia." Proc Natl Acad Sci U S A **99**(11): 7576-81.

DeVico, A. L. and R. C. Gallo (2004). "Control of HIV-1 infection by soluble factors of the immune response." Nat Rev Microbiol **2**(5): 401-13.

Devilder, M. C., S. Maillet, et al. (2006). "Potentiation of antigen-stimulated V gamma 9V delta 2 T cell cytokine production by immature dendritic cells (DC) and reciprocal effect on DC maturation." J Immunol **176**(3): 1386-93.

Diefenbach, A. and D. H. Raulet (2003). "Innate immune recognition by stimulatory immunoreceptors." Curr Opin Immunol **15**(1): 37-44.

Diefenbach, A., E. Tomasello, et al. (2002). "Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D." Nat Immunol **3**(12): 1142-9.

6. REFERENCES

Dieli, F., F. Poccia, et al. (2003). "Differentiation of effector/memory Vdelta2 T cells and migratory routes in lymph nodes or inflammatory sites." J Exp Med **198**(3): 391-7.

Dieli, F., G. Sireci, et al. (2002). "Selective depression of interferon-gamma and granulysin production with increase of proliferative response by Vgamma9/Vdelta2 T cells in children with tuberculosis." J Infect Dis **186**(12): 1835-9.

Dieli, F., M. Troye-Blomberg, et al. (2000). "Vgamma9/Vdelta2 T lymphocytes reduce the viability of intracellular Mycobacterium tuberculosis." Eur J Immunol **30**(5): 1512-9.

Dieli, F., M. Troye-Blomberg, et al. (2001). "Granulysin-dependent killing of intracellular and extracellular Mycobacterium tuberculosis by Vgamma9/Vdelta2 T lymphocytes." J Infect Dis **184**(8): 1082-5.

Eberl, M., B. Altincicek, et al. (2002). "Accumulation of a potent gammadelta T-cell stimulator after deletion of the lytB gene in Escherichia coli." Immunology **106**(2): 200-11.

Eberl, M., R. Engel, et al. (2005). "Human Vgamma9/Vdelta2 effector memory T cells express the killer cell lectin-like receptor G1 (KLRG1)." J Leukoc Biol **77**(1): 67-70.

Eberl, M., M. Hintz, et al. (2003). "Microbial isoprenoid biosynthesis and human gammadelta T cell activation." FEBS Lett **544**(1-3): 4-10.

Ensslin, A. S. and B. Formby (1991). "Comparison of cytolytic and proliferative activities of human gamma delta and alpha beta T cells from peripheral blood against various human tumor cell lines." J Natl Cancer Inst **83**(21): 1564-9.

Feurle, J., E. Espinosa, et al. (2002). "Escherichia coli produces phosphoantigens activating human gamma delta T cells." J Biol Chem **277**(1): 148-54.

Fisch, P., M. Malkovsky, et al. (1990). "Recognition by human V gamma 9/V delta 2 T cells of a GroEL homolog on Daudi Burkitt's lymphoma cells." Science **250**(4985): 1269-73.

Fisch, P., E. Meuer, et al. (1997). "Control of B cell lymphoma recognition via natural killer inhibitory receptors implies a role for human Vgamma9/Vdelta2 T cells in tumor immunity." Eur J Immunol **27**(12): 3368-79.

Fu, Z., M. Wang, et al. (2002). "The structure of a binary complex between a mammalian mevalonate kinase and ATP: insights into the reaction mechanism and human inherited disease." J Biol Chem **277**(20): 18134-42.

Furukawa, S., K. Sasai, et al. (1992). "Increase in T cells expressing the gamma/delta receptor and CD4+CD8+ double-positive T cells in primary immunodeficiency complicated by human T-cell lymphotropic virus type I infection." Blood **80**(12): 3253-5.

Gan, Y. H., C. D. Pauza, et al. (1995). "Gamma delta T cells in rhesus monkeys and their response to simian immunodeficiency virus (SIV) infection." Clin Exp Immunol **102**(2): 251-5.

Gao, Y., W. Yang, et al. (2003). "Gamma delta T cells provide an early source of interferon gamma in tumor immunity." J Exp Med **198**(3): 433-42.

Garcia, V. E., P. A. Sieling, et al. (1997). "Single-cell cytokine analysis of gamma delta T cell responses to nonpeptide mycobacterial antigens." J Immunol **159**(3): 1328-35.

Garrity, D., M. E. Call, et al. (2005). "The activating NKG2D receptor assembles in the membrane with two signaling dimers into a hexameric structure." Proc Natl Acad Sci U S A **102**(21): 7641-6.

Gilfillan, S., E. L. Ho, et al. (2002). "NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation." Nat Immunol **3**(12): 1150-5.

Gioia, C., C. Agrati, et al. (2002). "Lack of CD27-CD45RA-V gamma 9V delta 2+ T cell effectors in immunocompromised hosts and during active pulmonary tuberculosis." J Immunol **168**(3): 1484-9.

Gioia, C., C. Agrati, et al. (2003). "Different cytokine production and effector/memory dynamics of alpha beta+ or gamma delta+ T-cell subsets in the peripheral blood of patients with active pulmonary tuberculosis." Int J Immunopathol Pharmacol **16**(3): 247-52.

Girardi, M., D. E. Oppenheim, et al. (2001). "Regulation of cutaneous malignancy by gammadelta T cells." Science **294**(5542): 605-9.

Glatzel, A., D. Wesch, et al. (2002). "Patterns of chemokine receptor expression on peripheral blood gamma delta T lymphocytes: strong expression of CCR5 is a selective feature of V delta 2/V gamma 9 gamma delta T cells." J Immunol **168**(10): 4920-9.

Gober, H. J., M. Kistowska, et al. (2003). "Human T cell receptor gammadelta cells recognize endogenous mevalonate metabolites in tumor cells." J Exp Med **197**(2): 163-8.

Groh, V., A. Bruhl, et al. (2003). "Stimulation of T cell autoreactivity by anomalous expression of NKG2D and its MIC ligands in rheumatoid arthritis." Proc Natl Acad Sci U S A **100**(16): 9452-7.

Groh, V., R. Rhinehart, et al. (2001). "Costimulation of CD8alphabeta T cells by NKG2D via engagement by MIC induced on virus-infected cells." Nat Immunol **2**(3): 255-60.

Groh, V., R. Rhinehart, et al. (1999). "Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB." Proc Natl Acad Sci U S A **96**(12): 6879-84.

Groh, V., A. Steinle, et al. (1998). "Recognition of stress-induced MHC molecules by intestinal epithelial gammadelta T cells." Science **279**(5357): 1737-40.

Groh, V., J. Wu, et al. (2002). "Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation." Nature **419**(6908): 734-8.
Gueddari, N., G. Favre, et al. (1993). "Evidence for up-regulated low density lipoprotein receptor in human lung adenocarcinoma cell line A549." Biochimie **75**(9): 811-9.
Hampton, R. Y. (2002). "Proteolysis and sterol regulation." Annu Rev Cell Dev Biol **18**: 345-78.
Hara, T., Y. Mizuno, et al. (1992). "Predominant activation and expansion of V gamma 9-bearing gamma delta T cells in vivo as well as in vitro in Salmonella infection." J Clin Invest **90**(1): 204-10.
Harwood, H. J., Jr., I. M. Alvarez, et al. (1991). "In vivo regulation of human leukocyte 3-hydroxy-3-methylglutaryl coenzyme A reductase: increased enzyme protein concentration and catalytic efficiency in human leukemia and lymphoma." J Lipid Res **32**(8): 1237-52.
Hayday, A. and R. Tigelaar (2003). "Immunoregulation in the tissues by gammadelta T cells." Nat Rev Immunol **3**(3): 233-42.
Hayday, A. C. (2000). "[gamma][delta] cells: a right time and a right place for a conserved third way of protection." Annu Rev Immunol **18**: 975-1026.
Heilig, J. S. and S. Tonegawa (1986). "Diversity of murine gamma genes and expression in fetal and adult T lymphocytes." Nature **322**(6082): 836-40.
Hintz, M., A. Reichenberg, et al. (2001). "Identification of (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate as a major activator for human gammadelta T cells in Escherichia coli." FEBS Lett **509**(2): 317-22.
Ho, M., H. K. Webster, et al. (1990). "Increased gamma delta T cells in acute Plasmodium falciparum malaria." Immunol Lett **25**(1-3): 139-41.
Holoshitz, J. (1999). "Activation of gammadelta T cells by mycobacterial antigens in rheumatoid arthritis." Microbes Infect **1**(3): 197-202.
Holoshitz, J., L. M. Vila, et al. (1992). "Dual antigenic recognition by cloned human gamma delta T cells." J Clin Invest **89**(1): 308-14.
Houten, S. M., M. S. Schneiders, et al. (2003). "Regulation of isoprenoid/cholesterol biosynthesis in cells from mevalonate kinase-deficient patients." J Biol Chem **278**(8): 5736-43.
Jameson, J., D. Witherden, et al. (2003). "T-cell effector mechanisms: gammadelta and CD1d-restricted subsets." Curr Opin Immunol **15**(3): 349-53.
Jamieson, A. M., A. Diefenbach, et al. (2002). "The role of the NKG2D immunoreceptor in immune cell activation and natural killing." Immunity **17**(1): 19-29.
Janssen, O., S. Wesselborg, et al. (1991). "T cell receptor/CD3-signaling induces death by apoptosis in human T cell receptor gamma delta + T cells." J Immunol **146**(1): 35-9.
Jiang, K., B. Zhong, et al. (2000). "Pivotal role of phosphoinositide-3 kinase in regulation of cytotoxicity in natural killer cells." Nat Immunol **1**(5): 419-25.
Jiang, K., B. Zhong, et al. (2002). "Syk regulation of phosphoinositide 3-kinase-dependent NK cell function." J Immunol **168**(7): 3155-64.
Johnson, G. L. and R. Lapadat (2002). "Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases." Science **298**(5600): 1911-2.
Jomaa, H., J. Feurle, et al. (1999). "Vgamma9/Vdelta2 T cell activation induced by bacterial low molecular mass compounds depends on the 1-deoxy-D-xylulose 5-phosphate pathway of isoprenoid biosynthesis." FEMS Immunol Med Microbiol **25**(4): 371-8.
Jouen-Beades, F., E. Paris, et al. (1997). "In vivo and in vitro activation and expansion of gammadelta T cells during Listeria monocytogenes infection in humans." Infect Immun **65**(10): 4267-72.
Kabelitz, D. (1999). "Effector functions and control of human gammadelta T-cell activation." Microbes Infect **1**(3): 255-61.
Kabelitz, D., A. Glatzel, et al. (2000). "Antigen recognition by human gammadelta T lymphocytes." Int Arch Allergy Immunol **122**(1): 1-7.
Kabelitz, D., D. Wesch, et al. (2004). "Characterization of tumor reactivity of human V gamma 9V delta 2 gamma delta T cells in vitro and in SCID mice in vivo." J Immunol **173**(11): 6767-76.
Kabelitz, D., D. Wesch, et al. (2004). "Potential of human gammadelta T lymphocytes for immunotherapy of cancer." Int J Cancer **112**(5): 727-32.
Kamath, A. B., L. Wang, et al. (2003). "Antigens in tea-beverage prime human Vgamma 2Vdelta 2 T cells in vitro and in vivo for memory and nonmemory antibacterial cytokine responses." Proc Natl Acad Sci U S A **100**(10): 6009-14.
Kato, Y., Y. Tanaka, et al. (2001). "Targeting of tumor cells for human gammadelta T cells by nonpeptide antigens." J Immunol **167**(9): 5092-8.
Kato, Y., Y. Tanaka, et al. (2003). "Requirement of species-specific interactions for the activation of human gamma delta T cells by pamidronate." J Immunol **170**(7): 3608-13.
Kaufmann, S. H., B. Schoel, et al. (1991). "Heat-shock protein 60: implications for pathogenesis of and protection against bacterial infections." Immunol Rev **121**: 67-90.
Klas, C., K. M. Debatin, et al. (1993). "Activation interferes with the APO-1 pathway in mature human T cells." Int Immunol **5**(6): 625-30.

Kroca, M., A. Johansson, et al. (2001). "V gamma 9V delta 2 T cells in human legionellosis." Clin Diagn Lab Immunol **8**(5): 949-54.
Kuhnlein, P., R. Mitnacht, et al. (1996). "The canonical T cell receptor of dendritic epidermal gamma delta T cells is highly conserved between rats and mice." Eur J Immunol **26**(12): 3092-7.
Kunzmann, V., E. Bauer, et al. (2000). "Stimulation of gammadelta T cells by aminobisphosphonates and induction of antiplasma cell activity in multiple myeloma." Blood **96**(2): 384-92.
Kunzmann, V., E. Bauer, et al. (1999). "Gamma/delta T-cell stimulation by pamidronate." N Engl J Med **340**(9): 737-8.
Kunzmann, V., E. Kretzschmar, et al. (2004). "Polyinosinic-polycytidylic acid-mediated stimulation of human gammadelta T cells via CD11c dendritic cell-derived type I interferons." Immunology **112**(3): 369-77.
Kurane, I., J. Janus, et al. (1992). "Dengue virus infection of human skin fibroblasts in vitro production of IFN-beta, IL-6 and GM-CSF." Arch Virol **124**(1-2): 21-30.
Lafont, V., J. Liautard, et al. (2001). "Production of TNF-alpha by human V gamma 9V delta 2 T cells via engagement of Fc gamma RIIIA, the low affinity type 3 receptor for the Fc portion of IgG, expressed upon TCR activation by nonpeptidic antigen." J Immunol **166**(12): 7190-9.
Lai, X., Y. Shen, et al. (2003). "Immune biology of macaque lymphocyte populations during mycobacterial infection." Clin Exp Immunol **133**(2): 182-92.
Latinis, K. M., L. L. Carr, et al. (1997). "Regulation of CD95 (Fas) ligand expression by TCR-mediated signaling events." J Immunol **158**(10): 4602-11.
Lena, P., F. Villinger, et al. (2002). "Co-immunization of rhesus macaques with plasmid vectors expressing IFN-gamma, GM-CSF, and SIV antigens enhances anti-viral humoral immunity but does not affect viremia after challenge with highly pathogenic virus." Vaccine **20 Suppl 4**: A69-79.
Li, B., H. Bassiri, et al. (1998). "Involvement of the Fas/Fas ligand pathway in activation-induced cell death of mycobacteria-reactive human gamma delta T cells: a mechanism for the loss of gamma delta T cells in patients with pulmonary tuberculosis." J Immunol **161**(3): 1558-67.
Li, B., M. D. Rossman, et al. (1996). "Disease-specific changes in gammadelta T cell repertoire and function in patients with pulmonary tuberculosis." J Immunol **157**(9): 4222-9.
Loh, E. Y., M. Wang, et al. (1994). "Gene transfer studies of T cell receptor-gamma delta recognition. Specificity for staphylococcal enterotoxin A is conveyed by V gamma 9 alone." J Immunol **152**(7): 3324-32.
Lozupone, F., D. Pende, et al. (2004). "Effect of human natural killer and gammadelta T cells on the growth of human autologous melanoma xenografts in SCID mice." Cancer Res **64**(1): 378-85.
Maccalli, C., D. Pende, et al. (2003). "NKG2D engagement of colorectal cancer-specific T cells strengthens TCR-mediated antigen stimulation and elicits TCR independent anti-tumor activity." Eur J Immunol **33**(7): 2033-43.
Meresse, B., Z. Chen, et al. (2004). "Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease." Immunity **21**(3): 357-66.
Meylan, P. R., C. A. Spina, et al. (1993). "In vitro differentiation of monocytoid THP-1 cells affects their permissiveness for HIV strains: a model system for studying the cellular basis of HIV differential tropism." Virology **193**(1): 256-67.
Miyagawa, F., Y. Tanaka, et al. (2001). "Essential requirement of antigen presentation by monocyte lineage cells for the activation of primary human gamma delta T cells by aminobisphosphonate antigen." J Immunol **166**(9): 5508-14.
Modlin, R. L., C. Pirmez, et al. (1989). "Lymphocytes bearing antigen-specific gamma delta T-cell receptors accumulate in human infectious disease lesions." Nature **339**(6225): 544-8.
Morita, C. T., E. M. Beckman, et al. (1995). "Direct presentation of nonpeptide prenyl pyrophosphate antigens to human gamma delta T cells." Immunity **3**(4): 495-507.
Morita, C. T., H. K. Lee, et al. (1999). "Recognition of nonpeptide prenyl pyrophosphate antigens by human gammadelta T cells." Microbes Infect **1**(3): 175-86.
Morita, C. T., H. K. Lee, et al. (2001). "Structural features of nonpeptide prenyl pyrophosphates that determine their antigenicity for human gamma delta T cells." J Immunol **167**(1): 36-41.
Morita, C. T., S. Verma, et al. (1991). "Functionally distinct subsets of human gamma/delta T cells." Eur J Immunol **21**(12): 2999-3007.
Moser, B. and P. Loetscher (2001). "Lymphocyte traffic control by chemokines." Nat Immunol **2**(2): 123-8.
Moser, B. and K. Willimann (2004). "Chemokines: role in inflammation and immune surveillance." Ann Rheum Dis **63 Suppl 2**: ii84-ii89.
Moser, B., M. Wolf, et al. (2004). "Chemokines: multiple levels of leukocyte migration control." Trends Immunol **25**(2): 75-84.
Munk, M. E., A. J. Gatrill, et al. (1991). "In vitro activation of human gamma/delta T cells by bacteria: evidence for specific interleukin secretion and target cell lysis." Curr Top Microbiol Immunol **173**: 159-65.

Munk, M. E. and S. H. Kaufmann (1991). "The immune response to Mycobacterium tuberculosis." Behring Inst Mitt(88): 27-35.
Munz, C., R. M. Steinman, et al. (2005). "Dendritic cell maturation by innate lymphocytes: coordinated stimulation of innate and adaptive immunity." J Exp Med **202**(2): 203-7.
Nakashima, H., N. Yamamoto, et al. (1993). "Defensins inhibit HIV replication in vitro." Aids **7**(8): 1129.
Osborn, L., S. Kunkel, et al. (1989). "Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B." Proc Natl Acad Sci U S A **86**(7): 2336-40.
Patterson, J. B., D. C. Thomis, et al. (1995). "Mechanism of interferon action: double-stranded RNA-specific adenosine deaminase from human cells is inducible by alpha and gamma interferons." Virology **210**(2): 508-11.
Pavic, I., B. Polic, et al. (1993). "Participation of endogenous tumour necrosis factor alpha in host resistance to cytomegalovirus infection." J Gen Virol **74 (Pt 10)**: 2215-23.
Pfeffer, K., B. Schoel, et al. (1990). "Primary responses of human T cells to mycobacteria: a frequent set of gamma/delta T cells are stimulated by protease-resistant ligands." Eur J Immunol **20**(5): 1175-9.
Poccia, F., C. Agrati, et al. (2006). "Anti-severe acute respiratory syndrome coronavirus immune responses: the role played by V gamma 9V delta 2 T cells." J Infect Dis **193**(9): 1244-9.
Poccia, F., C. Agrati, et al. (2005). "Antiviral reactivities of gammadelta T cells." Microbes Infect **7**(3): 518-28.
Poccia, F., C. Agrati, et al. (2005). "Vgamma9Vdelta2 T cell-mediated non-cytolytic antiviral mechanisms and their potential for cell-based therapy." Immunol Lett **100**(1): 14-20.
Poccia, F., L. Battistini, et al. (1999). "Phosphoantigen-reactive Vgamma9Vdelta2 T lymphocytes suppress in vitro human immunodeficiency virus type 1 replication by cell-released antiviral factors including CC chemokines." J Infect Dis **180**(3): 858-61.
Poccia, F., S. Boullier, et al. (1996). "Peripheral V gamma 9/V delta 2 T cell deletion and anergy to nonpeptidic mycobacterial antigens in asymptomatic HIV-1-infected persons." J Immunol **157**(1): 449-61.
Poccia, F., B. Cipriani, et al. (1997). "CD94/NKG2 inhibitory receptor complex modulates both anti-viral and anti-tumoral responses of polyclonal phosphoantigen-reactive V gamma 9V delta 2 T lymphocytes." J Immunol **159**(12): 6009-17.
Poccia, F., M. L. Gougeon, et al. (2002). "Innate T-cell immunity in HIV infection: the role of Vgamma9Vdelta2 T lymphocytes." Curr Mol Med **2**(8): 769-81.
Poquet, Y., P. Constant, et al. (1996). "A novel nucleotide-containing antigen for human blood gamma delta T lymphocytes." Eur J Immunol **26**(10): 2344-9.
Poquet, Y., M. Kroca, et al. (1998). "Expansion of Vgamma9 Vdelta2 T cells is triggered by Francisella tularensis-derived phosphoantigens in tularemia but not after tularemia vaccination." Infect Immun **66**(5): 2107-14.
Poupot, M. and J. J. Fournie (2004). "Non-peptide antigens activating human Vgamma9/Vdelta2 T lymphocytes." Immunol Lett **95**(2): 129-38.
Qin, Z., J. Schwartzkopff, et al. (2003). "A critical requirement of interferon gamma-mediated angiostasis for tumor rejection by CD8+ T cells." Cancer Res **63**(14): 4095-100.
Randolph-Habecker, J., M. Iwata, et al. (2002). "Interleukin-1-mediated inhibition of cytomegalovirus replication is due to increased IFN-beta production." J Interferon Cytokine Res **22**(7): 765-72.
Raulet, D. H. (2003). "Roles of the NKG2D immunoreceptor and its ligands." Nat Rev Immunol **3**(10): 781-90.
Raziuddin, S., N. A. Mir, et al. (1994). "Gamma delta T lymphocytes and proinflammatory cytokines in bacterial meningitis." J Allergy Clin Immunol **93**(4): 793-8.
Raziuddin, S., A. W. Telmasani, et al. (1992). "Gamma delta T cells and the immune response in visceral leishmaniasis." Eur J Immunol **22**(5): 1143-8.
Reed, L. J. (2001). "A trail of research from lipoic acid to alpha-keto acid dehydrogenase complexes." J Biol Chem **276**(42): 38329-36.
Rincon-Orozco, B., V. Kunzmann, et al. (2005). "Activation of V gamma 9V delta 2 T cells by NKG2D." J Immunol **175**(4): 2144-51.
Rohmer, M., M. Knani, et al. (1993). "Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate." Biochem J **295 (Pt 2)**: 517-24.
Rosen, D. B., M. Araki, et al. (2004). "A Structural basis for the association of DAP12 with mouse, but not human, NKG2D." J Immunol **173**(4): 2470-8.
Rossol-Voth, R., S. Rossol, et al. (1991). "In vivo protective effect of tumour necrosis factor alpha against experimental infection with herpes simplex virus type 1." J Gen Virol **72 (Pt 1)**: 143-7.
Rothenfusser, S., A. Buchwald, et al. (2002). "Missing HLA class I expression on Daudi cells unveils cytotoxic and proliferative responses of human gammadelta T lymphocytes." Cell Immunol **215**(1): 32-44.
Rust, C. J., F. Verreck, et al. (1990). "Specific recognition of staphylococcal enterotoxin A by human T cells bearing receptors with the V gamma 9 region." Nature **346**(6284): 572-4.

Sambhi, S. K., M. R. Kohonen-Corish, et al. (1991). "Local production of tumor necrosis factor encoded by recombinant vaccinia virus is effective in controlling viral replication in vivo." Proc Natl Acad Sci U S A **88**(9): 4025-9.

Santini, D., B. Vincenzi, et al. (2002). "Pamidronate induces modifications of circulating angiogenetic factors in cancer patients." Clin Cancer Res **8**(5): 1080-4.

Scalise, F., R. Gerli, et al. (1992). "Lymphocytes bearing the gamma delta T-cell receptor in acute toxoplasmosis." Immunology **76**(4): 668-70.

Sciammas, R. and J. A. Bluestone (1998). "HSV-1 glycoprotein I-reactive TCR gamma delta cells directly recognize the peptide backbone in a conformationally dependent manner." J Immunol **161**(10): 5187-92.

Scotet, E., L. O. Martinez, et al. (2005). "Tumor recognition following Vgamma9Vdelta2 T cell receptor interactions with a surface F1-ATPase-related structure and apolipoprotein A-I." Immunity **22**(1): 71-80.

Selin, L. K., S. Stewart, et al. (1992). "Reactivity of gamma delta T cells induced by the tumour cell line RPMI 8226: functional heterogeneity of clonal populations and role of GroEL heat shock proteins." Scand J Immunol **36**(1): 107-17.

Sheehy, M. E., A. B. McDermott, et al. (2001). "A novel technique for the fluorometric assessment of T lymphocyte antigen specific lysis." J Immunol Methods **249**(1-2): 99-110.

Shefer, S., G. S. Tint, et al. (2000). "Is there a relationship between 3-hydroxy-3-methylglutaryl coenzyme a reductase activity and forebrain pathology in the PKU mouse?" J Neurosci Res **61**(5): 549-63.

Shen, Y., D. Zhou, et al. (2002). "Adaptive immune response of Vgamma2Vdelta2+ T cells during mycobacterial infections." Science **295**(5563): 2255-8.

Shrestha, N., J. A. Ida, et al. (2005). "Regulation of acquired immunity by gamma delta T-cell/dendritic-cell interactions." Ann N Y Acad Sci **1062**: 79-94.

Sicard, H., T. Al Saati, et al. (2001). "Synthetic phosphoantigens enhance human Vgamma9Vdelta2 T lymphocytes killing of non-Hodgkin's B lymphoma." Mol Med **7**(10): 711-22.

Sicard, H., S. Ingoure, et al. (2005). "In vivo immunomanipulation of V gamma 9V delta 2 T cells with a synthetic phosphoantigen in a preclinical nonhuman primate model." J Immunol **175**(8): 5471-80.

Sin, J. I., J. J. Kim, et al. (1998). "Enhancement of protective humoral (Th2) and cell-mediated (Th1) immune responses against herpes simplex virus-2 through co-delivery of granulocyte-macrophage colony-stimulating factor expression cassettes." Eur J Immunol **28**(11): 3530-40.

Sing, G., L. Butterworth, et al. (1998). "Composition of peripheral blood lymphocyte populations during different stages of chronic infection with hepatitis B virus." J Viral Hepat **5**(2): 83-93.

Smyth, M. J., J. Swann, et al. (2005). "NKG2D function protects the host from tumor initiation." J Exp Med **202**(5): 583-8.

Struyf, S., P. Proost, et al. (1998). "Enhanced anti-HIV-1 activity and altered chemotactic potency of NH2-terminally processed macrophage-derived chemokine (MDC) imply an additional MDC receptor." J Immunol **161**(6): 2672-5.

Sturm, E., E. Braakman, et al. (1989). "Coordinated V gamma and V delta gene segment rearrangements in human T cell receptor gamma/delta+ lymphocytes." Eur J Immunol **19**(7): 1261-5.

Sturm, E., E. Braakman, et al. (1991). "Daudi cell specificity correlates with the use of a V gamma 9-V delta 2 encoded TCR gamma delta." Curr Top Microbiol Immunol **173**: 183-8.

Sturm, E., E. Braakman, et al. (1990). "Human V gamma 9-V delta 2 T cell receptor-gamma delta lymphocytes show specificity to Daudi Burkitt's lymphoma cells." J Immunol **145**(10): 3202-8.

Sumida, T., T. Maeda, et al. (1992). "Predominant expansion of V gamma 9/V delta 2 T cells in a tularemia patient." Infect Immun **60**(6): 2554-8.

Sunder-Plassmann, R., W. F. Pickl, et al. (1995). "Crosslinking of CD27 in the presence of CD28 costimulation results in T cell proliferation and cytokine production." Cell Immunol **164**(1): 20-7.

Sutherland, C. L., N. J. Chalupny, et al. (2002). "UL16-binding proteins, novel MHC class I-related proteins, bind to NKG2D and activate multiple signaling pathways in primary NK cells." J Immunol **168**(2): 671-9.

Takayama, H., G. Trenn, et al. (1987). "A novel cytotoxic T lymphocyte activation assay. Optimized conditions for antigen receptor triggered granule enzyme secretion." J Immunol Methods **104**(1-2): 183-90.

Tanaka, Y., C. T. Morita, et al. (1995). "Natural and synthetic non-peptide antigens recognized by human gamma delta T cells." Nature **375**(6527): 155-8.

Tikhonov, I., C. O. Deetz, et al. (2006). "Human Vgamma2Vdelta2 T cells contain cytoplasmic RANTES." Int Immunol **18**(8): 1243-51.

Trichet, V., C. Benezech, et al. (2006). "Complex interplay of activating and inhibitory signals received by Vgamma9Vdelta2 T cells revealed by target cell beta2-microglobulin knockdown." J Immunol **177**(9): 6129-36.

6. REFERENCES

Upshaw, J. L., L. N. Arneson, et al. (2006). "NKG2D-mediated signaling requires a DAP10-bound Grb2-Vav1 intermediate and phosphatidylinositol-3-kinase in human natural killer cells." Nat Immunol **7**(5): 524-32.

Upshaw, J. L. and P. J. Leibson (2006). "NKG2D-mediated activation of cytotoxic lymphocytes: unique signaling pathways and distinct functional outcomes." Semin Immunol **18**(3): 167-75.

Van Lier, R. A., M. Brouwer, et al. (1988). "Signals involved in T cell activation. T cell proliferation induced through the synergistic action of anti-CD28 and anti-CD2 monoclonal antibodies." Eur J Immunol **18**(1): 167-72.

Verneris, M. R., M. Karami, et al. (2004). "Role of NKG2D signaling in the cytotoxicity of activated and expanded CD8+ T cells." Blood **103**(8): 3065-72.

Vincent, M. S., K. Roessner, et al. (1996). "Apoptosis of Fashigh CD4+ synovial T cells by borrelia-reactive Fas-ligand(high) gamma delta T cells in Lyme arthritis." J Exp Med **184**(6): 2109-17.

Vincent, M. S., K. Roessner, et al. (1998). "Lyme arthritis synovial gamma delta T cells respond to Borrelia burgdorferi lipoproteins and lipidated hexapeptides." J Immunol **161**(10): 5762-71.

Vivier, E., E. Tomasello, et al. (2002). "Lymphocyte activation via NKG2D: towards a new paradigm in immune recognition?" Curr Opin Immunol **14**(3): 306-11.

von Lilienfeld-Toal, M., J. Nattermann, et al. (2006). "Activated gammadelta T cells express the natural cytotoxicity receptor natural killer p 44 and show cytotoxic activity against myeloma cells." Clin Exp Immunol **144**(3): 528-33.

Wallace, M., S. R. Bartz, et al. (1996). "Gamma delta T lymphocyte responses to HIV." Clin Exp Immunol **103**(2): 177-84.

Wang, L., A. Kamath, et al. (2001). "Antibacterial effect of human V gamma 2V delta 2 T cells in vivo." J Clin Invest **108**(9): 1349-57.

Watzl, C. (2003). "The NKG2D receptor and its ligands-recognition beyond the "missing self"?" Microbes Infect **5**(1): 31-7.

Weissman, A. M., L. E. Samelson, et al. (1986). "A new subunit of the human T-cell antigen receptor complex." Nature **324**(6096): 480-2.

Wesch, D., S. Beetz, et al. (2006). "Direct costimulatory effect of TLR3 ligand poly(I:C) on human gamma delta T lymphocytes." J Immunol **176**(3): 1348-54.

Wilhelm, M., V. Kunzmann, et al. (2003). "Gammadelta T cells for immune therapy of patients with lymphoid malignancies." Blood **102**(1): 200-6.

Wong, G. H. and D. V. Goeddel (1986). "Tumour necrosis factors alpha and beta inhibit virus replication and synergize with interferons." Nature **323**(6091): 819-22.

Workalemahu, G., M. Foerster, et al. (2004). "Expression and synthesis of fibroblast growth factor-9 in human gammadelta T-lymphocytes. Response to isopentenyl pyrophosphate and TGF-beta1/IL-15." J Leukoc Biol **75**(4): 657-63.

Workalemahu, G., M. Foerster, et al. (2003). "Human gamma delta-T lymphocytes express and synthesize connective tissue growth factor: effect of IL-15 and TGF-beta 1 and comparison with alpha beta-T lymphocytes." J Immunol **170**(1): 153-7.

Worku, S., G. J. Gorse, et al. (2001). "Canarypox vaccines induce antigen-specific human gammadelta T cells capable of interferon-gamma production." J Infect Dis **184**(5): 525-32.

Wu, J., Y. Song, et al. (1999). "An activating immunoreceptor complex formed by NKG2D and DAP10." Science **285**(5428): 730-2.

Yang, K. Y., Y. Liu, et al. (2001). "Activation of a mitogen-activated protein kinase pathway is involved in disease resistance in tobacco." Proc Natl Acad Sci U S A **98**(2): 741-6.

Yang, O. O., E. A. Garcia-Zepeda, et al. (2002). "Monocyte chemoattractant protein-2 (CC chemokine ligand 8) inhibits replication of human immunodeficiency virus type 1 via CC chemokine receptor 5." J Infect Dis **185**(8): 1174-8.

7 ABBREVIATIONS

Ab	- Antibody
Ag	- Antigen
APC	- Antigen Presenting Cell
BAL	- Bronchoalveolar lavage fluid
BCG	- Bacillus calmette-guerin
BSA	- Bovine Serum Albumin
BSS	- Balanced Salt Solution
cDNA	- Complementary DNA
CD	- Cluster of differentiation
CDR	- Complementary Determining Region
CIAP	- Calf Intestine Alkaline Phospatase
ConA	- Concanavalin A
DC	- Dendritic cell
DEPC	- Diethylpyrocarbonate
DMSO	- Dimethyl sulfoxide
DNA	- Deoxyribonucleic acid
dNTP	- 100 mM solution of each dATP, dCTP, dGTP, dTTP
DP	- Double Positive
EDTA	- Ethylendiamintetracetic acid
FACS	- Fluorescence activated cell scan
FCS	- Fetal Calf Serum
FcεRI	- Fc epsilon receptor I
FITC	- Fluoresceinisothiocyanate
FL	- Fluorescence
FSC	- Forward scatter
G418	- Geneticin
h	- Hour
Ig	- Immunoglobulin
IFN-γ	- Interferon γ
IL	- Interleukin
Ig	- Immunglobulin
kDa	- Kilo Dalton
LN	- Lymph Node
M	- Molar
mAb	- Monoclonal Antibody
MHC	- Major Histocompatibility Complex
min	- Minutes
mRNA	- Messenger RNA

7. ABBREVIATIONS

NK - Natural Killer cell

NMIg - Normal mouse immunglobulin

ON - Overnight incubation

PBS - Phosphate Buffered Saline

PCR - Polymerase chain reaction

PE - Phycoerythrin

RNA - Ribonucleic acid

RNAase - Ribonuclease A

RT - Room Temperature

S - Streptavidin

sec - Seconds

SP - Single Positive

SSC - Side Scatter

TCR - T-cell Receptor

TRIS - N, N, N', N'-Tetramethylethylenediamine

TLR - Toll-like receptor

TNF-α - Tumor necrosis factor-α

V - Variable segment of the T cell receptor variable domain

Vγ9Vδ2 T-STL-Vg9Vd2 T cells Short Term Line

8 CURRICULUM VITAE

May 2007

BLADIMIRO RINCON OROZCO

Clinical Microbiologist,

MSc Immunology

Privat
Helmholzt Str. 20

D-69120, Heidelberg

M: +49-176-21728133

Email: blrincon@hotmail.com,

blrincon@gmail.com

Personal dates

Place and date of birth:	Bucaramanga, Colombia, 19 January 1975
Nationality:	Colombian
Familiar Status:	Single

EDUCATION

2004- 2007	**Dissertation: TCR and co-receptors mediated activation of Vγ9Vδ2 T cells, Summa Cum Lauden** Virology and Immunobiology Institute Würzburg University, Germany, Degree expected January 2007 Advisor: Prof. Dr. Thomas Herrmann
1999-2001	**Master Biomedicine area Immunology** Department of Microbiology and Parasitology Medicine School, Universidad de Antioquia Medellin, Colombia Advisor: Prof. Dr. Pablo Patino Grajales
1992-1997	**Clinical Microbiologist**

Health Faculty, Universidad Industrial de Santander
Bucaramanga, Colombia:

Professional experience

2007 until now	Post Doc position Viral Transformaion Division DKFZ- Heidelberg
2005 – 2007	Teaching Assistant, practical course of Immunogenetics Virology and Immunobiology Institute Würzburg University, Germany
2001 – 2003	Associated Researcher. Group of Primary Immunodeficiency Section of Immunology and Immunotherapy Medicine School, Universidad de Antioquia Medellin, Colombia
1999 – 2003	Teaching Assistant, practical course of Biochemistry Biochemical Section, Department of Chemical Pharmaceutics Universidad de Antioquia Medellin, Colombia.
1998 – 1999	Teaching Assistant, practical course of Immunology Microbiology Department Universidad Industrial de Santander Bucaramanga, Colombia.
1998 – 1999	Associated Researcher. Laboratory of Immunology and Virology Microbiology Department Universidad Industrial de Santander Bucaramanga, Colombia.
1997	Health Social Service, Hospital San Jose de Puerto Wilches Santander, Colombia
1994 – 1996	Research training rotation in Immunohystocompatibility Laboratory of Immunology and Virology Microbiology Department

Universidad Industrial de Santander
Bucaramanga, Colombia.

PUBLICATIONS

International Journals

Bladimiro Rincon-Orozco, Volker Kunzmann, Philine Wrobel, Dieter Kabelitz, Alexander Steinle, and Thomas Herrmann. Activation of Vγ9Vδ2 T Cells by NKG2D. **Journal of Immunology**, 2005, 175: 2144

M.T. Rugeles, **B. Rincon**, C. Rugeles, C.J. Montoya, M. Hernandez, C. Estrada, M.M. Olivares and P.J. Patino. Normal expression of IFN-γR in four patients with uncommon mycobacterial infection phenotypes. **Braz J Med Biol Res**. 2004 Sep;37(9):1353

Colombian Journals

Rincón O. B., Rugeles M.T., Patiño P.J. Asociación entre la Deficiencia del Receptor de Interferón Gamma y la susceptibilidad a infecciones por patógenos intracelulares, **Salud UIS** 2001, 1: 64.

Ramírez G., Pradilla G. Cabrales C.C., **Rincón O. B**. Neurocisticercosis, **Médicas UIS**, 1999 (13) p. 152

Rincón O. B.,Cabrales C.C.,Gonzalez G. Dengue y Dengue Hemorrágico, **Médicas UIS**, 1998 (12) p. 327

Professional Affiliations

German Association of Immunology
Colombian Association of Immunology

PRESENTATIONS AND ABSTRACTS

T Herrmann, M. Kreiß**,** , V. Kunzmann, and **B. Rincon-Orozco.** Antigen Recognition by Vgamma9 Vdelta2 TCR requires presentation by human cells. 16th European congress of immunology, Paris, September 6-9, 2006

B. Rincon-Orozco, Activation of Vγ9Vδ2 T cells by NKG2D. Oral presentation at Joint Meeting of the "EuroPathoGenomics Graduate Academy" NoE "EuroPathoGenomics" and the European Graduate College 587 Würzburg – Umea. Würzburg, December 2-3, 2005.

B. Rincon-Orozco, A. Steinle, V. Kunzmann, D. Kabelitz and T. Herrmann. Activation of Vγ9Vδ2 T cells by NKG2D. Poster presentation at Joint Annual Meeting of the German and Scandinavian Societies for Immunology. **Immunobiol**. 210 (2005) 6-8 p. 458.

B. Rincon-Orozco, A. Steinle, V. Kunzmann, and T. Herrmann. Activation of Vγ9Vδ2 T cells by NKG2D Ligation. Poster presentation at Luminy Advanced Course In Immunology, and The Road Ahead: Future Directions in Fundamental and Clinical Immunology, Marseille-Luminy, France, January 11-15 (2005).

B. Rincon-Orozco, A. Steinle, V. Kunzmann, and T. Herrmann. Activation of Vγ9Vδ2 T cells by NKG2D ligation. Poster presentation at Joint Annual Meeting of the German and Dutch Societies for Immunology. **Immunobiol**. 209 (2004) 4-6 p. 354.

B. Rincon-Orozco, A. Steinle, V. Kunzmann, and T. Herrmann. Activation of Vγ9Vδ2 T cells by NKG2D ligation. Poster presentation at International Symposium Threat of Infection, Microbes of High Pathogenic Potential – Strategies for Detection, Control and Eradication. Würzburg, July 25-28, 2004

M. Kreiß**, B. Rincon-Orozco**, V Kunzmann, and T Herrmann. Tissue and species specificity of presentation of ligands of the human Vγ9δ2 TCR. Poster presentation at Joint Annual Meeting of the German and Dutch Societies for Immunology. **Immunobiol**. 208 (2003) 1-3 p. 56

Courses

EMBO Course. Luminy Advanced Course In Immunology, and The Road Ahead: Future Directions in Fundamental and Clinical Immunology, Marseille-Luminy, France, January 11-15 (2005).

Network of Competence "pathogenomics" summer course. Proteome analysis of bacterial pathogens, Würzburg, Germany, July 28-30, 2004

EMBO workshop on Molecular Microbial Phatogenesis, Umea, Sweden, June 12-15, 2003

Awards

Associated member of the Graduiertenkolleg "Immunomodulation". Würzburg, Germany 2006. Speaker Prof. Dr. T. Hünig

Alejandro Ángel Escobar mention, category Physics and Biological Sciences. Program for Study and Treatment of Primary Immunodeficiency: From Molecular Characterization to Integral Treatment. Immunology Section, Antioquia University, Colombia. 2002

Founder Member of Colombian Students Association of Microbiology, Bucaramanga, Colombia, 1996.

Academic Excellent Award Scholarship, University Industrial of Santander, Bucaramanga Colombia, 1994, 1995, 1996, 1997.

Fellowships

Fellowship Internationales Graduiertenkolleg 587/2 Würzburg-Umea "Gene Regulation in and by Microbial Pathogens". Würzburg. Germany, 2004-2006. Speaker Prof. Dr. J. Morschhäuser

Fellowship Young Research of Colciencias, Medellin, Colombia, 2002.

Languages

Mother Tongue: Spanish

Other Languages English

- Reading skills: good
- Writing skills: good
- Verbal skills: good

German

- Reading skills: basic
- Writing skills: basic
- Verbal skills: basic

9 ACKNOWLEDGEMENTS

I would like first of all to express my deepest gratitude to Prof Dr. Thomas Herrmann for giving me the opportunity to do my PhD study and to make an interesting research at the Virology and Immunology Institute. Many thanks for his supervision and support to my work, and of course for kind advices and helps to me during these years.

My deep gratitude to Prof. Dr. Hacker and Prof Dr. Morschhäuser, first of all for giving me the opportunity to be a member of Graduate College of ``Gene Regulation in and by Microbial Pathogens``. For they useful and interesting scientific discussions. It was always a great pleasure for me to attend an interesting scientific seminars and presentations during these years. Many thanks for creating such a positive atmosphere and stimulating support.

My deep gratitude to Prof. Hünig, for all his supports, scientific discussions and kindness during being at the Immunology Institute, and for giving me the opportunity to participated in the Graduate College of ``Immunomodulation``. It was always a great pleasure for me to attend an interesting scientific seminars and presentations during these years. Many thanks for creating such a positive atmosphere and stimulating support. Many, many thanks.

My gratitude to Ingrid for helping me in my PhD work, and the rest of the peoples from the lab. Many thanks to Lisa, Holger, Elisa, Pablo, Mr Li, and Cristian. for creating such a positive atmosphere and stimulating support.

I also would like to thank all students of graduate college for nice friendship and interesting discussions.

Por supuesto no me podria olvidar de todos mis amigos que me hicieron feliz mi estadia en Alemania durante todos estos anos, al principo la invaluable ayuda de la colonia colombiana conformada por Juan Carlos, Robinson, Edwin, Marcela, Marta y Claudia, por los gratos y divertidos momentos y su ayuda.

A mi familia sudamericana Alicia y Horacio, gracias por su ayuda incondicional, colaboracion y amistad, a mis gueyes mexicanos Marcos y Oscar, mis pibes uruguayos don Martin y dona Silvia y los argentinos Rodrigo,

9. ACKNOWLEDGEMENTS

Fernando por los divertidisimos momentos y ayuda en todos las buenas y malas situaciones. A mi amigo Samuel Gracias (el taiwanes), por su agradable compania y ayuda. A toda la pandilla Espanola con la que disfrute estenuantes parrandas Laura, Jose, Las Sevis, Saul y todos los demas que ahora olvido.

Agradezco profundamente a las senoras del guest house (Ingerborg, Brigitte, Ursula, Monika, Hanna, Hella, Inge, Lotte, Gudrun, Brigitte and Doris) por su desinterasada ayuda y agradable compania durante todos estos anos y las interesantes experiencia adquiridas inolvidables. Ademas todas las interesantes personas que durante esas reuniones conoci.

A mi familia colombiana que a pesar de la distancia siempre estuvieron interesados en mi y me ayudaron en los dificiles momentos con una voz de consolacion y esperanza (Alberto, Jose, primos y primas)

Finalmente queria agradecer a las dos mujeres que mas quiero en mi vida la primera por traerme a este mundo y fomentar en mi el gran deseo del saber, a pesar de sus limitaciones inculcarme lo valioso de la responsabilidad, la honradez y la tenacidad para alcanzar todas mi metas, muchas gracias Mama, y por ultimo a una hermosa nina alemana que ha hecho mi mananas hermosas por tenerla a mi lado cuando despierto y mis noche felices por estar conmigo y ayudarme incondicionalmente, muchas gracias Melanie.

10 PUBLICATIONS

Parts of the thesis have been published

- **Bladimiro Rincon-Orozco**, Volker Kunzmann, Philine Wrobel, Dieter Kabelitz, Alexander Steinle, and Thomas Herrmann. Activation of Vγ9Vδ2 T Cells by NKG2D. **Journal of Immunology**, 2005, 175: 2144