Antigen recognition and thymic maturation of human TCR Vgamma9-Vdelta2 cells

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

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Basel 2007

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

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Basel, den 13.11.2007

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Niniejszą pracę dedykuję moim Rodzicom

(I dedicate this work to my Parents)

Acknowledgments

Foremost, I would like to thank my supervisor Gennaro for giving me the opportunity to work in his lab. Thanks for the guidance, support, inspiration and encouragement all throughout my thesis work. To have had a teacher with such a passion for science will surely play an important role in motivating me in my future career.

I also would like to thank everyone who shared with me the time in the institute, in particular those who worked with me in “Little Italy”. Special words of gratitude go to those who directly helped me with this work: Hans-Jürgen, Lucia, Lena and Nino.

Moreover I would like to thank my friends with whom I have shared unforgettable moments both within and outside of the lab:

Zaima, one of the most positive people I have ever met, for making beginning much easier;

Gabriel, the “coffee angel”, for always having something good to say;

Sabry, my hood-mate, for all the successful operations and for hours of valuable talking;

Manu, my “personal” biochemist, for all his help and for his contagious zest for life;

Fede, the “shopping queen”, for her almost unbreakable optimism that helped me in many moments;

Sami, the chemotaxis expert, for all the support;

In addition I would like to thank Vreni, the cell-sorter master and a good friend.

Last but not least I would like to thank one very special person, my best friend and life companion, Łukasz:

Dziękuję za to, że zawsze jesteś przy mnie...

Table of content

Abbreviations

7-DHC	7-dehydrocholesterol
ABC	ATP-binding cassette transporters
AP	alkaline phosphatase
APC	antigen presenting cell
APM	antigen-presenting molecule
APS	ammoniumpersulfate
ATP	adenosine triphosphate
BrHPP	bromohydrine pyrophosphate
BSA	bovine serum albumin
CA	calyculin A
CCR7	CC-chemokine receptor 7
CFTR	cystic fibrosis transmembrane conductance regulator
cDNA	complemantary deoxyribonucleic acid
CDR	complementarity determining region
cpm	counts per minute
DC	dendritic cells
DETC	dendritic epithelial T cells
DIDS	4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid
DMAPP	dimethylallylpyrophosphate
DMSO	dimethylsulfoxide
DN	double negative
DP	double positive
EDTA	ethylenediamine-tetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FPP	farnesylpyrophosphate
GGPP	geranylgeranylpyrophosphate
GM-CSF	granulocyte-macrophage colony-stimulating-factor
cGMP	cyclic guanosine monophosphate
GPP	geranylpyrophosphate
h	hour(s)

HLA	human leukocyte antigen
HMB-PP	(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
HMGR	3-hydroxymethyl-3-glutaryl-CoenzymeA-reductase
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
HS	human serum
HSA	human serum albumin
IELs	intraepithelial lymphocytes
IFNγ	interferon gamma
Ig	immunoglobulin
IL	interleukin
IPP	isopentenylpyrophosphate
i.t.	intra thymic
kDa	kilo Dalton
KGF	keratinocyte growth factor
LC-ESI-MS	liquid chromatography-electrospray-mass spectrometry
mAb	monoclonal antibody
MCT	monocarboxylate transporters
MEP	2-*C*-methyl-**D**-erythritol 4-phosphate
MEV	mevastatin
MHC	major histocompatibility complex
min	minute(s)
MOI	multiplicity of infection
Mon	monensin
MDR	multi-drug resistance protein
MRP	multi-drug related protein
MVD	diphosphomevalonate decarboxylase
MVK	mevalonate kinase
MVL	mevalonolacton
nBP	nitrogen-containing bisphosphonate drugs
NK	natural killer
ND	not determined
OA	okadaic acid
OATP	organic anion-transpoting polypeptide

PAM	pamidronate
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline solution
PCR	polymerase chain reaction
PHA	phytohemagglutinin
PMSF	phenylmethylsulfonylfluoride
PMVK	phosphomevalonate kinase
Rag	recombination activating gene
SBA	*sec*-butylamine
SCID	severe combined immunodeficiency
SDS	sodiumdodecylsulfate
shRNA	small hairpin RNA
SLC	secondary lymphoid-tissue chemokine
SP	single positive
SUR	sulfonylurea receptors
TAP	trasporter associated with antigen processing
TCR	T cell receptor
TE	Tris-EDTA buffer
TEC	thymic epithelial cells
TEA	triethylammonium-acetate
TEMED	N,N,N',N'-tetramethylethylenediamine
Tg	transgenic
TNFα	tumor necrosis factor alpha
U	unit(s)
Wt	wild type
ZOL	zoledronate

Summary

T cells are divided into two populations according to the type of TCR used for antigen recognition. One population uses a TCR heterodimer, which is composed by the non-covalently associated alpha and beta chains. This TCR recognizes protein and lipid antigens, which are presented by MHC and CD1 antigen-presenting molecules, respectively. A second population uses a TCR heterodimer composed by the gamma and delta chains and recognizes non-peptidic ligands in the absence of MHC and CD1 restriction. In humans the major population of TCR γδ cells uses the Vγ9-Vδ2 TCR. This is a unique population because it is present only in primates and constitutes >50% of peripheral TCR γδ cells. TCR Vγ9-Vδ2 cells are activated by microbial phosphorylated metabolites and by so far unknown ligands expressed by a group of tumor cells. The principal microbial antigen is (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), an intermediate metabolite generated in 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway of isoprenoids biosynthesis.

Despite these cells were described in 1986, many aspects remain unclear, including the nature of the stimulatory ligands present in tumor cells, the mechanisms of their activation during infection, the molecular mechanisms involved in antigen presentation, and the requirements for thymic maturation. In this dissertation we have addressed these important issues using *ex vivo* cells, biochemical approaches for ligand identification, T cell activation assays and generation of transgenic mice expressing this human TCR.

We have identified endogenous metabolites generated in the mevalonate pathway as the tumor ligands which stimulate TCR Vγ9-Vδ2 lymphocytes. We have found that tumor cells show altered mevalonate pathway which leads to accumulation of intermediate metabolites. This is novel mechanism utilized by the immune system to monitor the metabolic integrity of cells and to react to those which have a dysregulation of this important metabolic pathway.

In a second series of studies we have investigated how TCR Vγ9-Vδ2 cells are activated during bacterial infections. Despite published studies identified HMB-PP as a potent stimulatory ligand *in vitro*, there was no formal evidence that this compound participates in cell activation during infection. Unexpectedly, we found that HMB-PP is not the major stimulatory ligand during infection and instead endogenous mevalonate metabolites are the stimulatory ligands. We describe how infection modifies the 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), which is the key enzyme of the mevalonate pathway, and promotes increased synthesis of stimulatory metabolites. We show that infection induces a transient increase in HMGR protein levels and dephosphorylation, leading to increased enzymatic activity. This alteration occurs already within 1 hour after infection, thus representing a rapid mechanism reacting to infection. Thus, like with recognition of tumor cells, also during infection, the immune system of primates utilizes a mechanism which detects alterations of an important metabolic pathway.

We also investigated the mechanisms how mevalonate metabolites traffic within cells. We found that these ligands, which are generated within the

cytoplasm, are transported to the cell surface, where they interact with the TCR γδ, by the MRP5 transporter. We showed that MRP5-blocking drugs inhibit presentation to TCR γδ cells that over expression and knocking down of MRP5 protein increase and inhibit ligand presentation, respectively. These results show that like peptides, which are transported from cytoplasm to the ER through the ABC transporters TAP1 and TAP2, also TCR γδ ligands utilize ABC transporters to become immunogenic. We also found that MRP5 is not involved in forming complexes presented to the TCR γδ and that other unknown ubiquitous and non-polymorphic molecules are involved in this process.

In the last part of these studies we investigated the requirements for thymic maturation and peripheral expansion of TCR Vγ9-Vδ2 cells. We generated a transgenic (Tg) mouse model in which T cells express a TCR composed by human Vγ9-Vδ2 chains. Tg thymocytes express molecules characteristic of partially mature thymocytes together with high levels of Tg TCR. Tg cells do not acquire a mature phenotype and do not exit the thymus in the absence of TCR triggering. However, upon injection of TCR-specific mAbs, Tg thymocytes undergo maturation and colonize peripheral lymphoid organs. Mature Tg T cells remain in the periphery for up to 6 months, with a phenotype of naïve T cells and strongly react to physiological ligands when stimulated by human antigen-presenting cells, which express the restriction element. Thus, Tg T cells expressing the human TCR Vγ9-Vδ2 resemble TCR αβ cells since they also require selection events during thymic maturation.

Our studies suggest that TCR Vγ9-Vδ2 cells by reacting to cells which accumulate mevalonate metabolites provide an early immune response during the time when antigen-specific TCR αβ cells have not yet been recruited and expanded. Thus, TCR γδ cells fulfill the role of sentinel cells which monitor the metabolic integrity of other cells. These cells undergo thymic selection events and require the presence of unique molecules for efficient antigen presentation. Our studies indicate novel aspects of some of these important processes.

Introduction

TCR γδ cells represent a lymphocyte population phenotypically and functionally diverse from TCR αβ cells. They have been found in all vertebrates examined so far including humans (Brenner et al., 1986), monkeys (Malkovsky et al., 1992), mice (Saito et al., 1984; Raulet et al., 1991), rats (Lawetzky et al., 1990), rabbits (Isono et al., 1995), sheep (Hein and Mackay, 1991), cattle (Mackay and Hein, 1989), horses (Schrenzel and Ferrick, 1995), pigs (Hirt et al., 1990; Saalmuller et al., 1990) and chicken (Bucy et al., 1988; Sowder et al., 1988).

They usually represent a small proportion (1-10%) of circulating lymphocytes in most adult animals, while they represent a major proportion in certain extra-lymphoid sites. TCR γδ cells differ from classical MHC restricted T cells in terms of TCR diversity, requirements for antigen recognition and their role in immunity and tissue homoeostasis.

Genetic organization of TCR γ and δ loci

TCR γ and TCR δ chains genes, like TCR α, TCR β and immunoglobulins chain genes, are assembled during somatic rearrangement processes.
In human the TCR γ locus maps on chromosome 7 (Murre et al., 1985) and is composed of two constant gene segments (Cγ), five joining elements (Jγ) and fourteen variable (Vγ) genes of which six encode functional proteins and eight are pseudogenes (Lefranc and Rabbitts, 1990), (Figure 1A). These six genes can be

subdivided into two families: the VγI family composed of Vγ2, 3, 4, 5 and 8 genes and VγII consisting of Vγ9 (Vγ2 in other nomenclature) gene. The TCR γ chain undergoes Vγ-Jγ rearrangement and its variability at junctions is crated by addition of not germline encoded nucleotides (N nucleotides) during recombination process by terminal deoxynucleotidyl transferase (Tdt), (Strauss et al., 1987; Huck et al., 1988).

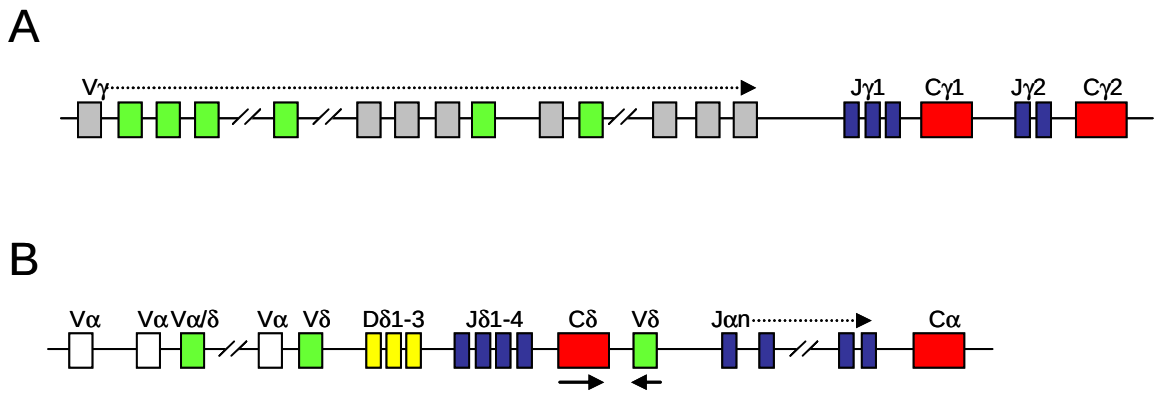

Figure 1. Genetic organization of human (A) TCR γ locus and (B) TCR δ locus adapted from (Hayday, 2000). TCR V segments are green and pseudogens are grey; C segments are red; J segments are blue and D segments are yellow. Only some Vγ and Vα are indicated.

TCR δ locus is closely linked to TCR α locus in contemporary mammals (Hayday et al., 1985; Hayday, 2000). In humans the δ locus is located within the TCR α locus on chromosome 14 (Collins et al., 1985), between Jα and Vα gene segments (Griesser et al., 1988), (Figure 1B). TCR δ chain is assembled via V-D-J rearrangement. The TCR δ locus is composed of single Cδ gene segment, four different Jδ segments preceded by three diversity (Dδ) elements (Chien et al.,

1987; Hata et al., 1987; Takihara et al., 1989). Some of V segments are used both as Vδ and Vα (Guglielmi et al., 1988; Takihara et al., 1989). Although eight distinct Vδ genes have been localized, only six of them were found expressed on the cell surface (Arden et al., 1995; Migone et al., 1995). The variability of TCR δ chain can be extremely high due to the Dδ segments that can undergo tandem rearrangement (Boehm et al., 1988) and flexible reading frame usage (Hata et al., 1988) creating diverse length and composition of the joining regions. Therefore, despite the limited number of germline encoded elements for the TCR γδ, the potential repertoire of this TCR is at least three orders of magnitude higher than TCR αβ repertoire due to the extremely high variability in the CDR3 regions (Davis and Bjorkman, 1988; Hata et al., 1988). Comparison of the CDR3 length reveled that TCR γδ is more similar to immunoglobulins than to TCR αβ (Rock et al., 1994).

Assembly of TCR γδ chains

The particular feature of TCR γδ is the preferential association of Vδ chains with certain Vγ chains. In humans Vδ2 chain is usually associated with unique Vγ chain: Vγ9-JP-Cγ1 while Vδ1 and Vδ3 chains are mostly paired with various Vγ elements from VγI gene family using Cγ2 (Sturm et al., 1989; Hayday, 2000). Cγ1 gene segment usage allows formation of disulphate bond between TCR chains while usage of Cγ2 results in non-disulphate linkage (Krangel et al., 1987; Littman et al., 1987).

TCR γδ structure

Crystal structure of human TCR Vγ9-Vδ2 revealed distinct differences as compared to TCR αβ or to antibody Fab fragments. TCR γδ has an unusual shape due to the small elbow angle and a small Vγ-Cγ inter-domain angle. The elbow angle, between pseudo two-fold symmetry axes that relate V to V and C to C (Lesk and Chothia, 1988), of TCR γδ is 110° while Fabs and TCR αβ have elbow angles in the range of 125-225° and 140-159°, respectively (Allison et al., 2001), (Figure 2).

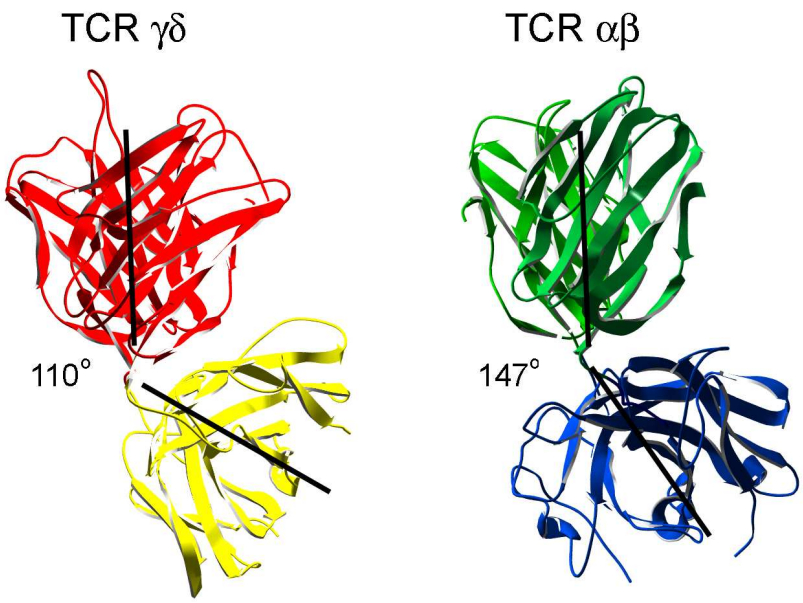

Figure 2. TCR γδ and TCR αβ structures aligned to the V domains.
The C domains of TCR γδ are yellow while V domains are red. In the TCR αβ blue are the C domains and green are V domains. Black lines indicate pseudo two-fold symmetry axes. The values for elbow angles are indicated. PDB accession numbers for TCR γδ and TCR αβ are 1hxm and 1qsf, respectively.

The inter-domain angles, between the long axis of the C domains and long axis of the V domains, of TCR γδ have 42° for Cγ-Vγ and 101° for Cδ-Vδ. In contrast antibodies and TCR αβ have average inter-domain angles of 92° (V_L-C_L, σ=9°), 76° (V_H-C_H, σ=11°), 100° (Cα-Vα, σ=4°) and 67° (Cβ-Vβ, σ=3°). Therefore, the 42° of Cγ-Vγ is the smallest inter-domain angle among these receptors. Moreover TCR γδ differ from TCR αβ also in the structure of C domains. The FG loop of Cγ is much smaller that the one of Cβ suggesting different binding with CD3ε subunit. The secondary structure of Cδ is composed of a regular immunoglobulin-like domain with three-stranded β-sheet as its outer face and therefore differs from the one or Cα (Allison et al., 2001)

The structural difference between TCR αβ and TCR γδ most likely reflects that these receptors recognize structurally different molecules.

TCR γδ-CD3 complex

The main difference between TCR αβ- and TCR γδ-CD3 complexes is their requirement for CD3δ chain. TCR γδ-CD3 complex, unlike TCR αβ, does not associate with CD3δ and contains only CD3γε dimers (Dave et al., 1997; Hayes and Love, 2002). The lack of CD3δ chains, unlikely for TCR αβ (Delgado et al., 2000), does not affect the ERK activation occurring upon TCR stimulation (Hayes and Love, 2002).

The difference in the recruitment of signaling molecules provides a difference in signaling potential of these T cell receptors. In fact TCR γδ cells,

upon CD3ε stimulation, have better proliferative response than TCR αβ cells (Hayes and Love, 2002). Therefore enhanced signaling capacity of TCR γδ cells together with their localization and recognition of native antigens allows these cells to respond rapidly and acquire effector functions faster than TCR αβ cells (Hiromatsu et al., 1992; Ferrick et al., 1995; Hayday, 2000; Hayes and Love, 2002).

Development of TCR γδ cells

TCR αβ and γδ cells develop in the thymus from the pluripotent $CD34^+$ precursor cells deriving from bone marrow but distinct from stem cells (Res et al., 1996). In humans immature thymocytes can be divided accordingly to the expression of CD34, CD38 and CD1a (Spits et al., 1998; Spits, 2002). The earliest thymic progenitors are $CD34^+CD38^-CD1a^-$, followed by $CD34^+CD38^+CD1a^-$ and $CD34^+CD38^+CD1a^+$ cells, with CD1a expression correlating with T linage commitment (Sanchez et al., 1994). In the next stage cells start to express CD4, but not CD8, and they are referred to as $CD4^+$ immature single positive (CD4 ISP) cells (Kraft et al., 1993; Spits, 2002). Importantly, this population contains precursors for both TCR αβ and γδ cells meaning that these cells are before β-selection checkpoint (Ramiro et al., 1996; Blom et al., 1999). The CD4 ISP stage is followed by cells that express CD4 and CD8α chain, and referred to as early double positive (EDP) cells (Spits, 2002). Recently it has been shown that within the population of EDP there are still

present cells uncommitted to the linage. The TCR γδ developmental potential is only lost on double positive (DP) stage (Joachims et al., 2006).

Up to date there are no clear evidences that TCR γδ cells are undergoing selection process in the thymus. However, the TCR γδ repertoire generated in the human thymus is much more diverse than the one present in the periphery where TCR Vγ9 chain pairs only with TCR Vδ2 chain (Casorati et al., 1989; Krangel et al., 1990), thus suggesting that certain selection of TCR γδ cells takes place in the thymus.

More extensive studies concerning TCR γδ cells development have been performed using mouse models. In mice TCR γδ cells and TCR αβ cells also develop from a common thymic double negative (DN) precursor but they diverge into separate lineages very early in ontogeny (Petrie et al., 1992; Dudley et al., 1995).

Immature αβ lineage cells expressing pre-TCR undergo proliferation and transition to CD4 CD8 DP stage (Fehling et al., 1995). DP thymocytes which express mature TCR αβ, follow positive and/or negative selection and emerge as CD4 or CD8 single positive (SP) thymocytes (Fehling et al., 1995). In contrast γδ lineage cells during differentiation do express mature TCR γδ complex, remain mainly DN and undergo limited proliferation (Pardoll et al., 1988).

The developmental stage at which γδ lineage diverges from αβ lineage presumably occurs between the $CD44^{+}CD25^{+}$ (DN2) and $CD44^{-}CD25^{-}$ (DN4) stage (Shortman et al., 1991; Petrie et al., 1992; Kang et al., 2001). There are indications that TCR γδ-dependent developmental checkpoint take place already

at DN3 stage (Prinz et al., 2006; Taghon et al., 2006), (Figure 3). Progression through this checkpoint is marked by high expression of CD27 on TCR γδ cells (Taghon et al., 2006).

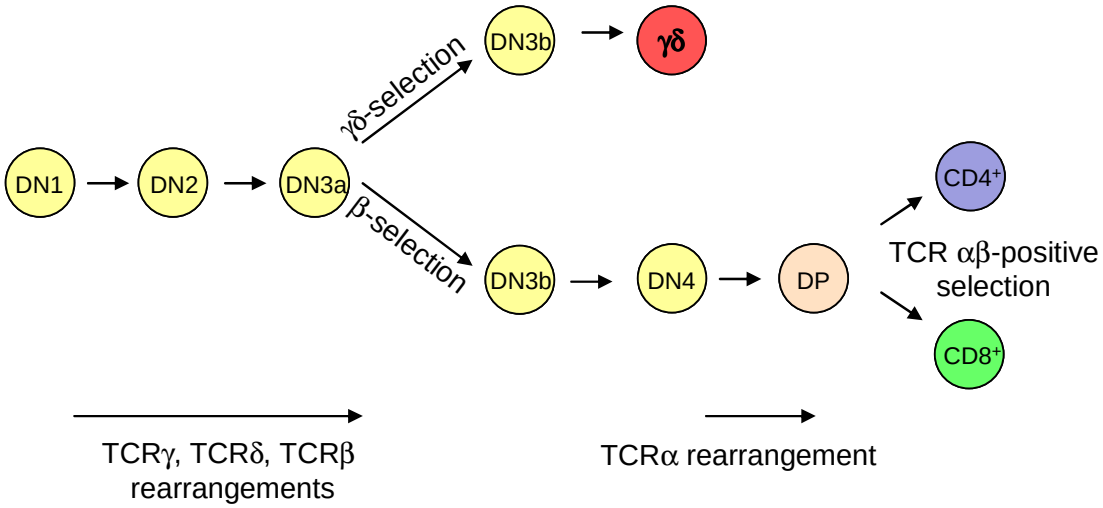

Figure 3. Schematics of thymic T cell development of TCR αβ and TCR γδ cells modified from (Hayday and Pennington, 2007).

TCR γ and δ gene rearrangements are initiated at DN2 stage whereas rearrangement of TCR β gene is slightly delayed and begins between DN2 and DN3 stages (Livak et al., 1999).

The αβ/γδ lineage choice is mediated by single TCR and regulated by the strength of TCR signal (Hayes et al., 2003; Haks et al., 2005; Hayes et al., 2005) and by Notch signaling (Garbe and von Boehmer, 2007). Strong TCR signals through TCR γδ or TCR αβ direct either the development of DN γδ lineage cells or MHC independent development of TCR αβ cells with γδ DN phenotype

(Terrence et al., 2000; Garbe and von Boehmer, 2007), (Figure 4A). Instead weak TCR signals (from pre-TCR or TCR γδ or TCR αβ) in synergy with Notch signaling would favor the αβ lineage commitment (Figure 4B), (Garbe and von Boehmer, 2007).

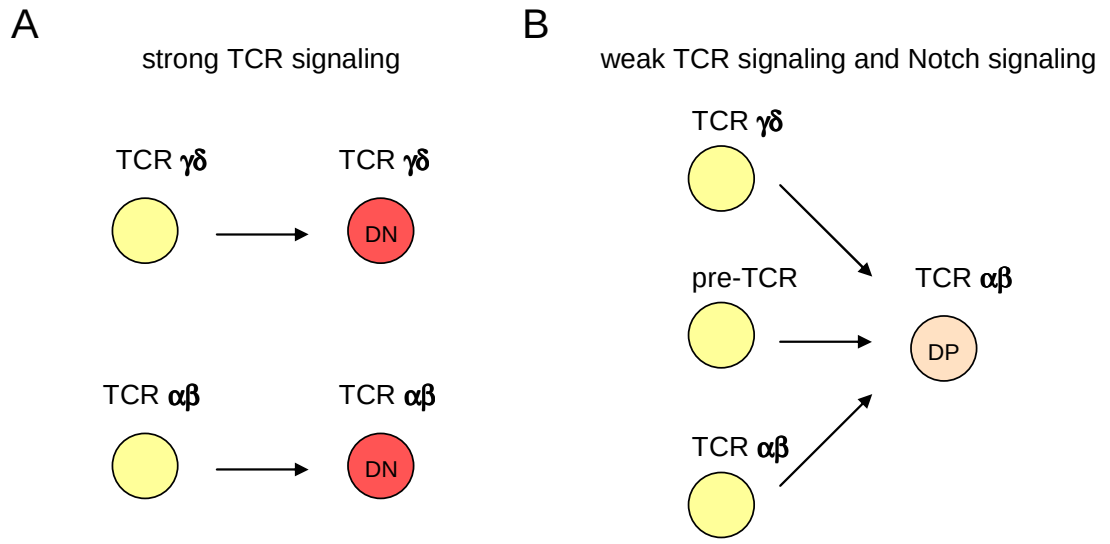

Figure 4. Influence of TCR signal strength and Notch signaling on the αβ/γδ lineage choice adapted from (Garbe and von Boehmer, 2007).
(A) Strong signaling by the TCR γδ or the TCR αβ results in differentiation into DN γδ lineage cells or DN cells with a γδ lineage phenotype but TCR αβ expression.
(B) Weak signal by the TCR γδ, pre-TCR or TCR αβ together with Notch signaling results in differentiation into αβ lineage cells.

Recently involvement of *Sox13* transcription factor in regulation of αβ/γδ lineage differentiation has been shown. *Sox13* is essential for the proper development of TCR γδ cells, but not TCR αβ cells. It is highly expressed on DN1 and DN2 cells and subsequently downregulated in αβ-lineage differentiating cells. Significant

levels of *Sox13* are maintained in peripheral TCR γδ cells. Since *Sox 13* expression by DN2 cells is heterogenous (50% of DN cells are positive for *Sox13*) it has been suggested that some lineage separation occurs even before TCR rearrangement (Melichar et al., 2007).

TCR γδ cells developing in adult, but not fetal thymus, require a significant number of DP thymocytes which *trans* regulate differentiation of TCR γδ cells through involvement of transcription factor RORγt and lymphotoxin β receptor (LTβR). The proper signaling from LTβR is essential for correct TCR γδ biased gene expression which is required for the proper function of TCR γδ cells. Thus the *trans* conditioning overall influences rather cell's functional competences than commitment to the lineage (Pennington et al., 2003; Silva-Santos et al., 2005; Hayday and Pennington, 2007).

Another factor important for the proper development of TCR γδ cells is IL-7 receptor signaling which promotes the expansion and survival of TCR γδ cells in the thymus. Moreover, it is also required for the proper recombination of TCR γ locus (Ikuta et al., 2001).

An important question which remains to be answered is whether TCR γδ requires ligand engagement for the proper TCR γδ cells development. Up to date only indirect evidence suggest the requirement for ligand-mediated positive selection in fetal thymus of the dendritic epidermal T cells (DETC), (Xiong et al., 2004; Lewis et al., 2006). This subset of TCR γδ cells populates mouse skin.

TCR γδ cell tissue distribution

TCR γδ cells comprise 1-5% of circulating T cells while they are very abundant in tissues. In human TCR γδ cells expressing TCR Vδ1 or Vδ3 chains are predominant in the epithelium of the intestine (De Libero et al., 1993) where they comprise the majority of intraepithelial lymphocytes (IELs). Cells bearing TCR Vδ2 chain localize mainly in secondary lymphoid organs, tonsils and peripheral blood where they represent 5-10% of total lymphocytes (Casorati and Migone, 1990; Parker et al., 1990; Haas et al., 1993). Importantly, in humans and some primates about 50-80% of all TCR γδ express restricted TCR composed by the Vγ9 and Vδ2 chains (Porcelli et al., 1991). In the postnatal thymus TCR Vγ9-Vδ2 cells constitute a minor population (up to 1% of total thymocytes), (Casorati et al., 1989; Falini et al., 1989) but they expand in the periphery, probably due to the continuous stimulation by unknown factors (Parker et al., 1990).

Also in mice certain populations of TCR γδ cells localize in epithelia of particular organs. In the epidermis DETC account for ~100% of resident IELs. They have TCR composed of Vγ3 and Vδ1 chains lacking junctional diversity (Havran and Allison, 1990). TCR Vγ4 chain is predominantly expressed in the reproductive tract, lung and tongue (Itohara et al., 1990). In the small intestine TCR γδ cells preferentially use Vγ5 and Vγ1.1 chains (Pereira et al., 2000) while in the secondary lymphoid organs TCR γδ cells mainly express Vγ2, Vγ1.1 and Vγ1.2 chains (Raulet et al., 1991; Pereira et al., 2000).

The tissue specific distribution of cells expressing particular TCR γδ heterodimers most likely is associated with recognition of ligands present at the localization site.

TCR γδ stimulatory antigens

Natural non-peptidic phosphorylated antigens

The main population of human TCR γδ cells expressing TCR Vγ9-Vδ2 respond *in vitro* to pathogen derived (both bacterial and parasite) extracts (Morita et al., 2000). The stimulatory components, obtained form mycobacteria cell lysates, have small molecular weight (less than 500 Da), are protease-resistant and contain critical phosphate residue (Pfeffer et al., 1990; Constant et al., 1994; Schoel et al., 1994; Tanaka et al., 1994). The analysis of *Mycobacterium smegmatis* culture supernatants resulted in identification of isopentenylpyrophosphate (IPP) and its hydroxymethyl derivatives as natural TCR γδ ligands (Tanaka et al., 1995). These compounds are intermediate metabolites of isoprenoids biosynthesis. In eukaryotes, archaebacteria and certain eubacteria the biosynthesis of IPP proceeds via mevalonate pathway, while in many eubacteria and plastids of algae and higher plants, IPP is supplied by 2-*C*-methyl-**D**-erythritol 4-phosphate (MEP) pathway (Eberl et al., 2003), (Figure 5 and Table 1).

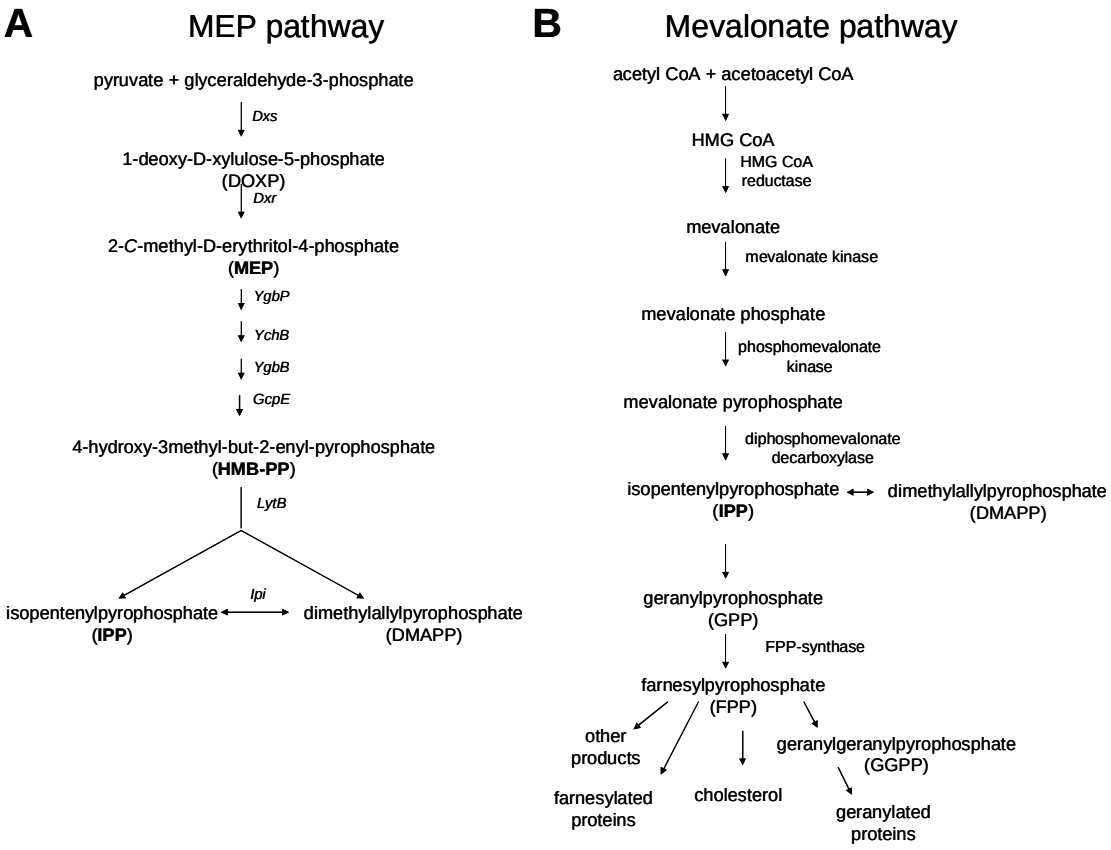

Figure 5. Schematics of prokaryotic MEP and eukaryotic mevalonate pathways of isoprenoids biosynthesis.

(A) MEP pathway with indicated genes coding enzymes.

(B) Mevalonate pathway with indicated selected enzymes.

Organism	MEP pathway	Mevalonate pathway
Prokaryotes		
Eubacteria		
Mycobacterium tuberculosis	+	-
Mycobacterium leprae	+	-
Escherichia coli	+	-
Haemophilus influenzae	+	-
Chlamydia pneumoniae	+	-
Pseudomonas aeruginosa	+	+
Listeria monocytogenes	+	+
Staphylococcus aureus	-	+
Streptococcus pneumoniae	-	+
Borrelia burgdorferi	-	+
Archaebacteria		
Pyrococcus horikowskii	-	+
Methanobacterium	-	+
Eukaryotes		
Apicomplexan parasites		
Plasmodium farciparum	+	-
Plants		
Arabidopsis thaliana	+	+
Fungi		
Saccharomyces ceravisiae	-	+
Mammals	-	+

Table 1. Utilization of MEP and mevalonate pathways by selected organisms, adapted from (Morita et al., 2000).

Beside IPP also other mevalonate pathway metabolites, including dimethylallylpyrophosphate (DMAPP), farnesylpyrophosphate (FPP), geranylgeranylpyrophosphate (GGPP) and geranylpyrophosphate (GPP), (Burk et al., 1995; Tanaka et al., 1995; Morita et al., 1999), (Figure 6) behave as TCR Vγ9-Vδ2 cells ligands, although with a potency 30-300 times lower than that IPP. Furthermore, an antigenic intermediate of bacterial MEP pathway, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) was identified as very strong TCR

γδ antigen (Hintz et al., 2001), (Figure 6). The reactivity of human peripheral blood mononuclear cells (PBMC) towards HMB-PP is restricted to TCR Vγ9-Vδ2 cells and leads to upregulation of activation markers, secretion of pro-inflammatory cytokines and expansion of these TCR γδ cells (Hintz et al., 2001; Eberl et al., 2002; Reichenberg et al., 2003).

The stimulatory capacity of HMB-PP for TCR Vγ9-Vδ2 cells is approximately four orders of magnitude higher than IPP. Therefore it was suggested that HMB-PP is the key activator of TCR Vγ9-Vδ2 cells during bacterial infection (Eberl et al., 2003). This aspect will be further discussed in this work.

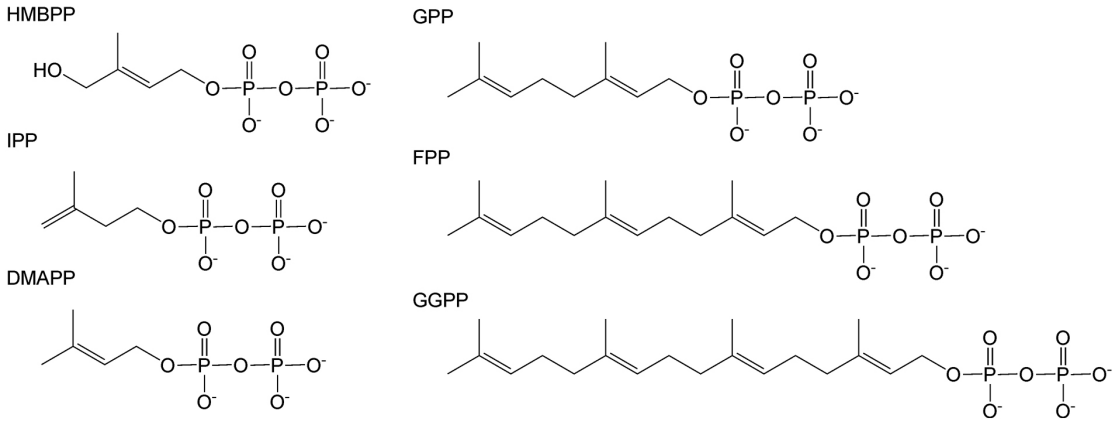

Figure 6. Structures of selected natural prenyl pyrophosphates.

Synthetic stimulatory ligands for TCR Vγ9-Vδ2 cells

Up to date a number of synthetic compounds (Figure 7) that are able to stimulate TCR Vγ9-Vδ2 cells have been synthesized, including methyl phosphate, monoethyl pyrophosphate (Tanaka et al., 1994), bromhydrin pyrophosphate (BrHPP, Phosphstim^{TM}), (Belmant et al., 2000; Espinosa et al., 2001) and 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP), (Tanaka et al., 2007). These compounds specifically activate TCR Vγ9-Vδ2 cells which upon stimulation release cytokines and induce target cell lysis (Belmant et al., 2000). As compared with naturally occurring ligands synthetic compounds are very strong agonist *i.e.* BrHPP have half-maximal activity in the nano molar concentration range (Belmant et al., 2000; Espinosa et al., 2001). Currently BrHPP and 2M3B1PP are produced in large scale amounts and are being used for clinical trials.

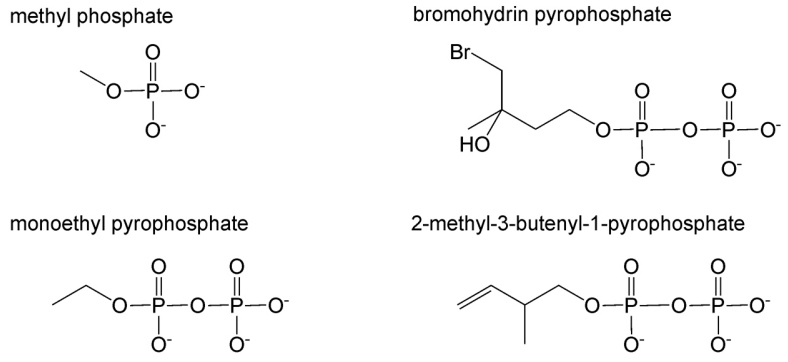

Figure 7. Structures of selected stimulatory synthetic phosphorylated antigens.

Another group of synthetic compounds are nitrogen-containing bisphosphonates (nBP). There drugs prevent bone resorption and are used for treatment of Paget's disease (Hosking, 2006), tumor-associated bone diseases (Sauty et al., 1996; Berenson et al., 2001; Tripathy et al., 2004) and osteoporosis (Boonen et al., 2005). They induce selective expansion of TCR Vγ9-Vδ2 cells in peripheral blood of human beings after intravenous administration (Kunzmann et al., 2000). Activated by nBP TCR γδ cells secrete pro-inflammatory cytokines and kill target tumor cells *in vitro* (Kunzmann et al., 2000). Structural similarities of nBP and bacterial antigens suggested that they might mimic natural ligands (Kunzmann et al., 1999), (Figure 8).

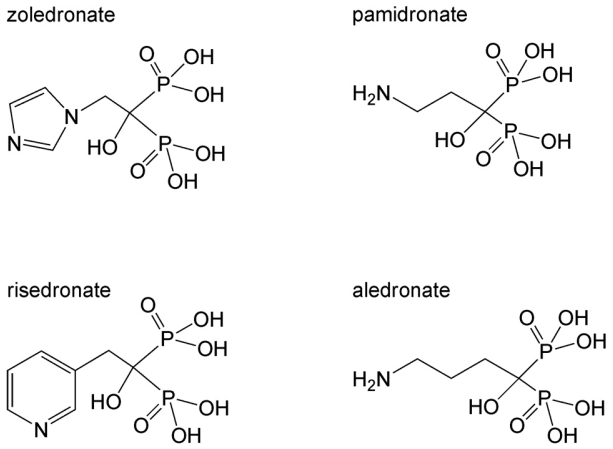

Figure 8. Structures of selected biological active nBP.

Importantly, nBP by blocking farneslypyrophosphate-synthase (FPP-synthase), (Thompson et al., 2002) one of the enzymes in the mevalonate pathway (Figure 5), lead to accumulation of IPP/DMAPP *in vitro* (Bergstrom et

al., 2000). Therefore, nBP could increase levels of intracellular ligands responsible for activation of TCR γδ cells (see this work).

Alkylamines

Different class of compounds activating TCR Vγ9-Vδ2 cells in an antigen specific manner are alkylamines (Bukowski et al., 1999). These small organic molecules contain short (two to five carbon atoms) alkyl chain linked to positively charged amine group (Figure 9). Alkylamines are produced and secreted by several bacterial strains (Daneshvar et al., 1989; Bukowski et al., 1999). They were also found in plants (apples), (Hartmann, 1967) and plant products (tea or wine), (Asatoor, 1966; Ibe et al., 1991). In order to stimulate TCR γδ cells, alkylamines must be present in milli molar concentrations which are unlikely present *in vivo*. This raises the question of the physiological relevance of alkylamines of TCR γδ activation and of the mechanisms how they are active.

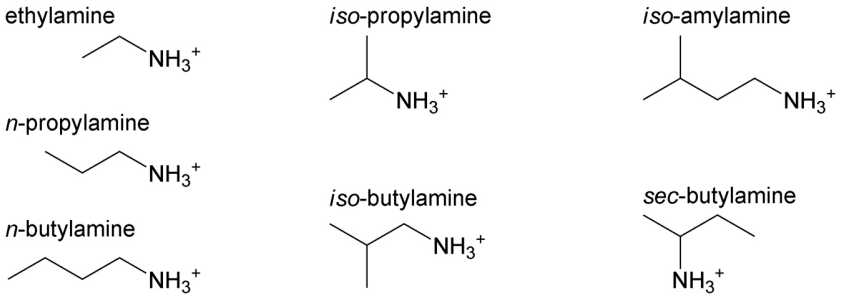

Figure 9. Structures of selected alkylamines.

TCR Vγ9-Vδ2 antigen recognition

TCR Vγ9-Vδ2 cells are activated in a crossreactive manner by variety of ligands like IPP, DMAPP, 2,3-diphosphogyceric acid (DPG), glycerol-3-phosphate (G3P), xylose-1-phosphate (Xyl-1P) , ribose-1-phosphate (Rib-1-P), (Burk et al., 1995). Optimal stimulation of TCR γδ cell is driven by cells of human origin (De Libero et al., 1991). The recognition of phosphate-containing antigens requires cell-cell interactions without the need for antigen processing (De Libero et al., 1991; Kabelitz et al., 1991; Lang et al., 1995; Morita et al., 1995). Taken together this suggests the existence of a dedicated antigen-presenting molecule. TCR Vγ9-Vδ2 cells recognize phosphorylated antigens in the absence of MHC or CD1 restriction since APC lacking MHC class I, β2-microglobulin, CD1, or MHC class II molecules are able to activate TCR Vγ9-Vδ2 cells (Morita et al., 1995). Moreover, tumor cell lines and normal PBMCs are able to activate TCR Vγ9-Vδ2 cells form different donors (Morita et al., 1995). Thus, the putative antigen-presenting molecule has rather limited or no polymorphism and is constitutively expressed in a variety of tissues.

TCR γδ cells reactivity to MHC and MHC–like molecules

<u>CD1c restricted TCR γδ cells</u>

Human TCR γδ composed of Vδ1 chain paired with either Vγ1 or Vγ2 chains recognize CD1c molecule which is mainly expressed on immature DC and B cells (Faure et al., 1990; Spada et al., 2000). CD1c is a member of CD1 family molecules which are non-MHC-encoded proteins sharing structural similarities

with MHC class I molecules. CD1 antigen-presenting molecules have little polymorphism and are specialized in the presentation of lipids and glycolipids (Beckman et al., 1994; Matsuda and Kronenberg, 2001; De Libero and Mori, 2003).

This recognition occurs in the absence of exogenous foreign antigen (Spada et al., 2000; Vincent et al., 2002) suggesting reactivity against self-lipid loaded into CD1c molecule (De Libero and Mori, 2003). TCR γδ cells upon recognition of CD1c secrete pro-inflammatory cytokines which contribute to the DC maturation process (Ismaili et al., 2002; Leslie et al., 2002). In addition, they have a cytolytic, Th1 effector phenotype and produce granulysin (Spada et al., 2000).

MIC and ULBP reactive TCR γδ cells

Subset of TCR Vδ1^{+} cells have been found to interact with cells expressing stress-induced MHC class I-related chain (MICA and MICB) molecules (Groh et al., 1998) and UL16-binding protein (ULBP family) molecules (Poggi et al., 2004). MIC are encoded within the MHC locus (Bahram et al., 1994) while ULBPs are encoded by genes on chromosome 6q25. MIC proteins are upregulated in response to cellular stress like heat shock in intestinal epithelium and on epithelial tumors (Groh et al., 1996; Groh et al., 1999) while ULBPs are expressed on various cancers of hematopoietic origin including acute myeloid and lymphoblastic leukemias (Salih et al., 2003).

Despite the structural similarities between MIC and MHC class I molecules, MIC do not associate with β2-microglobulin and do not present peptides, probably due to the limited size of the putative peptide binding grove and conformational differences in α1 and α2 domains (Groh et al., 1998; Li et al., 1999). TCR Vδ1$^+$ cells interact with MIC trough the TCR (TCR-dependent signal 1) and through natural killer activating receptor NKG2D (NKG2D dependent costimulatory signal 2), (Wu et al., 2002) while ULBPs most likely interact only through NK receptors (Cosman et al., 2001).

Other molecules

Among human TCR γδ cells a few T cell clones, expressing TCR Vδ1 chain, react to allo–MHC molecules including HLA-A2 (Spits et al., 1990), HLA-A24 (Ciccone et al., 1989), HLA-B27 (Del Porto et al., 1994) and some unspecified class I molecules (Rivas et al., 1989). In al these studies a cognate interaction of the TCR γδ with MHC molecules was not formally demonstrated.

In mouse TCR γδ cells recognizing T10/T22 MHC class Ib molecules (Matis et al., 1989; Van Kaer et al., 1991) or I-E^k MHC class II molecule (Matis et al., 1989) have been described. Recognition of these molecules is independent from the peptide binding and antigen processing (Schild et al., 1994; Crowley et al., 2000). In the case of I-E^k recognition depends on the post-translational changes in its glycosilation (Hampl et al., 1999).

Stimulation by bacterial superantigens

Bacterial superantigens are toxins secreted by several bacterial species, which activate T cells by binding to the non-polymorphic region of MHC class II molecules outside of the antigen binding groove (Kozono et al., 1995) and to the Vβ domain of the TCR αβ (Li et al., 1998). TCR Vγ9$^+$ cells are activated by the superantigen staphylococcal enterotoxin A (SEA), (Loh et al., 1994; Morita et al., 2001). This stimulation occurs in a MHC class II dependent but Vδ independent manner. TCR γδ cell require higher concentrations of SEA for stimulation as compared to TCR αβ cells (Morita et al., 2001). Therefore, due to the structural similarity between TCR Vγ and TCR Vβ (Allison et al., 2001) and comparable concentrations required for T cell stimulation (Surman et al., 1994) interaction of Vγ with SEA might resemble that of Vβ with staphylococcal enterotoxin B.

Effector functions of TCR γδ cells

Role of TCR γδ cells in microbial infections

TCR γδ cells were found to expand to high levels during a variety of bacterial, viral and protozoan infections. Elevated levels of TCR Vγ9-Vδ2 cells in the peripheral blood were observed in patients infected with *Mycobacterium tuberculosis* (Barnes et al., 1992), *Mycobacterium leprae* (Modlin et al., 1989), *Listeria monocytogenes* (Jouen-Beades et al., 1997), *Francisella tularensis* (Poquet et al., 1998), *Brucella melitensis* (Bertotto et al., 1993), *Salmonella typhimurium* (Hara et al., 1992), *Ehrlichia* (Caldwell et al., 1995). Also patients with the following parasite infections have increased number of TCR γδ cells:

Leishmania donovani (Raziuddin et al., 1992), T*oxoplasma* ssp (Scalise et al., 1992), *Plasmodium falciparum* (Ho et al., 1990). Moreover TCR Vγ9-Vδ2 cells may increase in case of Epstein-Barr virus (EBV), (De Paoli et al., 1990) and Herpes simplex virus (HSV), (Bukowski et al., 1994) infections.

During certain microbial infections TCR Vγ9-Vδ2 cells expand even up to 50-fold (Table 2).

Infectious disease	% of TCR γδ cells	Reference
bacterial		
tuberculosis	14 (35)	(Balbi et al., 1993)
tularemia	33	(Sumida et al., 1992; Poquet et al., 1998)
salmonellosis	18 (48)	(Hara et al., 1992)
brucellosis	29 (48)	(Bertotto et al., 1993)
ehrlichiosis	57 (97)	(Caldwell et al., 1995)
H. influence/meningitis	27 (37)	(Raziuddin et al., 1994)
N. meningititis/meningitis	25 (42)	(Raziuddin et al., 1994)
S. pneumoniae/meningitis	35 (46)	(Raziuddin et al., 1994)
legionellosis	15	(Kroca et al., 2001)
listeriosis	12 (33)	(Jouen-Beades et al., 1997)
Coxiella brunetii/Q-fever	16 (30)	(Schneider et al., 1997)
parasite		
acute malaria (non endemic)	18 (46)	(Schwartz et al., 1996)
toxoplasmosis	9 (15)	(Scalise et al., 1992)
leishmaniases	13 (18)	(Russo et al., 1993)

Table 2. Examples of human TCR γδ cells expansion in response to infection. Percentage of TCR γδ cells shows mean values detected in patients. Maximal number of detected cells is shown in brackets.

Expanded and activated TCR Vγ9-Vδ2 cells may directly participate in anti-microbial immune responses inducing killing of bacteria, through granulysine release, and bacteria-infected cells, through preforin and/or Fas-Fas ligand interactions (Hara et al., 1992; Dieli et al., 2000; Ottones et al., 2000). Activated TCR Vγ9-Vδ2 cells are able to produce significant amounts of Th1 cytokines: IFNγ and TNFα providing an important stimulus for macrophages attraction during the early stage of infection (Garcia et al., 1997; Wang et al., 2001). Moreover, TCR Vγ9-Vδ2 cells release large quantities of the β-chemokines such as macrophage inflammatory protein-1α (MIP-1α, CCL3) and MIP-1β (CCL4), (Cipriani et al., 2000). *In vitro* MIP-1α and MIP-1β attract TCR αβ $CD4^+$ and TCR αβ $CD8^+$ cells, respectively (Schall et al., 1993; Taub et al., 1993). Therefore, chemokines release by TCR Vγ9-Vδ2 cells might contribute to the pro-inflammatory microenvironment at the sites of infection.

The levels of TCR Vδ1/Vδ3 are elevated in renal allograph recipients developing cytomegalovirus (CMV) infection (Dechanet et al., 1999a). Moreover TCR $V\delta2^-$ cells activated by CMV-infected fibroblasts produce large amounts of TNFα and kill infected cells. This recognition is mediated by TCR independently of MHC class I presentation and without NKG2D engagement (Halary et al., 2005). Infection with human immunodeficiency virus-1 (HIV-1) leads to proliferation of TCR Vδ1 cells in the peripheral blood (Hinz et al., 1994). Most likely, these TCR γδ cells are activated in the intestinal epithelia and then migrate to the peripheral blood (Dechanet et al., 1999b).

In mice TCR γδ cells expand in response to mycobacteria (Janis et al., 1989), listeria (Hiromatsu et al., 1992) and salmonella (Emoto et al., 1992) infections. Importantly, mice lacking TCR γδ cells develop enhanced inflammation characterized by disruption of macrophage homeostasis and liver necrosis (Carding and Egan, 2002) and they do not survive infections with *Listeria monocytogenes* (Skeen et al., 2001) or *Klebsiella pneumoniae* (Moore et al., 2000). Furthermore, in TCR αβ deficient mice, TCR γδ cells provide early protective immune responses against listeriosis (Mombaerts et al., 1993) and malaria (Tsuji et al., 1994).

Tumor surveillance

TCR Vγ9-Vδ2 cells have been shown to recognize and kill tumor transformed cells like B cell lymphomas (Fisch et al., 1990; Selin et al., 1992), thymic lymphomas (De Libero et al., 1991) and erythroleukemia cells (Di Fabrizio et al., 1991). Moreover, this population of TCR γδ cells is expanded in blood and/or intra-lesions of patients with hemopoietic and solid tumors (Bonneville and Fournie, 2005).

The potent anti-tumor activities of TCR γδ cells have recently stimulated great interest in of TCR γδ cells cell-based cancer immunotherapy. TCR Vγ9-Vδ2 cells expanded *ex vivo*, with BrHPP and IL-2 and derived from patients with metastatic form of renal cell carcinoma have the ability to kill autologus primary renal tumor cells (Viey et al., 2005). Furthermore, nBP activated TCR Vγ9-Vδ2 cells produce cytokines, exhibit specific cytotoxicity against myeloma cell lines,

and lead to reduced survival of autologous myeloma cells (Kunzmann et al., 2000). TCR Vγ9-Vδ2 cells expanded *in vitro* in the presence of aledronate (another nBP) and IL-2 maintain their anti-tumor activity *in vivo* after adoptive transfer into mice with severe combined immunodeficiency (SCID), (Kabelitz et al., 2004).

Promising results in the treatment of patients with low-grade non-Hodgkin lymphoma and multiple myeloma were achieved by *in vivo* stimulation of TCR γδ cells using pamidronate and low-dose IL-2. This type of immunotherapy resulted in tumor regression (Wilhelm et al., 2003).

TCR Vγ9-Vδ2 cells have a phenotype of memory cells (Miyawaki et al., 1990; De Rosa et al., 2004) and the capacity to promptly release IFNγ and TNFα. These characteristics implicate that they can be rapidly recruited to the site of tumorgenesis and therefore contribute to early immune protection.

Human TCR Vδ1 cells exhibit a selective lytic activity against various tumor cell lines like colorectal cancer, esophageal cancer, renal cell cancer, pancreatic cancer, lung cancer (Ferrarini et al., 1994; Zocchi et al., 1994; Choudhary et al., 1995; Maeurer et al., 1996; Groh et al., 1999; Thomas et al., 2001). The recognition of tumor cells occurs via MIC or ULBP molecules which interact directly with NKG2D and possibly with TCR present on TCR Vδ1 cells (Groh et al., 1999; Wu et al., 2002; Poggi et al., 2004). Recently, it was reported that upon MICA-NKG2D interactions the antigen-dependent effector functions of TCR Vγ9-Vδ2 cells can be enhanced (Das et al., 2001a).

Tissue homeostasis and repair

TCR γδ cells from both human and mouse are able to produce keratinocyte growth factor (KGF), (Boismenu and Havran, 1994; Workalemahu et al., 2003), a cytokine promoting epithelial cell growth (Visco et al., 2004). Human TCR γδ cells from bronchoalveolar lavage in the presence of IPP are able to secrete fibroblast growth factor 9 (FGF-9), associated with epithelial cell proliferation (Workalemahu et al., 2004).

Furthermore, upon activation with IPP TCR γδ cells secrete metalloproteinase 7 (MMP7) which serves a key role in epithelium repair (Workalemahu et al., 2006). Therefore TCR γδ cells contribute to the maintenance of epithelial homeostasis and play a role in the restoring epithelial integrity.

Mouse DETCs can be activated *in vitro* by stressed or damaged keratinocytes. Upon activation they secrete KGF which implicates their role in the response to skin damage. They also participate in keratinocyte survival by constitutively secreting insulin-like growth factor 1 (IGF-1). In addition TCR $\delta^{-/-}$ mice have problems with tissue repair and keratinocyte homeostasis. The lung and intestine TCR γδ cells, exhibit similar roles in the maintenance epithelium integrity (Witherden et al., 2000; Jameson and Havran, 2007).

The fact that TCR γδ cells reside in epithelial tissues of all mammals, suggest that they play a conserved role in the monitoring of tissue integrity.

TCR γδ cells in autoimmune diseases and inflammations

There are findings suggesting that TCR γδ cells could be involved in the pathogenesis of autoimmune diseases. Accumulation of TCR γδ cells has been observed in the inflamed synovium of patients with rheumatoid arthritis (Holoshitz, 1999). By using a mouse model of collagen induced arthritis it was demonstrated that TCR γδ cells can have pro-inflammatory functions early in the disease but anti-inflammatory during the late stage of the disease (Peterman et al., 1993).

The number of TCR Vγ9-Vδ2 cells is increased in the peripheral blood of patients with diabetes mellitus (Lang et al., 1991) and in pre-diabetic and diabetic children (Gyarmati et al., 1999). The regulatory role of TCR γδ cells in diabetes was studied by using non-obese diabetic (NOD) mouse model. The intranasal inhalation of pro-insulin leads to the generation of a population of regulatory TCR γδ cells that can suppress the development of diabetes (Harrison et al., 1996).

In case of patients with multiple sclerosis (MS) elevated levels of TCR Vδ1 cells were found in the brain lesions (Wucherpfennig et al., 1992) and cerebrospinal fluid (Nick et al., 1995). The same population of TCR γδ cells has been found to be increased in the blood and intestine of patients with inflammatory bowel disease (Soderstrom et al., 1996; McVay et al., 1997).

Part 1

Intracellular endogenous ligands activating TCR Vγ9-Vδ2 cells

(These results have been published in *The Journal of Experimental Medicine*, **2003**, *197*, 163-168)

TCR Vγ9-Vδ2 cells recognize bacteria phosphorylated metabolites and also react to bone marrow-derived tumor cells such as Daudi Burkitt's lymphoma line (Fisch et al., 1990; Malkovska et al., 1992). In order to identify the nature of the tumor antigens we investigated whether TCR γδ cells recognize phosphorylated nonpeptidic ligands resembling those produced by microbes. One of the potent identified bacterial antigens is IPP, an intermediate product of isoprenoids biosynthesis which is present in prokaryotic as well as in eukaryotic cells (Burk et al., 1995; Tanaka et al., 1995). Bacteria produce IPP in either MEP or mevalonate pathways (Morita et al., 2000), (Figure 5), whereas in eukaryotes IPP is generated exclusively in the mevalonate pathway (Figure 5). It was previously reported that in some hematological malignancies (Harwood et al., 1991) and mammary carcinomas (Asslan et al., 1999) expression and function of 3-hydroxymethyl-3-glutaryl-CoenzymeA-reductase (HMGR), the rate-limiting enzyme in the mevalonate pathway, is increased. Based on these findings we investigated whether dysregulation of mevalonate pathway in tumors may lead to accumulation of mevalonate metabolites thus resulting in activation of TCR Vγ9-

Vδ2 cells. In the following studies we took advantage of drugs that influence enzymes of the mevalonate pathway (Figure 10).

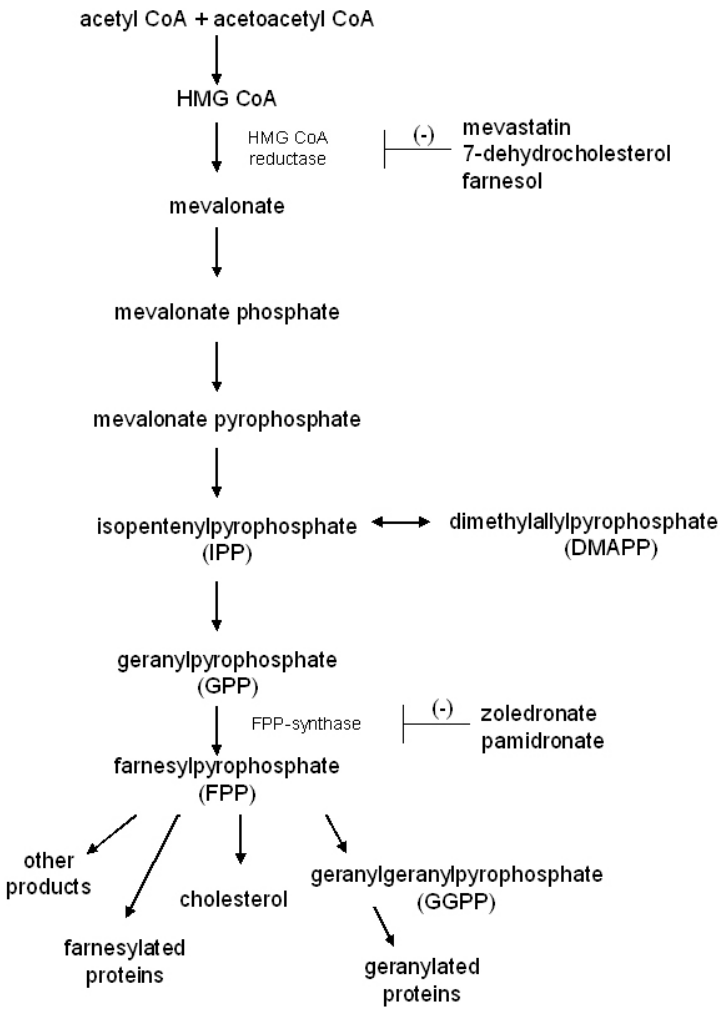

Figure 10. Mevalonate pathway with indicated compounds used in this study and the affected enzymes.

Results

Active HMGR in tumor cells is required for activation of TCR γδ cells

Various cell lines of human origin were tested for the capacity to activate TCR Vγ9-Vδ2 cells in the presence or absence of exogenous IPP and after treatment with ZOL (Table 3).

APC	Cell type	Medium	IPP	ZOL
Daudi	Bone marrow, B-cell lymphoma	2527*	14342	5647
THP-1	Bone marrow, monocytes	197	3135	16393
CEM 1.3	Bone marrow, T-cell lymphoma	310	6586	1564
K562	Bone marrow, erythroleukemia	36	2762	1145
A-375	Skin, melanoma	188	9169	15842
A-431	Skin, epidermoid carcinoma	89	3269	3067
Colo 201	Colon epithelia, coloncarcinoma	260	3103	4374
HEP G2	Liver parenchyma, hepatocarcinoma	96	12040	12417
HuH6	Liver parenchyma, hepatoblastoma	80	4329	3451
A-243	Central nervous system, astrocytoma	23	5415	7141
U118	Central nervous system, glioblastoma	33	2114	5575
BS 125.3.2	Central nervous system, glioblastoma	46	13942	8440
MRK-nu-1	Mammary gland, mammary carcinoma	35	3003	1965
HMC-1-8	Pleural effusion, mammary carcinoma	17	2011	1845
YMB-1	Mammary gland, mammary carcinoma	1162	3695	1525
Fibroblasts	isolated from primary, lung connective tissue	175	5085	8768

Table 3. Tumor cell lines of different tissue origin and primary lung fibroblasts were used as APC. Cells were either incubated with medium or IPP (10 µM) or were pulsed with ZOL (zoledronate, 50 µg/ml) for 3 h before T cell were added. * Numbers represent mean values in pg/ml of TNFα release by the G2B9 TCR γδ clone.

We found that not only Daudi cell but also YMB-1 cells, a solid breast carcinoma activate TCR γδ cells (Table 3). In order to investigate whether HMGR is involved in generation of TCR γδ stimulatory antigens we treated both, Daudi and YMB-1 cells with mevastatin (MEV), and inhibitor of the HMGR catalytic site (Istvan and Deisenhofer, 2001) 2 h before TCR γδ cells addition. Activation of TCR Vγ9-Vδ2 was strongly reduced in the presence of MEV treated Daudi or YMB-1 cells (Figure 11). To rule out toxicity and unspecific inhibitory effect of MEV stimulation with exogenous IPP and PHA was also performed (Figure 11).

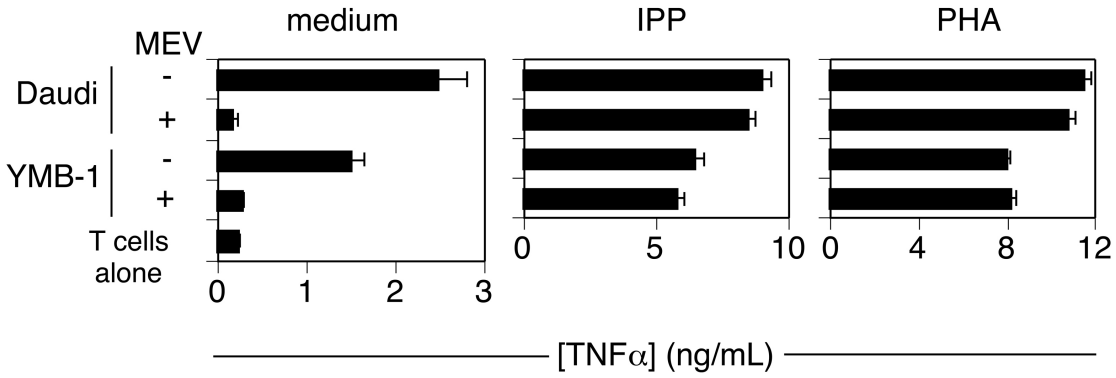

Figure 11. TCR γδ cell activation, by tumor cells, depends on active HMGR.
Daudi or YMB-1 cells, in the absence or presence of MEV, were used as APC in TCR γδ cell activation assay. In control experiment stimulation with exogenously added IPP or PHA was performed.

HMGR is one of the most tightly regulated enzyme in the cells (Goldstein and Brown, 1990). The activity of HMGR is controlled through protein synthesis, degradation and phosphorylation (Cheng et al., 1999). In order to further investigate the involvement of HMGR in the generation of TCR γδ stimulatory

ligands we treated Daudi cells with 7-DHC or farnesol, two endogenous metabolites that facilitate degradation of HMGR (Correll et al., 1994; Honda et al., 1998). Both compounds induced reduction of intracellular HMGR protein levels (Figure 12A) and significantly inhibited TCR γδ activation by Daudi cells (Figure 12B). In control experiments TCR γδ cell activation was not inhibited when stimulated with exogenous IPP in the presence of these compounds (Figure 12B).

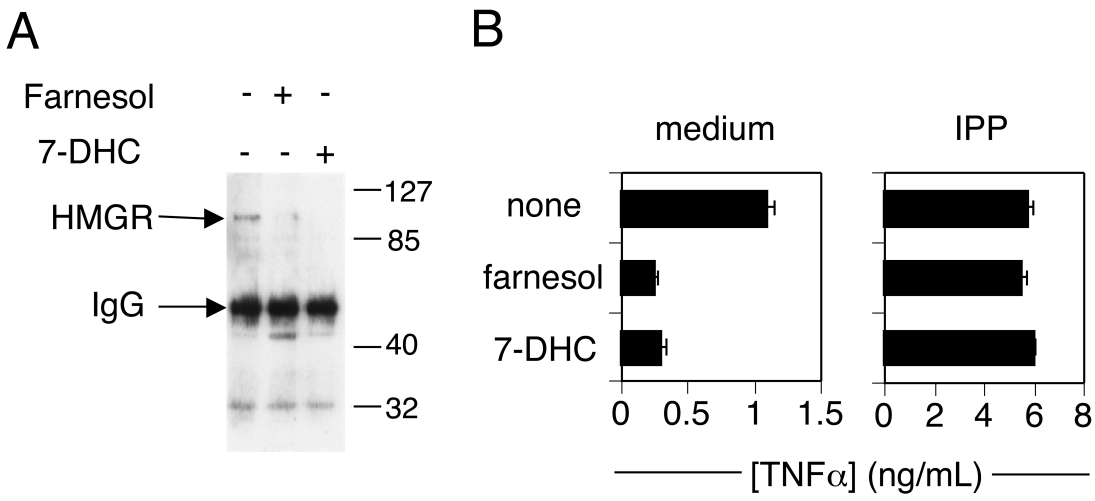

Figure 12. TCR γδ cell activation by tumor cells depends on the HMGR protein level.
(A) HMGR protein levels in lysates after treatment of Daudi cells with 7-DHC or farnesol detected by immunoprecipitation and Western blot.
(B) Daudi cells treated either with 7-DHC or farnesol were used to stimulate TCR γδ cells. As control, activation in the presence of exogenously added IPP was performed.

These experiments show that active HMGR is important for stimulation of TCR γδ cells by Daudi and YMB-1 cells. Moreover, the data suggest that indeed phosphorylated metabolites generated in the mevalonate pathway are the stimulatory ligands for TCR Vγ9-Vδ2 cells.

HMGR overexpressing cells are potent TCR γδ cells stimulators

Transfected cells expressed high levels of HMGR as shown by intracellular staining with a specific HMGR mAb (Figure 13)

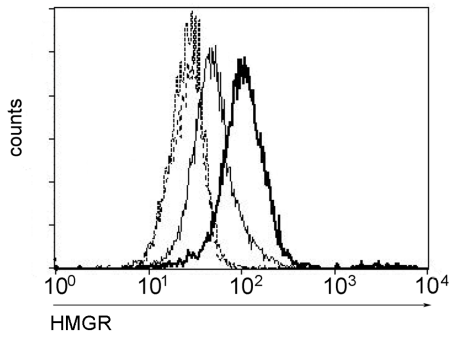

Figure 13. HMGR overexpression in transfected Daudi cells.
Intracellular level of HMGR protein in HMGR-trasfected (bold line) or non-transfected (thin line) Daudi cells was determined by staining with anti-HMGR specific mAb. Control staining was performed with irrelevant mAb (dotted lines).

These cells were used as APC in TCR γδ activation assay. The capacity to stimulate TCR γδ cells by Daudi-HMGR transfected cells was significantly increased as compared to the wild type Daudi cells (Figure 14). Moreover, when APC were treated with suboptimal doses of MEV, T cell activation was strongly

reduced. The complete inhibition, in case of Daudi-HMGR cells, was achieved only when MEV was added at least 12 h before the assay (Figure 14). HMGR-transfected cells might require longer incubation with inhibitor since the HMGR protein level in these cells is increased and also mevalonate metabolites might reach higher concentrations.

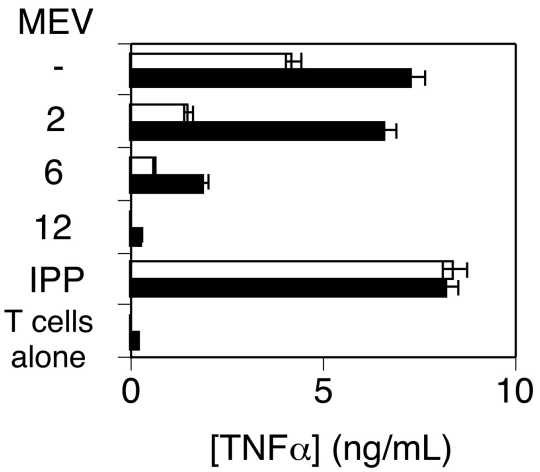

Figure 14. HMGR overexpression increases TCR γδ cell activation.
HMGR-transfected (closed bars) or wt Daudi cells (open bars) were used as APC in TCR γδ stimulation assay. A suboptimal dose (10 μM) of MEV was added 2, 6 or 12 h before incubation with T cells. Stimulation with exogenouse IPP was used as positive control.

Nitrogen-containing bisphosphonates treated APC activate TCR γδ cells

The involvement of endogenous mevalonate pathway in the generation of TCR γδ stimulatory ligands was further investigated in a series of experiments with nitrogen-containing bisphosphonate drugs (nBP). These drugs have a

capacity to inhibit farneslypyrophosphate-synthase (FPP-synthase), (Thompson et al., 2002), (one of the enzymes in the mevalonate pathway, Figure 5B) and *in vitro* lead to accumulation of IPP/DMAPP (Bergstrom et al., 2000) metabolites that are able to activate TCR γδ cells when exogenously added (Selin et al., 1992; Burk et al., 1995; Tanaka et al., 1995).

We tested two structurally different nBP, zoledronate (ZOL) and pamidronate (PAM) in TCR γδ activation assay. We compared their effect with etidronate, a non-nBP compound that does not block FPP-synthase (Bergstrom et al., 2000; Dunford et al., 2001). APC treated with either ZOL or PAM, but not with etidronate, stimulated TCR γδ cells very efficiently (Figure 15).

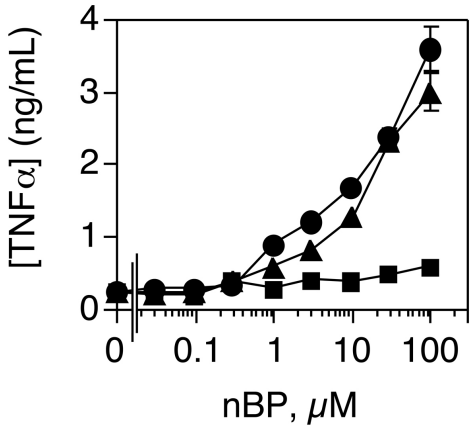

Figure 15. nBP treated APC stimulate TCR γδ cells.
Daudi cells treated with increasing doses of ZOL (▲) or PAM (●) or etidronate (■) were used as APC in TCR γδ activation assay.

These results show that not all compounds containing a phosphate group induce stimulation of TCR γδ cells and confirm previous studies performed with other nBP (Kunzmann et al., 2000; Das et al., 2001b).

nBP have different mechanisms of action than IPP

When stimulated with exogenously added phosphorylated metabolites TCR γδ cells require their permanent presence during the assay. When IPP is added to APC and then cells are washed, the capacity of APC to stimulate TCR γδ cells is lost. Presumably the activatory compounds are not stably associated on the surface of APC (Morita et al., 1995). We tested whether the same rule applies to nBP treated cells.

THP-1 cells were pulsed for 2 h with ZOL, PAM or IPP and then extensively washed before adding TCR γδ cells. After pulsing with ZOL or PAM, but not with IPP, THP-1 cells strongly activated TCR γδ cells (Figure 16). In control experiments all compounds were stimulatory when they were kept during the whole assay (Figure 16).

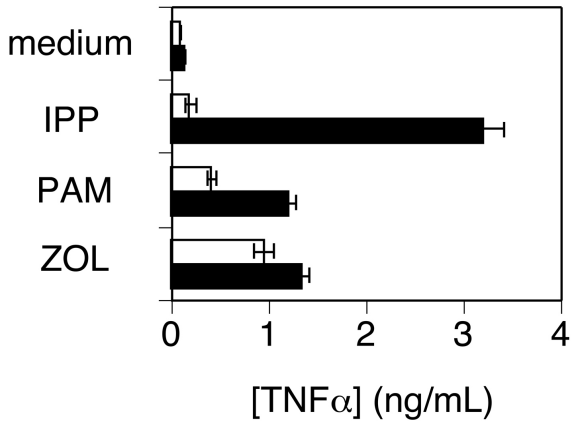

Figure 16. nBP drugs can be pulsed on APC.
THP-1 cells were pulsed for 2 h (open bars) with nBP or IPP and used for stimulation of TCR γδ cells. Overnight incubation (closed bars) with ZOL or PAM was used as a positive control.

These data show that nBP behave differently from IPP and confirm previous observations that nBP can be pulsed on primary monocytes and on different tumor cell lines (Das et al., 2001b; Kato et al., 2001).

We compared the kinetics of T cell activation by ZOL- or PAM-treated APC. Daudi cells were pulsed with ZOL or PAM for various periods of time before adding TCR γδ clone. ZOL treated APC reach 80% of maximal activation already after 2 h pulse (Figure 17). Nonetheless, cells treated with PAM reached the same stimulatory capacity after prolonged pulsing time.

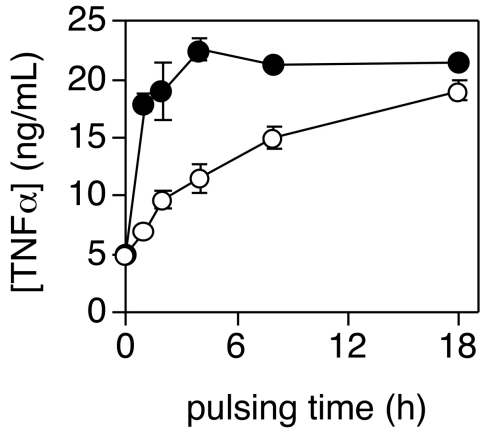

Figure 17. ZOL induces TCR γδ cells faster activation than PAM.
Daudi cells pulsed for 2 h either with ZOL (●) or PAM (○) were used to stimulate TCR γδ cells.

These results implicate that ZOL is more potent inhibitor of FPP-synthase (Dunford et al., 2001) but both compounds do not differ in their mechanism of action.

nBP require internalization for their activity

The fact, that APC treated with nBP remain stimulatory after pulsing and washing of the compounds raised the question whether their internalization into APC is mandatory for activation of TCR γδ cells. Therefore, to address this issue Daudi cells were pulsed with ZOL in conditions that inhibit pinocytosis and receptor mediated endocytosis (Schlegel et al., 1981; Wilcox et al., 1982). Pulsing the cells with ZOL either at 4°C (Figure 18A) or in the presence of monensin (Mon), (Figure 18B) strongly inhibited activation of TCR γδ cells. In

control experiments APC treated at 4°C before ZOL addition did not lose the capacity to stimulate T cells (Figure 18A) and Mon did not affect activation with exogenously added IPP (Figure 18B).

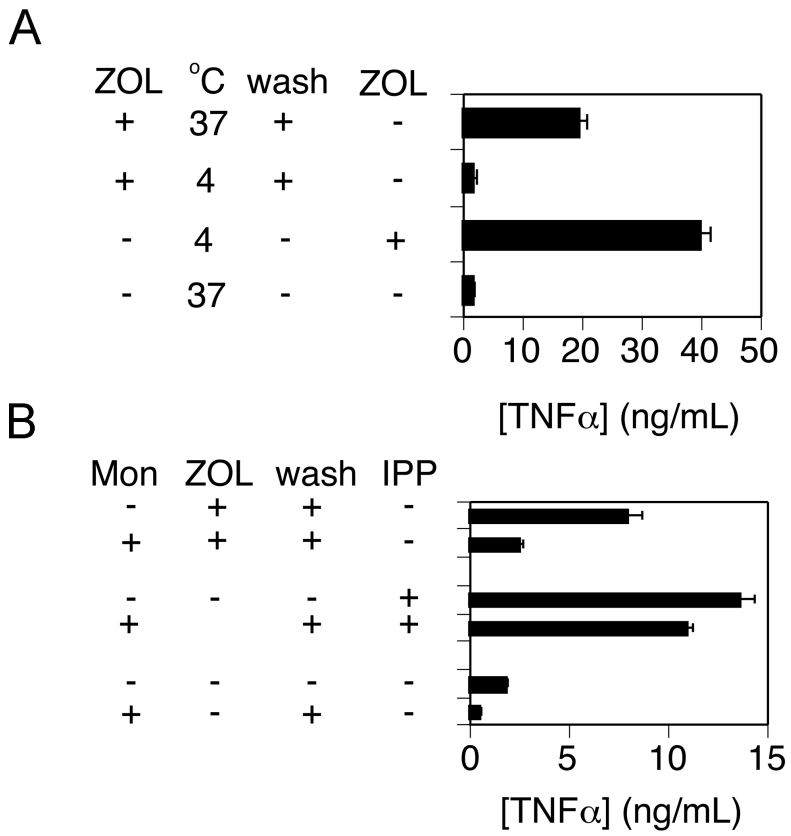

Figure 18. ZOL requires cellular internalization by APC, to induce TCR γδ cells activation.

(A) Daudi cells were pulsed with ZOL either at 4 or 37°C and used in TCR γδ activation assay. To exclude inhibitory effect of incubation at 4°C ZOL was added back to the control group.

(B) Daudi cells after pulsing with ZOL in the absence or presence of Mon were used to stimulate TCR γδ cells. Stimulation with exogenously added IPP was used as a positive control.

In order to further proof that nBP are internalized we pulsed Daudi cells with ^{14}C-labeled ZOL under similar experimental conditions and in the presence of NaN_3 in order to block ATP-dependent active uptake during pulsing time. Incubation either at 4°C or in the presence of monensin completely abolished the uptake of ^{14}C-ZOL (Figure 19).

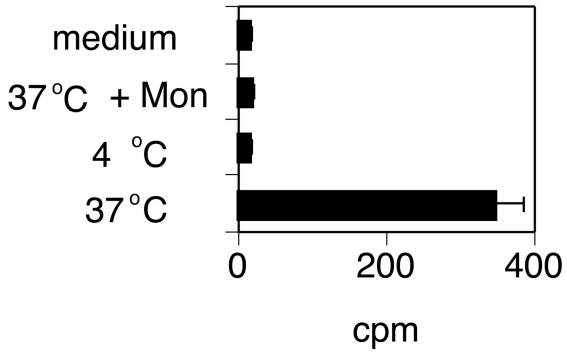

Figure 19. Intracellular uptake of ^{14}C-labeled ZOL by Daudi cells.
Daudi cells were pulsed with ^{14}C-labeled ZOL at 37°C in the absence or presence of Mon. Incubation at 4°C was performed in order to exclude surface binding of ZOL.

These data demonstrate that nBP need internalization in order to become active. This is another difference between this class of compounds and IPP which is active without being internalized (Morita et al., 1995).

nBP induce accumulation of endogenous TCR γδ ligands

The previous experiments strongly suggest that nBP induce modification in APC, which then become stimulatory for TCR γδ cells. Therefore, we

investigated the hypothesis that nBP induce intracellular accumulation of stimulatory metabolites.

In order to further examine this hypothesis we performed TCR γδ cells stimulation by Daudi cells pulsed with ZOL in the presence of MEV. The complete inhibition of TCR γδ cells activation was observed when ZOL was added simultaneously with MEV (Figure 20). One hour interval between ZOL and MEV addition reduced the inhibitory effect to 50%. When MEV was added 3 h after ZOL the inhibition was not observed anymore suggesting that accumulation of TCR γδ cells stimulatory ligands had already taken place.

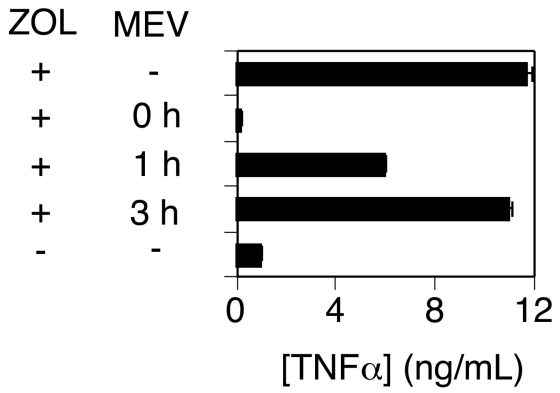

Figure 20. MEV inhibits T cell activation induced by nBP when added simultaneously with ZOL.
Daudi cells were pulsed with ZOL in the presence of MEV which was added at indicated time points after ZOL. Daudi cells were washed and used to stimulate TCR γδ cells.

This result suggests that TCR γδ cells stimulatory ligands, generated in the mevalonate pathway, must be downstream products of HMGR and upstream

intermediates of FPP-synthase. Therefore activatory metabolites are most likely prenylated pyrophosphates or mevalonic acid pyrophosphate derivatives.

Identification of metabolites important for tumor cell recognition

In order to identify stimulatory metabolites Daudi cells extracts were used to generate them *in vitro* utilizing mevalonic acid, the first metabolite in mevalonate pathway produced by HMGR. Purified products were treated with alkaline phosphatase and were tested in TCR γδ cells activation assay. The TCR γδ cell stimulatory capacity of generated ligands was sensitive to alkaline phosphatase treatment (Figure 21).

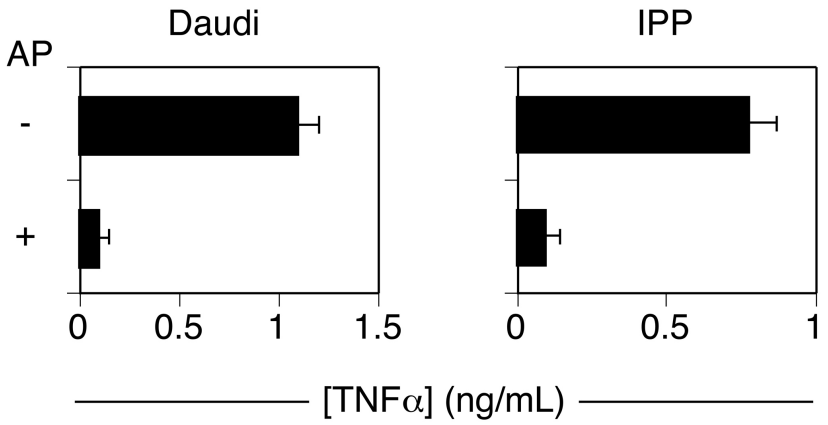

Figure 21. The stimulatory ligands present in Daudi extracts are alkaline phosphatase (AP) sensitive.
Daudi cell extracts were treated with AP and then tested for the capacity to activate TCR γδ cells. IPP treated identically was used as a positive control for AP activity.

In the next set of experiments Daudi cell extracts were incubated with ^{3}H-mevalonic and radioactive downstream products were detected in the HPLC fractions. The retention time obtained with Daudi cell extracts was identical as the reference ^{3}H-labeled IPP (Figure 22A, B and C). All obtained HPLC fraction was tested in TCR γδ cells activation assay. The same fractions from the Daudi cell extracts (which elute at 15-16 min) were also capable to stimulate TCR γδ cells (Figure 22D).

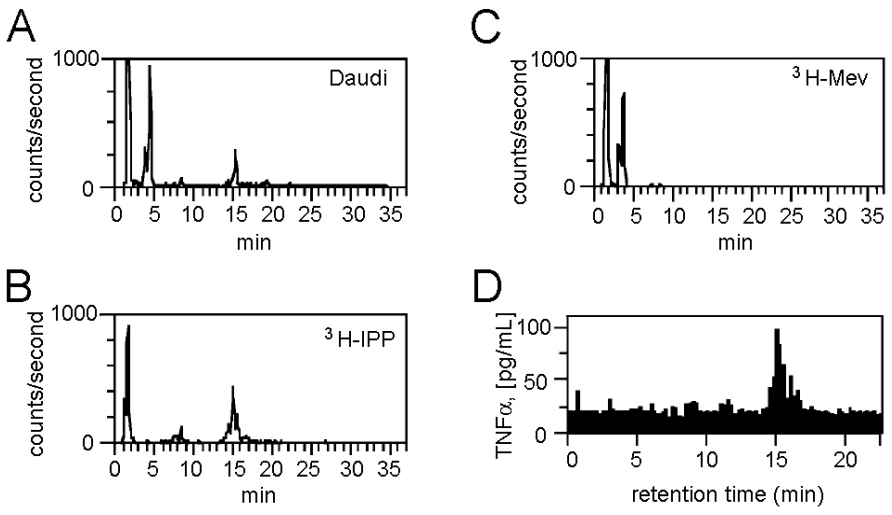

Figure 22. Mevalonate pathway metabolites are the intracellular Daudi ligands activating TCR γδ cells.

(A) Profile of ^{3}H-labeled compounds generated with Daudi cell extracts after addition of ^{3}H-mevalonic acid.

(B) ^{3}H-labeled IPP reference profile.

(C) ^{3}H-mevalonic acid reference profile.

(D) Bioactivity of TCR γδ cells stimulatory ligands from Daudi cell extracts after addition of ^{3}H-mevalonic acid. HPLC fractions were tested at final dilution 1:12 using THP-1 cells as APC in TCR γδ cells activation assay.

To identify the exact chemical structure of the active compounds mass spectrometry analysis was performed on the most active fraction eluting at 15.5 min (Figure 23).

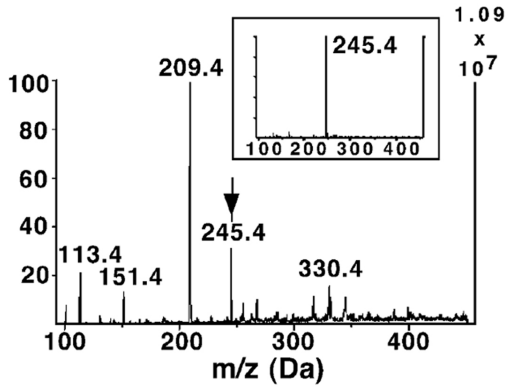

Figure 23. Identification of IPP in Daudi extracts by mass spectrometry.
Mass spectrometry analysis of active HPLC fraction eluting at 15.5 min. The arrow indicates the mass of IPP. The insert shows the mass of reference IPP.

The ion-spray mass spectrometry analysis of the HPLC-purified and TCR γδ stimulatory fraction confirmed the presence of a compound with the same mass as IPP.

Discussion

The population of TCR γδ cells expressing TCR composed of Vγ9 and Vδ2 chains is activated by small phosphorylated nonpeptidic metabolites of bacterial origin. In prokaryotes these stimulatory antigens are generated either in the MEP or mevalonate pathway of isoprenoids biosynthesis. Moreover TCR Vγ9-Vδ2 cells have also ability to recognize and eliminate certain lymphomas *in vitro* and in a SCID animal model *in vivo* (Fisch et al., 1990; Malkovska et al., 1992).
Since mevalonate pathway is present in all eukaryotic cells we investigated the involvement of this pathway in generation of TCR γδ stimulatory ligands.

Two tumor cell lines of different origin *i.e*. Daudi Burkitt's lymphoma and YMB-1 solid breast carcinoma lose their capacity to activate TCR Vγ9-Vδ2 cells when treated with mevastatin a potent inhibitor of the HMGR catalytic site (Istvan and Deisenhofer, 2001). In addition treatment with endogenous metabolites, 7-DHC and farnesol, which, facilitate HMGR degradation by negative feedback mechanism (Correll et al., 1994; Honda et al., 1998) reduced the level of endogenous HMGR and significantly inhibited TCR γδ activation. Upregulation of HMGR protein level, by overexpresion of the enzyme in Daudi cells, resulted in significant increase of TCR Vγ9-Vδ2 cells activation. These results indicate that active HMGR is important for generation of TCR γδ stimulatory ligands.

Furthermore the involvement of endogenous mevalonate pathway in generation of phosphorylated antigens was investigated using nBP drugs which inhibit FPP synthase (Bergstrom et al., 2000). The treatment with nBP increases

the capacity of various cells types (including human primary lung fibroblasts) to activate TCR Vγ9-Vδ2 cells. All tested cell lines were also very stimulatory when exogenous IPP was added. Presentation of both exogenous and endogenous ligands by many different cell types indicates the involvement of non polymorphic molecule(s) with a broad tissue distribution. However, the fact that nBP, in contrast to IPP, can be pulsed on antigen presenting cells suggest that they differ in their mechanisms of action. Indeed we showed that nBP in order to be active require internalization. Moreover, the effect of nBP depends on active HMGR since it is blocked when cells are treated with MEV. These results suggest that nBP by blocking FPP, induce accumulation of mevalonate metabolites which are directly responsible for activation of TCR γδ cells. Further analysis revealed that these metabolites contain phosphate residue and their mass corresponds to the one of IPP. However, since detected IPP originates from an *in vitro* enzymatic assay, it cannot be excluded that *in vivo* other phosphorylated metabolites of the mevalonate pathway might be involved in TCR γδ activation. Therefore, we can conclude that detection of certain tumors by TCR Vγ9-Vδ2 cells is mediated by cognate recognition of endogenous metabolites generated in these tumor cells. Currently we cannot discriminate whether in tumor cells generation of TCR γδ activatory ligands occurs due to the increased activity of HMGR, or due to the impairment in the metabolite utilization, or both. Nonetheless, the recognition of cells with altered mevalonate pathway that overproduce phosphorylated metabolites may allow the immune system to target the cells with metabolic abnormalities. Importantly, dysregulation of mevalonate pathway leading to TCR

γδ cells activation can be pharmacologically induced with nBP providing a novel approach in tumor immunotherapy.

Part 2

Transient dysregulation of the mevalonate pathway during early bacterial infection leads to TCR Vγ9-Vδ2 cells activation

Elevated levels of TCR Vγ9-Vδ2 cells in the peripheral blood of patients (Morita et al., 1999) and macaques (Shen et al., 2002) have been described in a variety of bacterial and protozoan infections. These TCR γδ cells have pro-inflammatory functions and it has been suggested that they participate in protective immunity (Morita et al., 1999).

The expansion of TCR Vγ9-Vδ2 cells has been associated with the production of HMB-PP by microbes. Indeed, infections displaying *in vivo* expansion of TCR γδ cells are caused by microorganisms that also utilize the MEP pathway (Eberl et al., 2003). The possibility that TCR Vγ9-Vδ2 cells are stimulated by HMB-PP during infections has not yet been tested experimentally and it has been assumed that only APC infected with HMB-PP-producing microorganisms stimulate TCR γδ cells (Eberl et al., 2003). Here we investigate the mechanisms by which TCR γδ cells get activated by bacteria-infected APC.

Results

Stimulation of TCR Vγ9-Vδ2 cells by bacteria-infected APC is MEP pathway independent

Two types of human cells, namely freshly isolated $CD14^+$ monocytes and immature dendritic cells (DC) were infected and used as antigen-presenting cells (APC) to stimulate TCR Vγ9-Vδ2 T cells. Infecting agents were the gram-negative *Escherichia coli*, which utilizes the MEP pathway, or the gram-positive *Staphylococcus aureus*, which lacks the MEP pathway and utilizes the mevalonate pathway (Morita et al., 2000), (Table 1). TCR Vγ9-Vδ2 cells were efficiently activated by infected APC (Figure 24A). The activation was inoculum-dependent and at a multiplicity of infection (MOI, bacteria: APC) of 0.1 an efficient stimulation could already be observed when using both bacteria species. Surprisingly, monocytes and DC infected with *S. aureus* were also strong stimulators, showing that MEP pathway negative bacteria also have the capacity to activate TCR γδ cells. MHC class II-deficient APC infected with *S. aureus* were also stimulatory, thus ruling out a superantigen effect during infection with these bacteria (Rust et al., 1990), (Figure 24B).

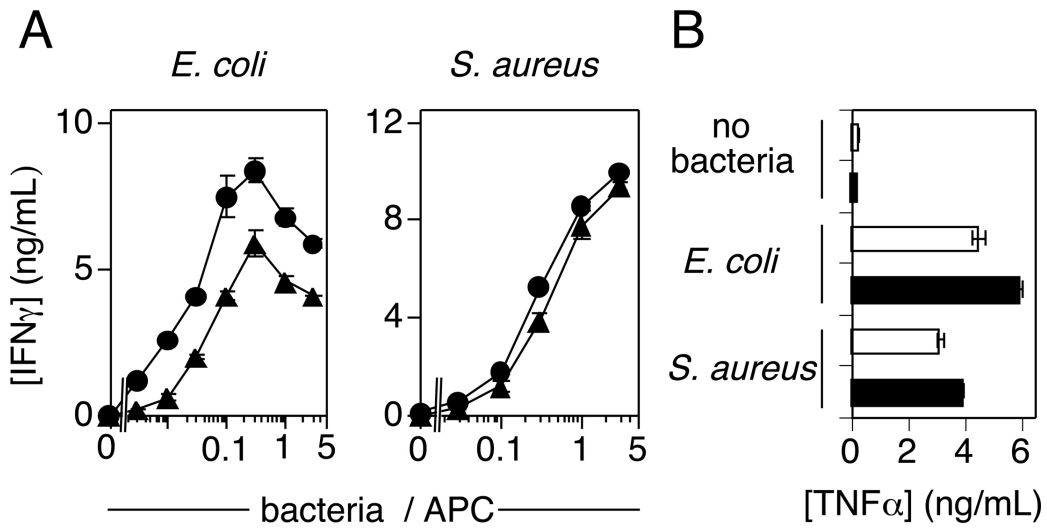

Figure 24. *E. coli*- and *S. aureus*-infected APC stimulate TCR γδ cells.
(A) Monocytes (▲) and DC (●) were infected with *E. coli* or *S. aureus*. MOI (bacteria/APC) is expressed on the X axis, IFNγ (ng/ml ± SD) released by TCR γδ cells on the Y axis.
(B) *S. aureus*-infected APC activate TCR γδ cells without superantigen effect. Raji cells (open bars) and RJ 2.2.5 cells (closed bars), a MHC class II-deficient Raji mutant, were infected (MOI = 3) with *E. coli* or *S. aureus* and used as APC.

The stimulatory effect was TCR γδ cell specific (Figure 25A) because, upon infection with the two bacterial strains of APC expressing the appropriate restriction molecules, TCR αβ peptide-specific and class II-restricted cells (Figure 25B) or TCR αβ lipid-specific and CD1-restricted cells (Figure 25C) were not activated (Figure 25A, 25B and 25C).

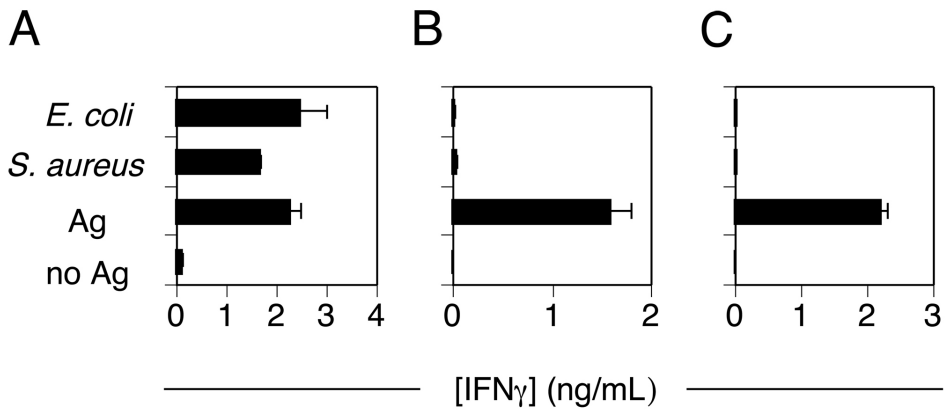

Figure 25. Bacteria infected APC specifialy activate TCR γδ cells.
(A and B) THP-1 or (C) CD1b-transfected THP-1 were infected with *E. coli* or *S. aureus* (MOI = 0.3) and used as APC to stimulate T cells. (A) TCR γδ cells were activated by bacteria-infected APC and also when exogenous IPP (10 µM) was added (Ag). (B) TCR γδ MHC class II-restricted cells specific for *M. tuberculosis* H37Ra protein antigen (Ag) responded only to heat-killed *M. tuberculosis*. (C) TCR γδ CD1b-restricted cells specific for the Ac_2SGL sulfoglycolipid (Ag) responded only to APC presenting Ac_2SGL.

Endogenous mevalonate pathway is involved in generation of TCR γδ ligands during infection

The strong stimulation observed when using APC infected with low numbers of bacteria raised the question of whether bacterial ligands are themselves responsible for TCR γδ cell stimulation. One possible explanation for these findings is that APC upregulate their mevalonate pathways during infection and accumulate endogenous TCR γδ-stimulatory ligands. To test this hypothesis, APC were incubated with zoledronate (ZOL), which induces accumulation of IPP by blocking the mevalonate pathway (this work, Figure 10), and infected with small numbers of bacteria, both at suboptimal doses, which are not stimulatory

alone. A strong synergistic effect was observed (Figure 26A), providing evidence that the stimulatory capacity of APC is positively modulated during bacterial infection.

Investigations were performed to understand the mechanisms whereby bacteria facilitate stimulation of TCR γδ cells. The synergistic effect was dependent on the presence of active HMGR as is shown by the complete loss of activation in the presence of the HMGR inhibitor mevastatin (MEV), (Figure 26B). We also investigated whether upon infection APC upregulate their capacity to present antigen to and costimulate TCR γδ cells. This was ruled out by the lack of a significant increase of TCR γδ cell response to exogenous IPP when infected cells were used as APC (Figure 26C).

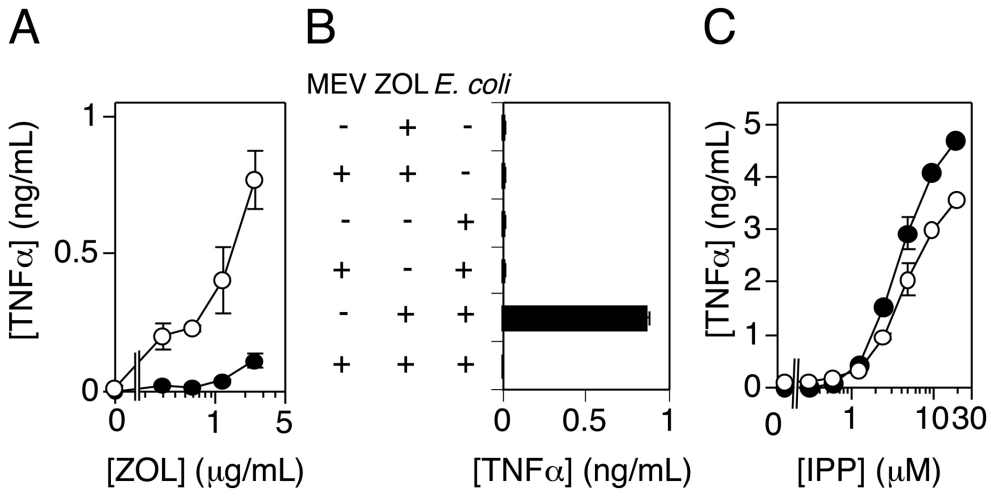

Figure 26. Synergistic effect of bacterial infection and ZOL depends on active mevalonate pathway.
(A) Infection and ZOL have synergistic effects. A-375 cells were treated with ZOL in the absence (●) or presence (○) of *E. coli* (MOI = 0.1).

(B) Synergistic effect of infection is HMGR-dependent. A-375 cells were treated with mevastatin (MEV) 2 h prior infection (MOI = 0.1) and addition of 2.5 μg/ml ZOL.
(C) Infection does not increase the APC antigen presentation capacity. IPP presentation by A-375 cells in the absence (●) or presence (○) of *E. coli* (MOI = 0.1).

Moreover we tested whether bacterial growth is modified in the presence of MEV and ZOL. None of the compounds influenced the growth of the bacteria used in the infection experiments (Figure 27)

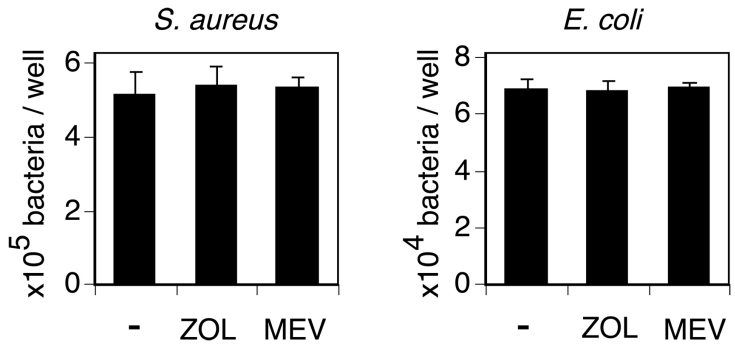

Figure 27. Mevalonate pathway modulators do not affect bacterial growth.
ZOL or MEV were incubated with *S. aureus* or *E. coli* and bacterial growth was determined by CFU counts.

Next the hypothesis was tested whether infection modulates HMGR activity in APC by using wild type or HMGR-transfected A-375 cells infected with either *E. coli* or with *S. aureus* (Figure 28A). HMGR-transfected APC showed a stronger stimulation capacity than wild type cells at all MOI and with both bacteria, suggesting the importance of an active endogenous mevalonate pathway. Moreover, MEV strongly reduced T cell activation in these experimental settings, thus revealing the importance of endogenous eukaryotic ligands (Figure

28B). MEV did not inhibit stimulation with exogenous IPP (Figure 28B), ruling out non-specific inhibitory effects.

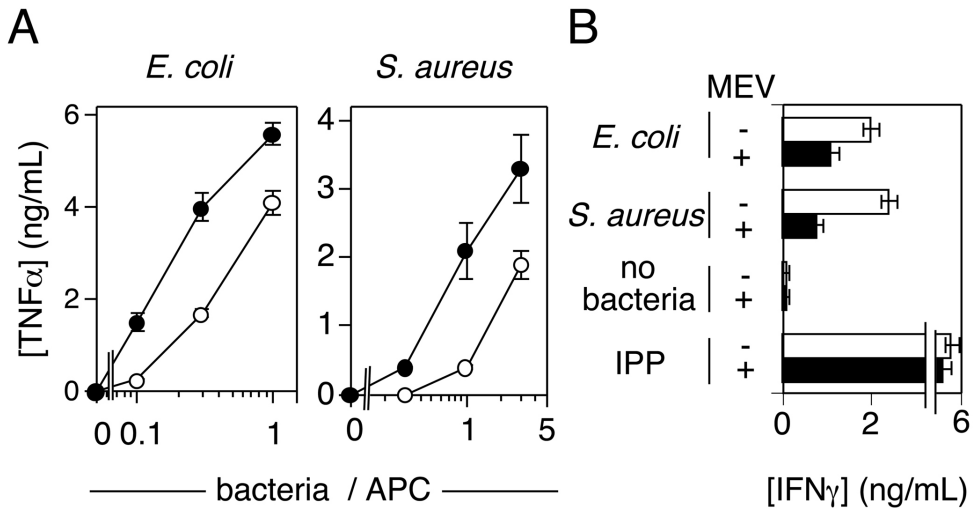

Figure 28. The mevalonate pathway is involved in the generation of the T cell stimulatory antigen during bacterial infection

(A) HMGR-overexpression facilitates TCR γδ cell response during infection. HMGR-transfected A-375 cells (●) infected with *E. coli* or *S. aureus* are more stimulatory than A-375 wt cells (○).

(B) Induction of endogenous ligands during bacterial infection. *E. coli*- or *S. aureus*-infected monocytes (MOI = 0.3) were used as APC with or without MEV.

Bacterial infections modulate HMGR protein levels and phosphorylation state

As HMGR is the rate-limiting enzyme of the mevalonate pathway, we investigated whether bacteria directly influence the intracellular levels and the function of HMGR. HMGR protein levels were evaluated by densitometry after immunoprecipitation and Western blotting of APC lysates. After infection with *E. coli* or with *S. aureus* (Figure 29A) a slight, but consistent, increase in the total levels of HMGR was already observable 1 h after infection, and was followed by a decline 24 h after infection. Analysis of HMGR gene transcription by semi-quantitative RT-PCR showed that HMGR mRNA levels do not change immediately after infection and that they increase significantly after 24 h (Figure 29B). This rapid increase in HMGR protein level at 1 h post infection, without a concomitant increase in mRNA levels, suggests that the regulation may occur through a reduced degradation of HMGR.

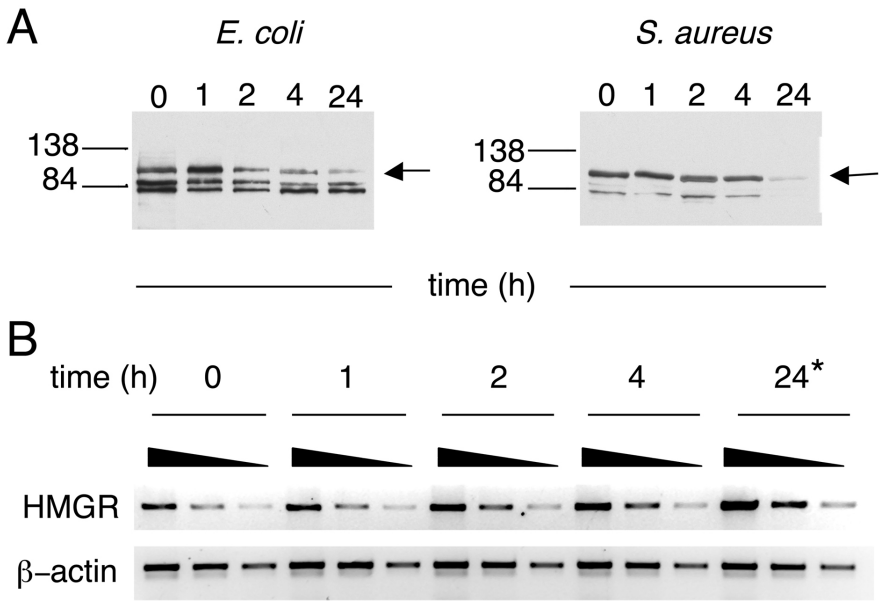

Figure 29**.** Bacterial infection modifies HMGR protein levels.
(A) HMGR was immunoprecipitated from THP-1 cells infected (MOI = 1) with *E. coli* or *S. aureus* for 1, 2, 4 and 24 h (gentamycin was added after 4 h) and analyzed by Western blot. The bands indicated with the arrow correspond to intact HMGR (97 kDa). They were scanned and the values compared to the one obtained in uninfected cells (time 0). (B) Bacterial infection influences HMGR gene transcription at later time points. Semi-quantitative RT-PCR analysis of HMGR (upper panel) was performed on THP-1 cells infected with *E. coli* (MOI = 1) for different time points. Serial 1:3 dilutions of cDNA are shown. As a control β-actin RT-PCR (lower panel) was performed. * indicates that the difference between time 0 and 24 h is significant ($p<0.05$).

As dephosphorylated HMGR is more active and generates more mevalonate than phosphorylated forms (Omkumar et al., 1994; Istvan and Deisenhofer, 2000), the possibility that rapid HMGR dephosphorylation occurs during bacterial infection was investigated. HMGR was immunoprecipitated from *E. coli*- or *S. aureus*-infected cells as well as from control cells and detected by Western blotting by using a phosphoserine-specific mAb (Figure 30A). The

percentage of HMGR represented by phosphorylated HMGR was calculated in each time point. An ~40% reduction of phosphorylated HMGR was already observed in APC 1 h after infection. Levels of phosphorylated HMGR decreased to 25% after 2 h and returned to normal levels 12-24 h post infection (Figure 30B), suggesting that there is a rapid increase of the HMGR enzymatic activity shortly after infection. This hypothesis is supported by the increased capacity of infected APC to stimulate TCR γδ cells, which peaked 5 h after infection and was reduced after 13 h (Figure 30C).

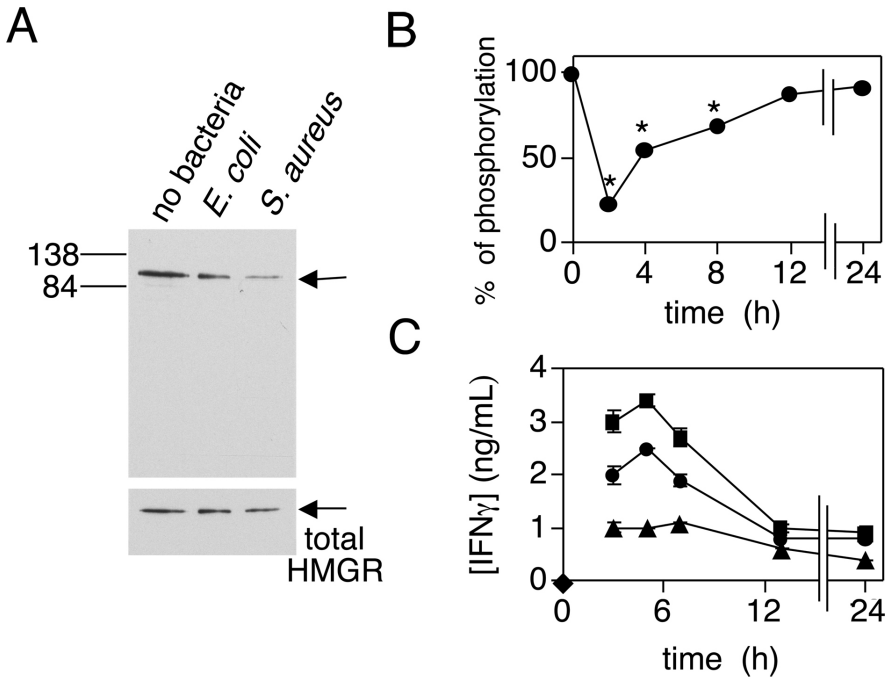

Figure 30. Infection induces transient HMGR dephosphorylation and TCR γδ cell activation.

(A) HMGR-transfected A-375 cells were infected (MOI = 10) with *E. coli* or *S. aureus* for 30 min, then gentamycin was added for 30 min. HMGR protein was immunoprecipitated

and phosphorylation was analyzed by Western blot. Control total HMGR protein levels are shown in the lower panel.
(B) HMGR phosphorylation is modulated during infection. HMGR-transfected A375 were infected with *E. coli* (MOI = 10) and HMGR phosphorylation and protein levels were analyzed. Results are expressed as a % of phosphorylated HMGR standardized for total HMGR signal in infected and uninfected cells (time 0, 100%).
(C) Duration of APC stimulatory capacity after bacterial infection. DC were infected with *E. coli* at MOI 1 (■), 0.3 (●), 0.1 (▲) or uninfected (◆). Gentamycin was applied after 3 h and T cells added at the indicated time points.

Increased PP2A activity leads to HMGR dephosphorylation induced by bacterial infection

The mechanism for serine 872 dephosphorylation in HMGR was investigated by using phosphatase inhibitors. Within the serine/threonine phosphatase family, PP2A is an enzyme with an ubiquitous distribution, large abundance and broad specificity (Wera and Hemmings, 1995). We took advantage of okadaic acid (OA), and calyculin A (CA) drugs, which block PP2A activity (Favre et al., 1997) to determine whether PP2A is required for activation of TCR γδ cells. We could not use the bacteria infection model to stimulate TCR γδ cells because preincubation of APC with these drugs leads to altered phagocytosis (Zhang et al., 2000; Sosroseno et al., 2003). Therefore, Daudi cells, which stimulate TCR Vγ9-Vδ2 cells without addition of exogenous ligands (Fisch et al., 1990), were used. Daudi cells pretreated with OA and CA were no longer able to activate TCR γδ cells as revealed by Ca^{2+} mobilization in the T cells (Figure 31A), showing the requirement for active PP2A. Unspecific effects of phosphatase inhibitors on antigen presentation were ruled out by showing that

Daudi cells pretreated with ZOL (to stimulate the accumulation of endogenous ligands) maintain their capacity to induce T cells Ca^{2+} flux (Figure 31B).

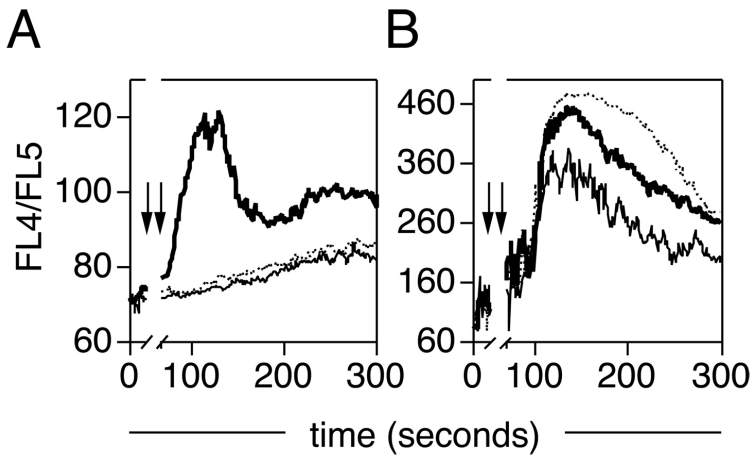

Figure 31. PP2A inhibitors block activation of TCR γδ.
(A and B) Daudi cells treated with OA (dotted line), CA (thin line) or untreated (bold line), in the absence (A) or presence (B) of ZOL, were used to induce Ca^{2+}-flux in TCR γδ cells. APC and T cells were mixed and immediately analyzed to detect the base line, then cells were removed, centrifuged to facilitate doublet formation and reapplied to the cytometer (arrow). The mean Ca^{2+}-bound and unbound Indo-1 ratio in T cells forming doublets with APC is plotted on Y-axis versus time in X-axis.

In order to confirm the activation of PP2A upon infection, we compared the enzymatic activity of PP2A as early as 2 h after inoculation of either *E. coli* or *S. aureus* (Figure 32) to that of uninfected cells. A significant increase was observed thus showing that upon bacterial infection, PP2A phosphatase is activated and suggesting participation in HMGR dephosphorylation.

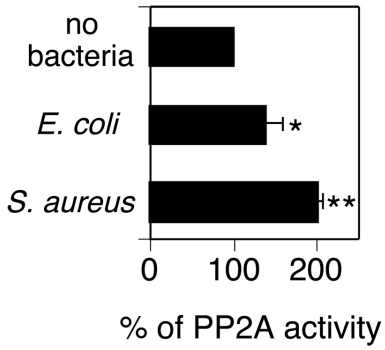

Figure 32. PP2A activity is increased upon bacterial infection.
Cell lysates from HMGR-transfected A-375 cells infected (MOI = 10) with *E. coli* or *S. aureus* for 2 h (30 min after infection gentamycin was added) were used to determine PP2A activity. Results are represented as % of PP2A activity. 100% was calculated for uninfected cells. Significant differences are indicated by *($p<0.05$) or **($p<0.005$).

HMGR activity is increased during early times of bacterial infection

To examine whether HMGR dephosphorylation and increased protein levels might be responsible for increased HMGR enzymatic activity, infected and control cells were lysed and the crude extracts were tested *in vitro* for their content of active HMGR, *i.e.* for the capacity to utilize the substrate hydroxymethylglutaryl-CoA (HMG-CoA), using NADPH as a co-substrate. The product of the reaction, mevalonic acid was monitored by liquid chromatography-electrospray-mass spectrometry (LC-ESI-MS) after conversion into the more stable mevalonolactone (MVL), (Gerber et al., 2004). In preliminary experiments aimed at determining the enzyme kinetics we found that a 60 min enzymatic reaction was optimal to generate measurable metabolite without reaching a plateau, thus permitting exact MVL quantization (Figure 33A and 33B). In the

absence of NADPH, MVL was not detectable, confirming the specificity of the assay. A kinetic analysis showed that HMGR activity increases 1 h after infection, peaks at 4 h (40% increase in MVL production), and then declines at 24 h (Figure 33C).

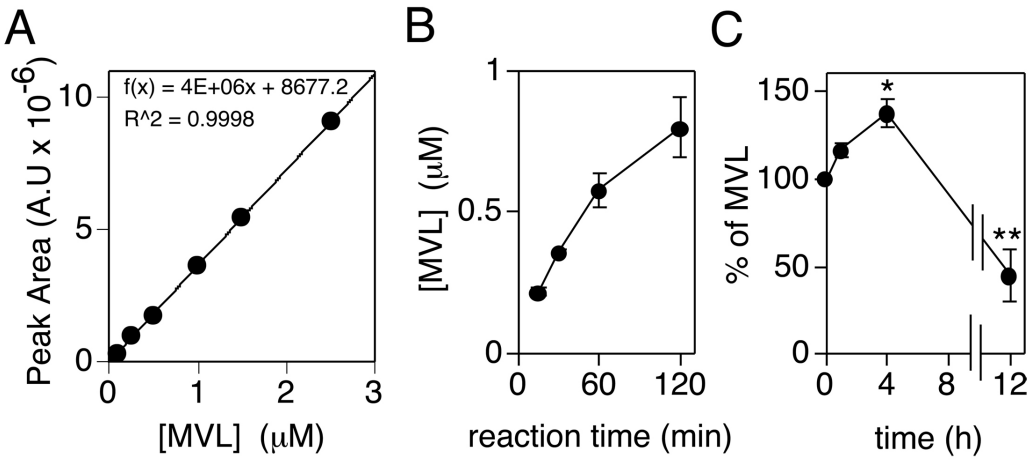

Figure 33. HMGR activity increases in early bacterial infection.
(A) Calibration curve of MVL. The area is calculated from the SIM of m/z 131 at 2.7 min.
(B) Reaction kinetics of HMGR activity in THP-1 homogenate. Values represent duplicate measurements ± SD.
(C) HMGR activity upon infection. THP-1 cells were infected with *E. coli* (MOI = 1) for 1, 4 and 24 h (gentamycin was added after 4 h) or uninfected (time 0). Values represent duplicate experiments ± SD, 100% of MVL (1.229 μM ± 0.05 per 10^7 cells) is calculated on uninfected cells. Significant differences are indicated by * ($p<0.05$) or ** ($p<0.01$).

Thus, the dephosphorylation of HMGR and its increased levels result in a transient, but high, upregulation of its enzymatic activity.

Activity of MVK, PMVK and MVD is not changed during bacterial infection

We next investigated whether bacterial infection also influences other enzymes in the mevalonate pathway. We tested the activity of the 3 enzymes that generate IPP downstream of HMGR in the pathway, *i.e.* mevalonate kinase (MVK), phosphomevalonate kinase (PMVK) and diphosphomevalonate decarboxylase (MVD), (Figure 5). The activity of each enzyme was determined in extracts from APC infected with *E. coli* and compared to control cells. Kinetic analysis of all three enzymes showed that in the activities remain unchanged between 1 and 24 h after infection (Figure 34A, 34B and 34C).

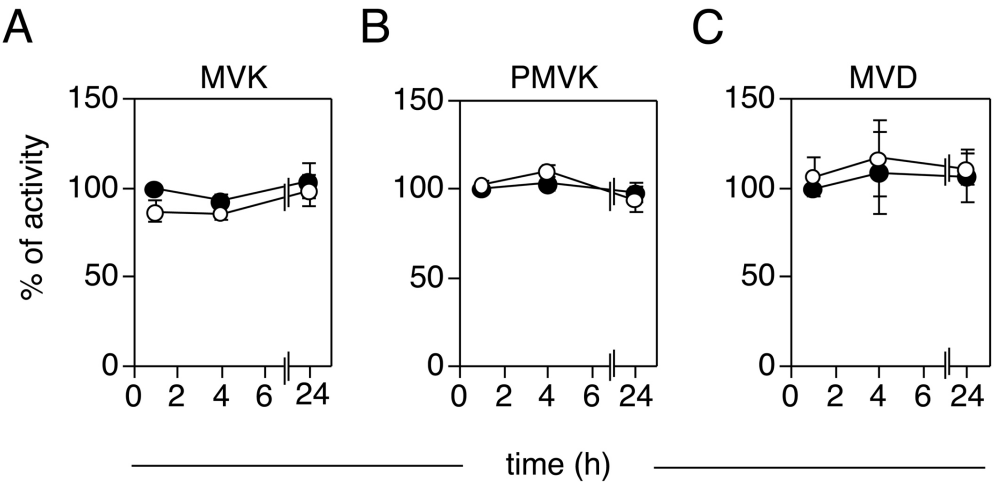

Figure 34. Enzymatic activity of MVK, PMVK and MVD is not modified upon bacterial infection.

(A) MVK, (B) PMVK and (C) MVD enzymatic activities upon infection. THP-1 cells were cultured for 1, 4 or 24 h (gentamycin was added after 4 h) in the presence (○) or absence (●) of E. coli (MOI=1). Values on the Y axis represent duplicate experiments ± SEM. 100% of each enzyme activity was calculated for 1 h cultured uninfected cells and corresponds to: 124.5±8 pmol/min/mg of total protein (2.85±0.5 x 10^5 cpm) for MVK,

157±19 pmol/min/mg of total protein (2.7±0.4 x 10^5 cpm) for PMVK, and 60±25 pmol/min/mg of protein (0.9±0.2 x 10^5 cpm) for MVD. The observed changes are not statistically significant ($p>0.05$).

Increased HMGR activity during bacterial infection is MyD88 independent

The early events leading to upregulation of HMGR activity during infection were further investigated by testing the possible involvement of Toll-like receptors (TLRs) triggering. For this purpose, HMGR activity was investigated in DC from MyD88-deficient mice. Crude extracts from *E. coli*-infected MyD88$^{-/-}$ and wild type C57BL/6 DC were tested for their content of active HMGR by LC-ECI-MS. A significant increase of HMGR activity in DC from both mice was already observed 1 h after infection, peaking at 4 h, and declining to normal levels after 18 h (Figure 35), thus indicating that the upregulation of HMGR activity is MyD88-independent.

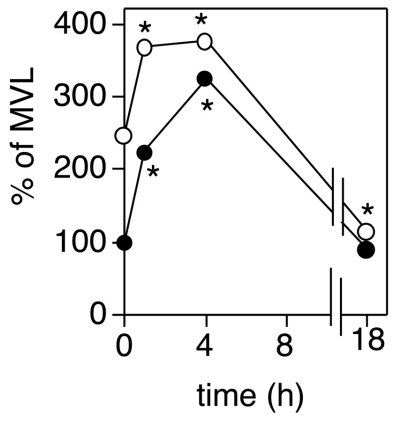

Figure 35. The increase of HMGR activity after infection is MyD88-independent.

DC from MyD88$^{-/-}$ (○) or C57BL/6 (●) mice were infected with *E. coli* (MOI = 1) for 1, 4 and 24 h (gentamycin was added after 4 h) or uninfected (time 0). Values on Y axis represent % of MVL production. 100% is calculated on C57BL/6 uninfected cells and corresponds to 0.4 μM of MVL per 10^7 cells. Significant differences are indicated by * ($p<0.01$).

From these experiments we conclude that HMGR enzymatic activity is increased in a MyD88-independent manner after bacterial infection, while the activities of other enzymes involved in IPP generation are unchanged.

Altogether these results point to the following chronological order of events occurring in bacteria infected cells. At the early time points of bacterial infection (1h) HMGR activity increases without concomitant increase of mRNA synthesis. Later on (24 h) the activity of HMGR is decreased and it is accompanied by an increase of mRNA levels. This suggests that HMGR feedback regulatory mechanism occurs. Immediately after infection bacteria-infected cells augment PP2A phosphatase activity, increase the amounts of dephosphorylated and total HMGR, raise the production of TCR γδ-stimulatory ligands and acquire the capacity to stimulate TCR γδ cells, which then declines 24 h post-infection (Figure 36).

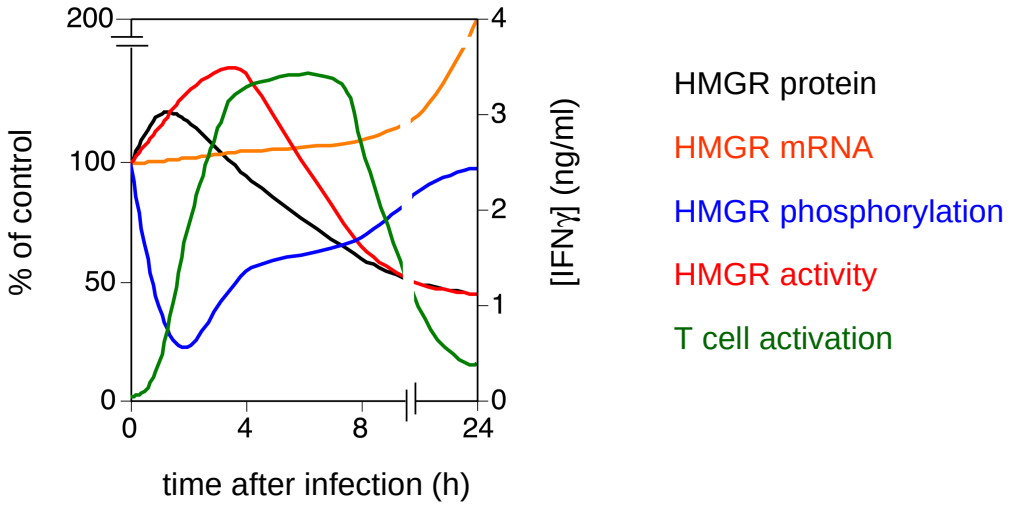

Figure 36. Schematic representation of the correlation between HMGR function and TCR γδ cell response during the early phase of infection

The various parameters measuring HMGR function in APC are expressed on the left Y axis as % of control represented by uninfected cells. The T cell response is expressed as INFγ release on the right Y axis.

Discussion

The selective expansion of TCR Vγ9-Vδ2 cells during infection with certain bacteria or parasites suggests that these T cells have a role in immune defense against pathogens. Whether endogenous or bacterial products stimulate TCR Vγ9-Vδ2 cells during infection was unknown. The discovery of HMB-PP, a metabolite product of the MEP pathway, as the most potent TCR γδ stimulatory ligand implicated its major role in T cell activation during microbial infection (Eberl et al., 2003). However, despite these compounds are active at low concentrations, it is questionable whether they reach the minimal concentration required for TCR γδ activation. Here we find that different phagocytic cell types, including freshly isolated monocytes and immature DC, immediately increase the production of mevalonate metabolites when infected with either MEP-positive *E. coli* or MEP-negative *S. aureus* bacteria and, as a consequence, acquire the capacity to stimulate TCR Vγ9-Vδ2 cells.

The TCR γδ-stimulatory ligands are not produced by the bacteria themselves, but rather by the host mevalonate metabolic pathway. In fact infection of APC with very low MOI efficiently stimulates TCR γδ cells. It is possible that bacterial HMB-PP represents an additional stimulatory ligand in a later phase of infection when large numbers of MEP-positive bacteria accumulate.

In this study we show that bacterial infections naturally modulate the mevalonate metabolism of APC and suggest that endogenous mevalonate metabolites accumulating within infected cells stimulate TCR γδ cells. Upon infection of APC

with low doses of bacteria, and in the presence of small amounts of ZOL, both at suboptimal and inactive doses, there is a strong stimulation of TCR γδ cells. Thus bacterial infection has a synergistic activity on the ZOL effects. In addition, the treatment of infected APC with mevastatin prevents the generation of the cellular stimulatory ligands and inhibits TCR γδ cell activation. Therefore, the HMGR activity in APC sets the threshold of TCR γδ response to infection.

Infection promotes two different mechanisms, both of which augment the function of this key enzyme. Firstly, there is a rapid rise in the HMGR protein levels within the first hour post-infection. Since there is no concomitant increase in HMGR mRNA levels, it is likely that HMGR protein rises as a consequence of its reduced degradation in infected cells. Secondly, infection also leads to increased amounts of the dephosphorylated form of HMGR, which is followed by enhanced enzymatic activity. This is in agreement with other studies showing that HMGR activity is negatively influenced by phosphorylation of serine 872 by AMP-activated protein kinase (Omkumar et al., 1994; Istvan and Deisenhofer, 2000). Mass Spectrometric quantitative analysis confirmed that there was increased HMGR activity in infected phagocytic cells, as compared to non-infected cells. The early modifications in HMGR protein levels and dephosphorylated forms immediately precede maximal T cell activation, which shows a peak 4-8 h after infection in our experimental system. This suggests a direct correlation of the three phenomena, which appear in a temporally ordered fashion (Figure 36). The other enzymes involved in IPP synthesis (*i.e.* mevalonate kinase, phosphomevalonate kinase and diphosphomevalonate decarboxylase), do not

increase their activity upon infection, which confirms the key role of HMGR as the main regulatory enzyme in the mevalonate pathway (Goldstein and Brown, 1990).

Our attempts to outline the mechanisms leading to HMGR dephosphorylation indicate an increased activity of PP2A in the early phases of bacterial infection and suggest that HMGR is a PP2A substrate. Activity of this serine/threonine phosphatase is required to maintain the stimulatory capacity of Daudi cells, as shown by inhibition with PP2A-blocking drugs, and this reveals a previously unrecognized role of this phosphatase in stimulating TCR γδ cells. PP2A is probably involved in the production of TCR γδ stimulatory ligands and not in antigen presentation, as indicated by experiments with ZOL-treated Daudi cells. Indeed, these cells accumulate endogenous ligands and maintain a strong TCR γδ cell stimulatory capacity in the presence of PP2A inhibitory drugs.

Previous studies have shown that other phosphatases, such as dual specificity phosphatases, are activated during innate immune responses (Liu et al., 2007). Importantly, these phosphatases are not active on serine as substrate as is the case with PP2A and therefore are not candidate to dephosphorylate HMGR *in vivo*.

HMGR activation following infection is independent from the MyD88 pathway, thus implying that this phenomenon is not associated with microbial activation of Toll-like receptors which utilize MyD88. Whether, other adaptor proteins of innate immunity are involved in HMGR activation remains to be investigated.

Different stimuli, including cholesterol depletion (Goldstein et al., 2006), growth factors and insulin signaling (Demoulin et al., 2004; Nadeau et al., 2004) exposure to hypotonic media, and ER calcium depletion (Lee and Ye, 2004), have been reported to induce upregulation of HMGR transcription. Interestingly, HMGR gene transcription also increases after internalization of latex beads (Castoreno et al., 2005), which, to some extent mimics phagocytosis of bacteria. Moreover, membrane pore formation by the bacterial aerolysin toxin, leads to upregulation of HMGR transcription and to increased cholesterol synthesis as a defence mechanism facilitating membrane repair (Gurcel et al., 2006). Therefore, upregulation of mevalonate pathway occurs after a variety of cell stressing signals, as a result of increased HMGR gene transcription and enzymatic activity. Here we add a novel mechanism involving HMGR dephosphorylation and PP2A activation occurring during the early phases of infection. Whether the other physiological conditions which upregulate HMGR transcription also induce stimulation of TCR γδ cells has not been investigated.

Overall these findings have revealed two important aspects relating to the manner in which infected cells activate TCR Vγ9-Vδ2 cells and therefore to the possible relevance of this T cell population in immune responses *in vivo*. Firstly, HMGR dephosphorylation and accumulation of endogenous (self) ligands is fast, being detectable within the first hour post infection. This rapid mechanism may contribute to the stimulation of TCR Vγ9-Vδ2 cell response during the very early stages of infection, when very low numbers of bacteria are present and when other bacteria-specific T cells are not available at the site of infection.

Another important issue is that the mevalonate dysregulation following infection is transient. This probably occurs because of the feedback regulation of both the mevalonate pathway and cholesterol metabolism, both of which are tightly controlled within cells (Goldstein and Brown, 1990). The temporary alteration of the mevalonate pathway is necessary to avoid continuous stimulation of TCR Vγ9-Vδ2 cells, which are very abundant and thus have to be activated with caution. Despite its short duration, the transient activation may explain the observed expansion of this TCR γδ population in the periphery during early post-natal life (Parker et al., 1990). This could be the result of repeated infections naturally occurring in this age.

Part 3

Multi-drug related protein 5 (MRP5, ABCC5) is involved in trafficking of phosphorylated mevalonate metabolites

The fact that TCR γδ cells are activated by endogenous mevalonate metabolites generated inside APC raised the question how these antigens are transported to cell surface in order to be presented to the T cells. Prenylated phosphorylated metabolites are polar molecules (Figure 6), and therefore a passive transportation through the cell membrane is very improbable despite their low molecular masses. Instead, it may be more likely an inside-out transport by a transmembrane transfer protein, whose activity is either ATP-dependent or driven by a concentration gradient.

Results

Transfer of TCR γδ stimulatory ligands

The hypothesis of ligand transfer was investigated using mouse cells that do not have the capacity to directly stimulate TCR γδ cell (De Libero et al., 1991; Kato et al., 2003) in co-culture with human dendritic cells (DC), that efficiently stimulate TCR γδ cells. The M12.4.1 mouse B cell lymphoma cell line, in which TCR γδ stimulatory ligands accumulation was induced by pulsing with

zoledronate (ZOL), were mixed with DC cells and used in T cells activation assay. We observed a significant increase of TCR γδ cells activation (Figure 37).

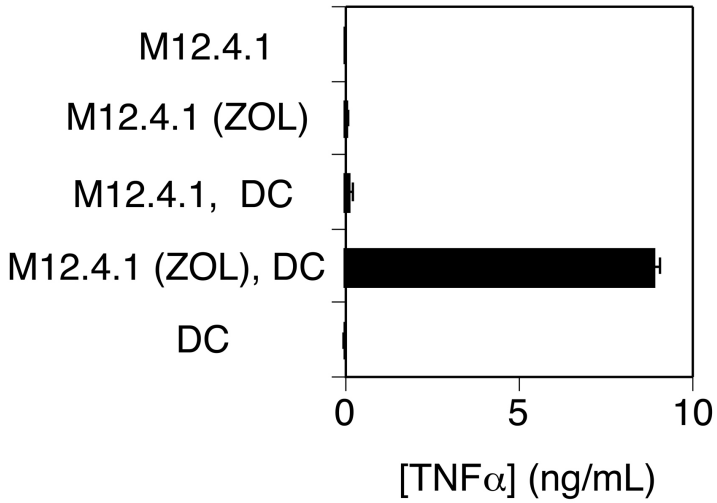

Figure 37. Transfer of endogenously generated antigens from mouse to human cells. Mouse M12.4.1 cells pulsed with ZOL were mixed with DC cells and used to stimulate TCR γδ clone. As a control not treated M12.4.1 cells mixed with DC cells were used.

We interpreted this result with a probable transfer of activatory metabolites from mouse cells to human APC.

The synthesis of TCR γδ stimulatory ligands takes place in the cytoplasm (Hogenboom et al., 2004c; Hogenboom et al., 2004b; Hogenboom et al., 2004a) where they need to be transported from in order to be presented. Thus, we hypothesized that the transport through the membrane of phosphorylated antigenic metabolites is mediated by a transporter protein located at the cell surface.

Involvement of ATP-binding cassette transporter-C (ABC-C) in transport of the TCR γδ ligands

In order to further investigate the protein mediating the transmembrane transport for TCR γδ ligands, a series of drugs inhibiting different transport proteins were used to block TCR γδ cell activation. The main targets of our investigation were the: ATP-binding cassette transporters (ABC), the monocarboxylate transporters (MCT) and the inorganic transporters. In addition, we also considered different classes of ATPases in our studies.

Firstly, we established a TCR γδ assay in the presence of both APC accumulating endogenous mevalonate metabolites and drugs known as inhibitors of different classes of transporters. Table 4 lists the drugs used in the assays and their target transporters.

Compound	Inhibited transporter
Isopentenol	Transporter for IPP/GPP/FPP?
Geraniol	
Farnesol	
Foscarnet	inorganic phosphate transporter
Lactate	various MCT
Phenylpyruvate	MCT1
Phloridzin	various MCT
DIDS	MCT2/MCT4/ABC??, irreversible chloride antiporter inhibitor
ARL-67156	ecto-ATPase inhibitor
β,γ–MAT	ecto-ATPase inhibitor
Oligomycin	mitochondrial F-type ATPase inhibitor, intracellular ATP-depletor
Omeprazol	gastric H^+-ATPase inhibitor
Bafilomycin A1	V-type ATPase inhibitor
Ouabain	Na/K transport ATPase inhibitor
Glybenclamide	MDR1, MRP2, OATP1/2, SUR1/2
PSC-833	MDR1, MDR2, many MRP
PKF-274	MDR1, MDR2, many MRP
Niflumic acid	MCT1, CFTR
Quercetin	MCT1, MDR1, many MRP
Sulfinpyrazone	MRP1-5
Benzbromarone	MRP1-5
Probenecid	MRP1, 2, 3
Methotrexate	MRP1, 2, 3, MRP4?
MK-571	MRP1, 2, 4, 5

Table 4. List of tested compounds with indicated groups of inhibited transporters. 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), multi-drug resistance protein (MDR), multi-drug related protein (MRP), organic anion-transpoting polypeptide (OATP), sulfonylurea receptors (SUR), cystic fibrosis transmembrane conductance regulator (CFTR).

Endogenous antigens in Daudi cells were increased by treatment with ZOL and in some experiments also with alkylamines (*sec*-butylamine, SBA, Figure 9). In control experiments activation in the presence of exogenously

added phytohemagglutinin (PHA) and activation of a CD1a-restricted TCR αβ clone was performed to exclude drug toxicity. Specific inhibition of TCR γδ activation was considered when activation with PHA or activation of TCR αβ clone was not affected. When drugs showed to be toxic when kept continuously in culture during entire assay, pulsing experiments were performed. All drugs were used at concentrations shown to be active in the literature. The percentage of inhibition of T cell activation was calculated for each tested drug considering the response in the absence of drugs as 100% (Table 5).

		TCR γδ				TCR αβ
		ZOL	SBA	IPP	PHA	Ag
Drug	Dose	% of inhibition				% of inhibition
Isopentenol	200 μM	0	ND	0	0	ND
Geraniol	40 μM	0	ND	0	0	ND
Farnesol	20 μM	0	ND	0	0	ND
Foscarnet	300 mM	0	0	0	0	0
Lactate	100 μM	0	ND	0	0	ND
Phenylpyruvate	100 μM	0	ND	0	0	ND
Phloridzin	100 μM	0	ND	0	0	ND
DIDS	300 μM	40	40	20	15	0
ARL-67156	100 μM	0	0	0	0	0
β,γ–MAT	300 μM	0	0	0	0	0
Oligomycin	100 μg/ml	30	50	20	10	0
Omeprazol	30 μg/ml	0	0	0	0	0
Bafilomycin A1	100 nM	0	0	0	0	ND
Ouabain	30 μM	0	0	0	0	0
Glybenclamide	100 μM	0	0	0	0	0
PSC-833	100 μM	100	100	70	40	35
PKF-274	100 μM	100	100	75	45	40
Niflumic acid	100 μM	0	0	0	0	0
Quercetin	100 μM	30	40	10	0	0
Sulfinpyrazone	10 mM	95	95	10	0	ND
Benzbromarone	100 μM	65	80	40	0	ND
Probenicid	3 mM	0	0	0	0	0
Methotrexate	100 μM	0	0	0	0	0
MK-571	300 μM	50	50	30	10	10

Table 5. List of drugs with doses used in the TCR γδ and TCR αβ activation experiments.

Specific inhibition of TCR γδ cells activation was determined by comparing activation in the absence and presence of the drug, (ND, not determined).

The activation of TCR γδ cells was not affected when isopentenol, geraniol or farnesol, the alcohol analogs of IPP, GPP and FPP, respectively, were used. This suggests that the putative transporter recognizes the phosphate group which is present in active ligands (Pfeffer et al., 1990; Constant et al., 1994; Schoel et al., 1994; Tanaka et al., 1994). Therefore, the inhibitors of inorganic phosphate transporters, foscarnet (Yusufi et al., 1986; Timmer and Gunn, 1998), and inhibitors of MTC, lactate, phloridzin, phenylpyruvate (Halestrap and Price, 1999), were tested. These compounds also did not show inhibition of TCR γδ cells activation. In contrast, two inhibitors of ABC transporters, PSC-833 and PKF-274, preferentially decreased activation of TCR γδ cells induced by ZOL or SBA. These findings indicated involvement of the protein(s) from the ABC-transporters family in the TCR γδ activation.

PSC-833 and PKF-274 drugs, which were chemically designed to inhibit multi-drug resistant protein 1 (MDR1), (Boesch et al., 1991; Lum et al., 1993), a member of the B subfamily of ABC transporters, also inhibit other ABC transporters (Paul et al., 1996). Therefore, their broad activity does not allow discrimination of the transporter involved in TCR γδ cells activation.

We have found two other drugs, sulfinpyrazone and benzbromarone, that specifically inhibit TCR γδ cells activation. The fact that these drugs inhibit various anion transporters belonging to the ABC family (Bakos et al., 2000; Evers et al., 2000) supported the hypothesis that the transporters involved in TCR γδ

activation recognize the anionic part of the stimulatory ligands. Additionally we found specific inhibition in the presence of quercetin, an inhibitor of ABC-C transporters (Walgren et al., 2000). Therefore, we narrowed our studies to the subfamily C of ABC transporters.

This subfamily is composed of 12 characterized members: 9 multi-drug related proteins (MRP1-9), two sulfonylurea receptors (SUR1/2) and a cystic fibrosis transmembrane conductance regulator (CFTR). The MRPs studied so far, MRP1-8, are all organic anion pumps but they differ in substrate specificity and tissue distribution. CFTR is a chloride channel (Schwiebert et al., 1999) and SURs are the regulatory subunits of the ATP- sensitive potassium (K_{ATP}) channel (Aguilar-Bryan et al., 1998). Table 6 lists the members of the ABC-C family of transporters, their chromosomal localization, tissue distribution and substrate specificity.

Name (other nomenclature)	Chromosomal localization	Tissue distribution	Substrate
MRP1 (ABCC1)	16p13.12-13	ubiquitous	GSH conjugates, organic sulfate and monoglutamate
MRP2 (ABCC2)	10q23-24	liver, kidney, gut	GSH conjugates, organic sulfate and monoglutamate
MRP3 (ABCC3)	17q21.3	liver, adrenals, pancreas, kidney, gut, gallbladder	bile salt (and conjugates), glucuronides, GSH conjugates
MRP4 (ABCC4)	13q31-32	prostate, lung, muscle, pancreas, testis, ovary, bladder, gallbladder	nucleoside-based drugs, conjugated steroids
MRP5 (ABCC5)	3q27	ubiquitous	nucleoside monophosphate analogs, cyclic nucleotides
MRP6 (ABCC6)	16p13.1	liver, kidney	acidic peptide
CFTR (ABCC7)	7q31.2	lung, gut, many epithelial tissues	
SUR1 (ABCC8)	11p15.1	pancreas	
SUR2 (ABCC9)	12p12.1	hart and skeletal muscles, with low levels in all other tissues	
MRP7 (ABCC10)	6p21	ubiquitous (low)	GSH and glucuronide conjugates
MRP8 (ABCC11)	16q11-12	liver, lung, kidney, number of fetal tissues	cyclic nucleotides
MRP9 (ABCC12)	16q11-12	Testis, pancreas, brain	unknown

Table 6. List of members of ABC transporter subfamily C indicating: chromosomal localization (Dean et al., 2001), tissue distribution (Kool et al., 1997; Yabuuchi et al., 2001; Bera et al., 2002) and examples of transported substrates (Borst et al., 2000; Chen et al., 2003; Borst et al., 2006).Glutathione (GSH).

Since TCR γδ cells are activated by many cell types from different tissue origin (Table 3), we assumed that the transporter involved in the antigen presentation has rather broad tissue distribution. Therefore, we reduced the number of candidate ABC-C transporters to those that are ubiquitously expressed. Furthermore, we excluded CFTR as a candidate because, niflumic acid, a potent inhibitor of CFTR (Scott-Ward et al., 2004) did not affect the activation of TCR γδ cells. We also eliminated MRP7 due to its low level of expression and our investigation focused on MRP1, MRP4 and MRP5.

The MK-571, an analog of leukotriens which specifically inhibits the transport activity of MRP1, MRP4 and MRP5 (Reid et al., 2003) reduced the activation of TCR γδ cells. However, other drugs which inhibit only MRP1, such as probenicid and methotraxate (Bakos et al., 2000) did not affect TCR γδ cell activation. Thus, we concluded that MRP1 is not responsible for IPP transport.

These results narrowed the studies to MRP4 and MRP5. Importantly, the tissue distribution of MRP5 was described as ubiquitous while MRP4 has been found in many but not all tissues (Kool et al., 1997). To further discriminate between MRP4 and MRP5, a real-time quantitative PCR analysis was performed to compare the mRNA levels of these two transporters in cell lines from various tissues (Table 7), which all stimulate TCR γδ cells when pulsed with ZOL.

Cell line	MRP4	MRP5
A-375	+	+
A-431	-	+
CEM 1.3	+	+
Colo-201	+	+
Daudi	-	+
HEP G2	+	+
HL-60	+	+
HuH6	+	+
K562	+	+

Table 7. MRP4, and MRP5 mRNA expression in different cell lines.
+, gene product was detected; -, no gene product.

The MRP5 mRNA was detected in all tested cell lines, while MRP4 mRNA was absent in A-431 and Daudi cells. Since Daudi and A-431cells, after pulsing with ZOL and SBA, are very strong activators of TCR γδ cells we excluded MRP4 and focused on MRP5 as a candidate transporter of endogenous antigens.

MRP5 overexpression increases stimulation of TCR γδ cells

In order to investigate the hypothesis that MRP5 is responsible for the transport of stimulatory ligands, a Daudi cell line overexpresing MRP5 protein was generated. The capacity to stimulate TCR γδ cells by Daudi MRP5-transfected cells was significantly increased as compared to the wild type Daudi cells (Figure 38).

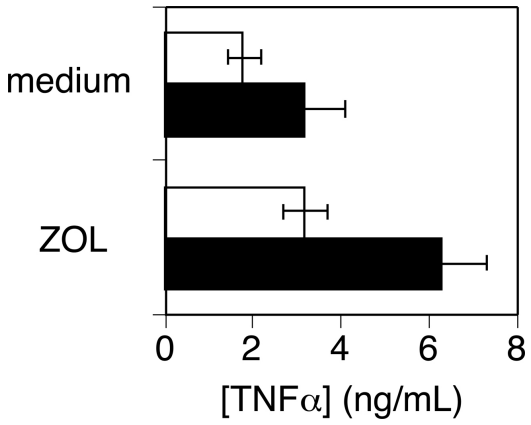

Figure 38. MRP5 overexpression increases TCR γδ cells activation.
MRP5-transfected (closed bars) or wt Daudi cells (open bars) were used as APC in TCR γδ cells stimulation assay.

To further investigate the effect of MRP5 overexpression on TCR γδ cells activation we transfected A-375 cell line, that requires treatment with ZOL in order to become stimulatory, with MRP5 gene and used them as APC (Figure 39A, 39B and 39C).

We found that MRP5 overexpression in APC facilitates not only presentation of endogenous antigens, accumulating after pulsing with ZOL (Figure 39A), but also improves presentation of exogenously added IPP (Figure 39B). As control we used the A375 MRP5-transfected cells to present peptide to MHC class I restricted T cells. We found that, the stimulatory effect of MRP5 was TCR γδ cell specific (Figure 39C) since presentation to the TCR αβ MHC class I restricted cells was not affected.

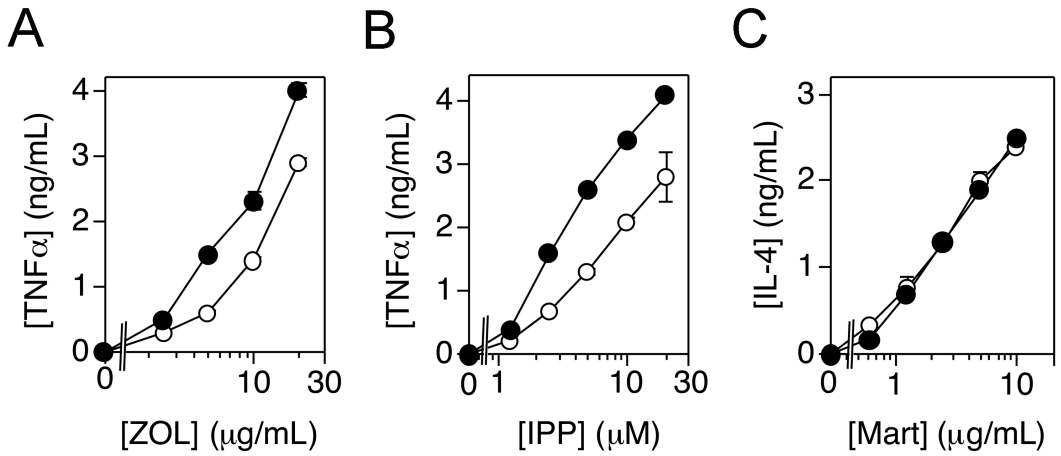

Figure 39. MRP5 participates in the transport of endogenous and exogenous TCR γδ ligands.
(A) MRP5 overexpression facilitates TCR γδ cells activation by ZOL treated APC. MRP5 transfected A-375 cells (●) stimulate TCR γδ more efficiently than A-375 wt cells (○).
(B) Exogenously added IPP is better presented by MRP5 transfected A-375 cells (●) than A-375 wt cells (○).
(C) MRP5 ovexpression does not influence stimulation of MHC class I restricted, Mart peptide specific TCR αβ clone. MRP5 transfected A-375 cells (●) and A-375 wt cells (○) were used as APC.

MRP5 downmodulation affects stimulation of TCR γδ cells

In order to further confirm the involvement of MRP5 in the presentation of TCR γδ stimulatory ligands we knocked down the MRP5 protein and performed antigen presentation experiment. We generated four different small hairpin RNA (shRNA) interference constructs targeting MRP5 mRNA. A-375 cells were transfected with each of these constructs and were used as APC in TCR γδ activation experiments. We found that A-375 cells transfected with MRP5 shRNA interference constructs, but not with control firefly luciferase shRNA interference

constructs (Ff1), stimulated TCR γδ cells less efficiently than wt cells. The decrease in T cell activation was observed when APC were treated with ZOL (Figure 40A) or IPP (Figure 40B).

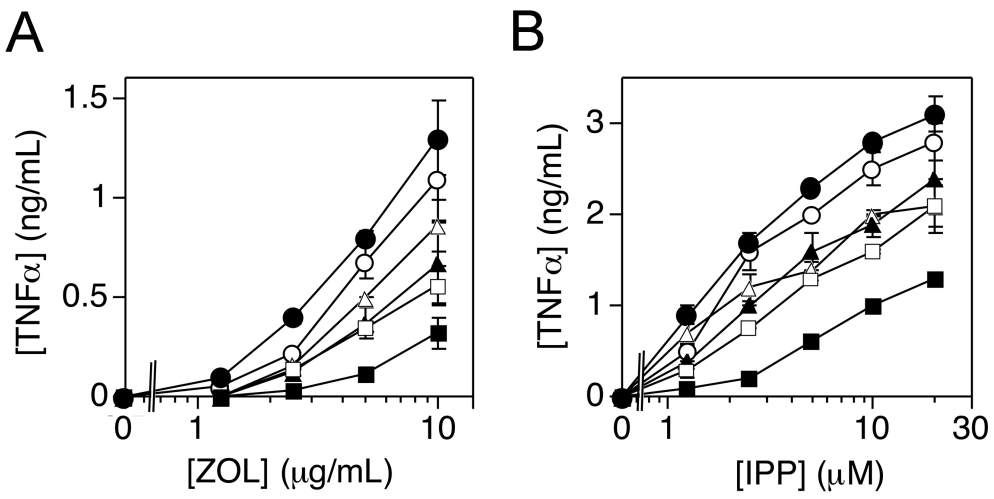

Figure 40. MRP5 gene specific silencing alters stimulation of TCR γδ cells.
(A and B) A-375 wt (○) cells and A-375 cells transfected with MRP5 specific shRNA interference silencing constructs pCMV_42 (△), pCMV_1501 (▲), pCMV_2526 (□), pCMV_3344 (■) or with irrelevant shRNA silencing construct Ff1 (●) were used as APC in endogenous (A) and exogenous (IPP) antigen presentation assays.

The MRP5 shRNA interference construct pCMV_3344 had the strongest inhibitory capacity. Therefore, we used cells transfected with this construct in additional antigen presentation assays utilizing two other TCR γδ clones (Figure 41). We found that knocking down of MRP5 completely inhibited presentation of endogenous ligand and strongly reduced presentation of exogenous IPP.

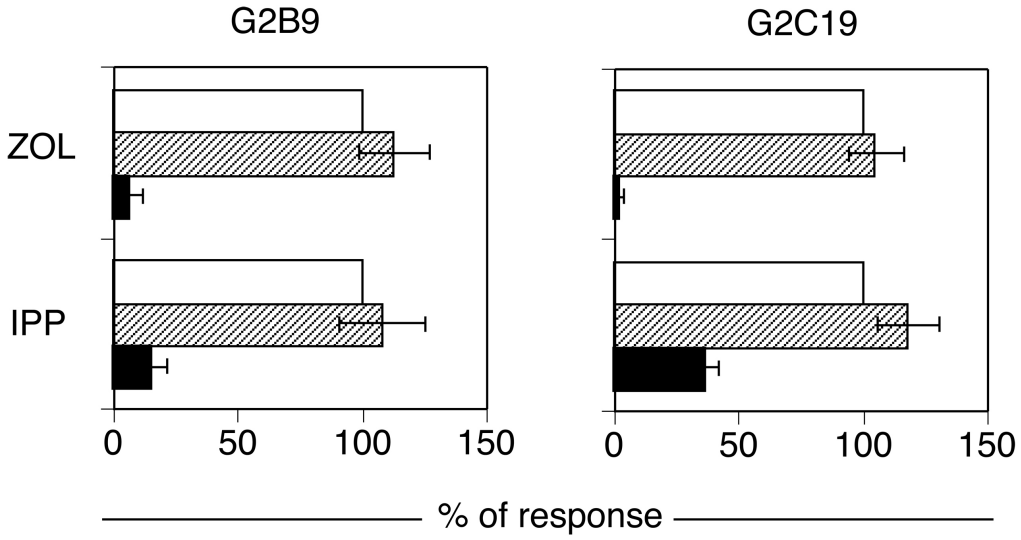

Figure 41. Presentation of endogenous and exogenous antigens TCR γδ clones (G2B9 and G2C19).
The percentage of T cell response induced by A-375 wt (100%, open bars), A-375 Ff1 (dashed bars) or A-375 3344 (closed bars) cells treated either with ZOL (2.5 μg/ml) or IPP (2.5 μM).

Knocking down of MRP5 mRNA in APC decreased TCR γδ cells activation regardless whether the antigen was induced inside APC by ZOL or was added from outside as IPP. The inhibition was TCR γδ specific since presentation of Mart peptide to the TCR αβ Mart-specific clone was not altered (Figure 42).

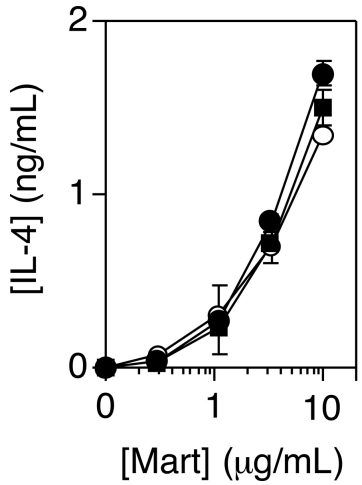

Figure 42. Presentation of Mart peptide to the TCR αβ clone.
A-375 wt (○) cells and A-375 cells transfected with MRP5 shRNA interference 3344 (■) or Ff1 (●) irrelevant shRNA interference were used as APC.

Taken together these data strongly suggest that MRP5 is the transporter involved in transfer TCR γδ stimulatory ligands generated in the mevalonate pathway.

Discussion

The fact that intracellular metabolites activate TCR Vγ9-Vδ2 cells raised the question of how they are transported to the cell surface in order to establish a cognate interaction with TCR. One possibility could be that inside APC, mevalonate ligands bind to a dedicated presenting molecule and then are transported on the cell surface. Thus it would resemble the biology of MHC-peptide and CD1-lipid complexes. However, the inability of IPP to bind to putative antigen-presenting molecules in a stable manner (IPP cannot be pulsed, Figure 16) makes this mechanism unlikely. If this would be the case, we might speculate that the antigen presenting molecule is loaded intracellularly, forms immunogenic complexes with mevalonate metabolites in a stable manner, and then traffick on the cell surface, where ligands are degraded or dissociate with a very fast kinetics.

Another possibility is that the mevalonate ligands are translocated across membranes by specific transporter proteins. Indeed, the polarity and high charge of pyrophosphorylated metabolites make unlikely a passive diffusion through membranes.

Here we identified MRP5, a member of ABC-C transporter family, as a protein required for activation of TCR Vγ9-Vδ2 cells. MRP5 is a multispecific organic anion pump which transports nucleotide analogs (Jedlitschky et al., 2000; Wijnholds et al., 2000). This protein has 12 transmembrane helices in each of two membrane spanning domains forming P-glycoprotein-like core (Persson and Argos, 1994; Belinsky et al., 1998). Therefore, together with MRP4 and MRP8,

MRP5 differs from other MRP proteins which contain additional transmembrane domain (TMD_0) composed of five transmembrane helices (Figure 43), (Borst et al., 1999; Guo et al., 2003; Ballatori et al., 2005).

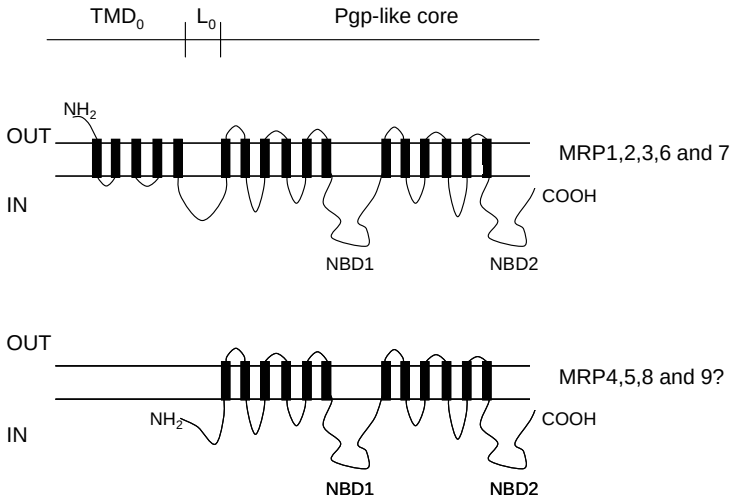

Figure 43. Schematics showing structures of MRP, adapted from (Borst et al., 2000; Kruh et al., 2007).
The common part of all MRP is the linker domain (L_0) and P-glycoprotein (Pgp)-like core composed of transmembrane domains with nucleotide binding domains (NBD1 and 2). MRP4, 5, 8 and possibly MRP9 are lacking extra N-terminal transmembrane domain (TMD_0).

Blocking of MRP5 significantly inhibits activation of TCR γδ cells when APC are loaded with ZOL or alkylamines and slightly decrease presentation of exogenous IPP but does not affect activation induced by PHA. Moreover, we also found that tumor cells overexpressing MRP5 acquire stronger TCR γδ stimulatory capacity than control cells when they present both endogenous and exogenous antigens. The fact that MPR5 improves also presentation of exogenous IPP can

be explained with two mechanismis i) MRP5 might participate in the loading of putative antigen presenting molecule directly on the cell surface or ii) IPP might partially internalized inside APC and then reexpressed on the cell surface with the help of MRP5.

The involvement of MRP5 in TCR γδ antigen presentation has been further confirmed by experiments with MRP5 specific shRNA interference. Cell lines with knock down MRP5 have much lower capacity to stimulate TCR γδ cells with endogenous and exogenous antigens. However, the decrease in presentation of exogenous IPP, when both specific MRP5 inhibitors and specific shRNA interference were used, is much smaller as compared with endogenous ligands. Therefore, it is likely that our second hypothesis is more accurate. If this is the case, then MRP5 would be involved in the presentation of the internalized pool of IPP.

Up to date the physiological function of MRP5 is not clear. It was described as transporter for cyclic nucleotides, especially cGMP and was suggested that MRP5 removes cGMP from the cytosol during signal transduction (Jedlitschky et al., 2000). It is possible that IPP and other prenylated metabolites generated in the mevalonate pathway mimic cGMP. Therefore, the role of MRP5 would be to recognize these substrates and facilitate their removal when they accumulate in the cytosol. It is unlikely that MRP5 behaves as an antigen-presenting molecule for the TCR γδ cells because the predicted extracellular MRP5 domains (Persson and Argos, 1994; Belinsky et al., 1998) are too small. We therefore, prefer the hypothesis that independent antigen-presenting

molecule interacts with TCR γδ. MRP5 may participate in antigen presentation to TCR γδ cells by transporting the mevalonate metabolites from the cytoplasm, where the metabolites are synthetised. MRP5 protein is localized on the cell membrane (McAleer et al., 1999) and perhaps in small amounts intracellularly (Wijnholds et al., 1997). These localizations might mediate two different mechanisms of action. In the first case, MRP5 may transport mevalonate metabolites outside the cell where they finally interact with antigen-presenting molecules. A second possibility is that MRP5 facilitates traffic of stimulatory metabolites from cytosol inside other compartments, *i.e*. ER. After transport in this compartment, metabolites could bind to the antigen-presenting molecules, form a complex and then be transported to the cell surface. This second mechanism of action would resemble that of ATP-dependent trasporters associated with antigen processing (TAP) involved in peptide traffic from cytosol into ER (Neefjes et al., 1993; Momburg et al., 1994). Interestingly, TAP molecules are part of complex protein multimers composed of several proteins which facilitate the assembly of MHC class I proteins and also provide transport and loading of peptides on these presenting molecules (Cresswell et al., 1999). At present it is not clear whether MRP5 constitutes multi-subunit complexes with other proteins. Interestingly, other transporters are associated with a second protein and form complexes which are localized on the cell surface. This is the case of MCT4- and MCT1 which form dimers with CD147 on the cell surface (Kirk et al., 2000).

Taken together our data show that MRP5 is involved in the presentation of TCR γδ stimulatory ligand generated in the mevalonate pathway. MRP5 is most likely participates in transfer of these phosphorylated ligands from the cytoplasm, where they are synthesized, to the cell surface where they are presented to the TCR γδ cells.

Part 4

Thymic development of TCR Vγ9-Vδ2 cells

Studies in mice have shown that there are several differences between human and mouse TCR γδ cells, mainly concerning their tissue distribution and antigen recognition. In particular, mice lack TCR genes homologous to the human Vγ9 and Vδ2 genes and therefore TCR Vγ9-Vδ2 cells, which represent the main population of TCR γδ cells in human peripheral blood, are absent in mice. The finding that TCR Vγ9-Vδ2 cells are restricted only to primates (Porcelli et al., 1991; Haas et al., 1993) has hampered the studies on the development of these cells as well as their relative importance *in vivo*. Therefore, we created a transgenic (Tg) mouse model in which mouse T cells express a TCR made by a human Vγ9-Vδ2 heterodimer.

In these studies we investigated which are the requirements for thymic maturation and colonization of peripheral lymphoid organs of this unique population of TCR γδ cells.

Results

Localization of Tg T cell in lymphoid organs

The expression of transgenic (Tg) human TCR chains on T lymphocytes from single TCR Vγ9-JP-Cγ1 (TCR γ) Tg or TCR Vδ2-Dδ3-Jδ1-Cδ (TCR δ) Tg

mice as well as from double TCR γδ Tg mice were analyzed by using monoclonal antibodies (mAbs) specific for the human TCR Vγ9 or Vδ2 chains. Analysis of the thymocytes from single Tg animals showed that the Tg TCR chains were not expressed at the cell surface (Figure 44), indicating that human TCR chains were not pairing with any of the endogenous mouse TCR chains. However, high expression of human TCR Vγ9 and TCR Vδ2 chains were detected on cell surface of double Tg animals, thus showing that the transgenic TCR chains could be expressed, pair to each other and assemble with the mouse CD3 complex at the surface of mouse cells.

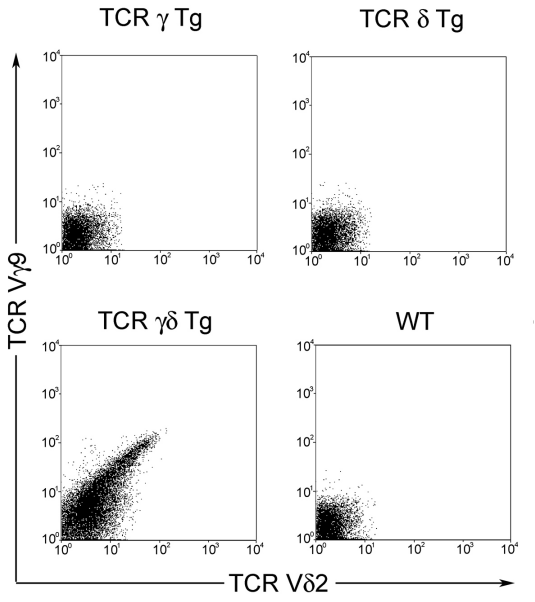

Figure 44. Expression of Tg TCR Vγ9-Vδ2.
TCR γδ analysis on the thymocytes isolates from single TCR γ, single TCR δ and double TCR γδ Tg animals and the WT control. Plots show double staining on total vital thymocytes using anti-TCR Vγ9 (Y axis) and anti-TCR Vδ2 (X axis) mAbs.

To have a clearer picture of the various cellular populations present in these animals, TCR γδ double Tg mice were further bred to recombination activating gene 2-deficient (Rag2 KO) mice, which lack endogenous B and T lymphocytes due to the impairment of V (D) J recombination (Shinkai et al., 1992).

High levels of human TCR γδ expression were found in the thymus of TCR γδ Rag-2-deficient Tg mice (Figure 45A), where approximately 65 ± 5% of cells were positive for the Tg TCR. The average cellularity of the Tg thymus was 6 x 10^7 cells (Figure 45B) thus in Tg animals a total of about 39 ± 3 x 10^6 cells/per thymus expressed the Tg TCR.

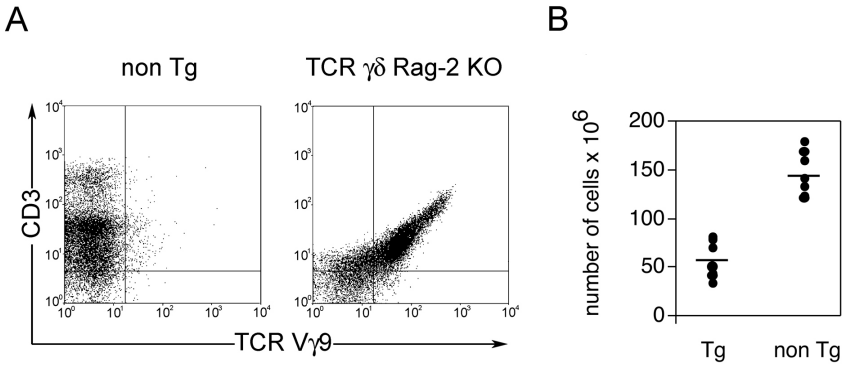

Figure 45. Tg TCR expression in the thymus.
(A) Surface expression of mouse CD3 and TCR Vγ9 chain on the total thymus of TCR γδ Rag-2 KO mice and non Tg littermate control.
(B) Total number of cells obtained from thymi of Tg TCR γδ Rag-2 KO mice (n=11) and non Tg littermates (n=7).

When we analyzed peripheral lymphoid organs, we found that, in the spleen (Figure 46) and in the lymph nodes (data not shown), only 0.1-0.9% cells bearing human TCR γδ were detected (the average of Tg T cells was 1.4 x 10^4/per spleen).

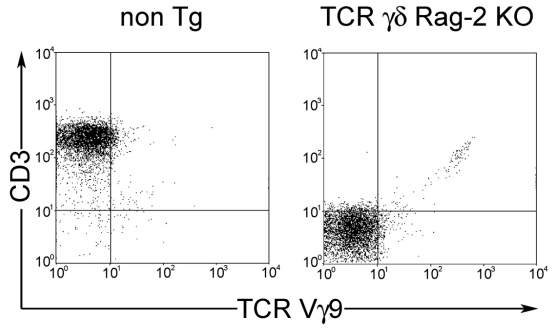

Figure 46. Surface expression of mouse CD3 and TCR Vγ9 chain on splenocytes. Analyzed cells were gated as Mac-1 and B220 negative from TCR γδ Rag-2 KO and non Tg control mice.

Tg thymocytes have a semi-mature phenotype

Impairment in the colonization of peripheral lymphoid organs prompted us to investigate whether Tg thymocytes were arrested during their development. Thymocytes from Rag-2-deficient TCR γδ Tg mice were stained with mAbs specific for surface markers characteristic of thymocyte differentiation. While Tg animals have similar percentages of thymocytes with DP phenotype, as compared to non Tg littermates, they have slightly increased numbers of DN cells, compensated by a decrease in the single CD4 and single CD8 positive

populations (Figure 47A). Within the thymus approximately 3% of the cells express high levels of CD62L (Figure 47B), a marker characteristic of mature thymocytes**,** but the population of CD62L^{int} cells which co-expresses CD69, the population of cells that undergoes positive or negative selections (Swat et al., 1993; Brandle et al., 1994), is absent in Tg animals (Figure 47B). Thymocytes were further analyzed for the expression of CD24 (heat stable antigen, HSA), a marker which is gradually lost during the maturation process (Lucas et al., 1993). We observed that Tg TCR γδ thymocytes do not downmodulate CD24 (Figure 47C), thus suggesting a block in thymocytes maturation. Moreover, DN Tg thymocytes do not acquire high levels of CD5, a negative regulator of TCR signaling (Figure 47D) which is upregulated after the TCR engagement (Tarakhovsky et al., 1995; Azzam et al., 1998). These findings show that Tg thymocytes express a pattern of surface molecules characteristic of only partially mature thymocytes (CD62L^{hi}, CD24^{hi}, CD5^{lo}) which depicts a maturation arrest and is accompanied by lack of egression from the thymus.

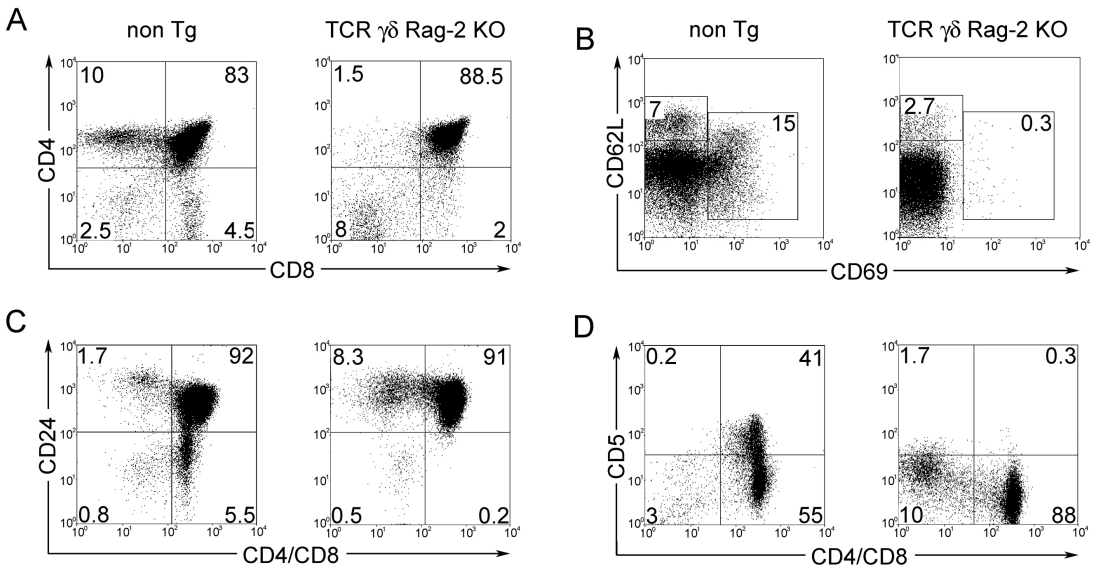

Figure 47. Analysis of transgenic thymocytes maturation stage as compared to non Tg cells.

(A) The expression of CD4 and CD8 on total thymocytes. Numbers represent percentage of cells in each quadrant.

(B) CD62L versus CD69 cell surface analysis on thymocytes. Numbers represent percentages of cells in each gated region.

(C) CD24 expression analyzed versus CD4 and CD8. Numbers represent percentage of cells in each quadrant.

(D) CD5 expression analyzed versus CD4 and CD8. Numbers represent percentage of cells in each quadrant.

Tg TCR is functional and induces T cell activation *in vitro*

We next investigated the possible reasons why the Tg TCR could not be appropriately stimulated *in vivo.* Firstly, to check whether the CD3-Tg TCR γδ complex was functional, we performed activation assays. Freshly isolated thymocytes were stimulated *in vitro* with the anti-TCR Vγ9 mAb (B3) specific for the human TCR Vγ9 region, with irrelevant mAb or with concanavalin A (Con A)

mitogen. This stimulation induced a strong Tg TCR γδ cells activation as demonstrated by upregulation of CD5 (Muller et al., 1997), and CD69 (Testi et al., 1989; Swat et al., 1993), comparable with that obtained with Con A (Figure 48). As control, the same treatment of thymocytes from non Tg littermates did not induce any upregulation of these activation markers. Thus, the TCR Vγ9-Vδ2 heterodimer expressed by Tg thymocytes is functional and after triggering induces T cell activation.

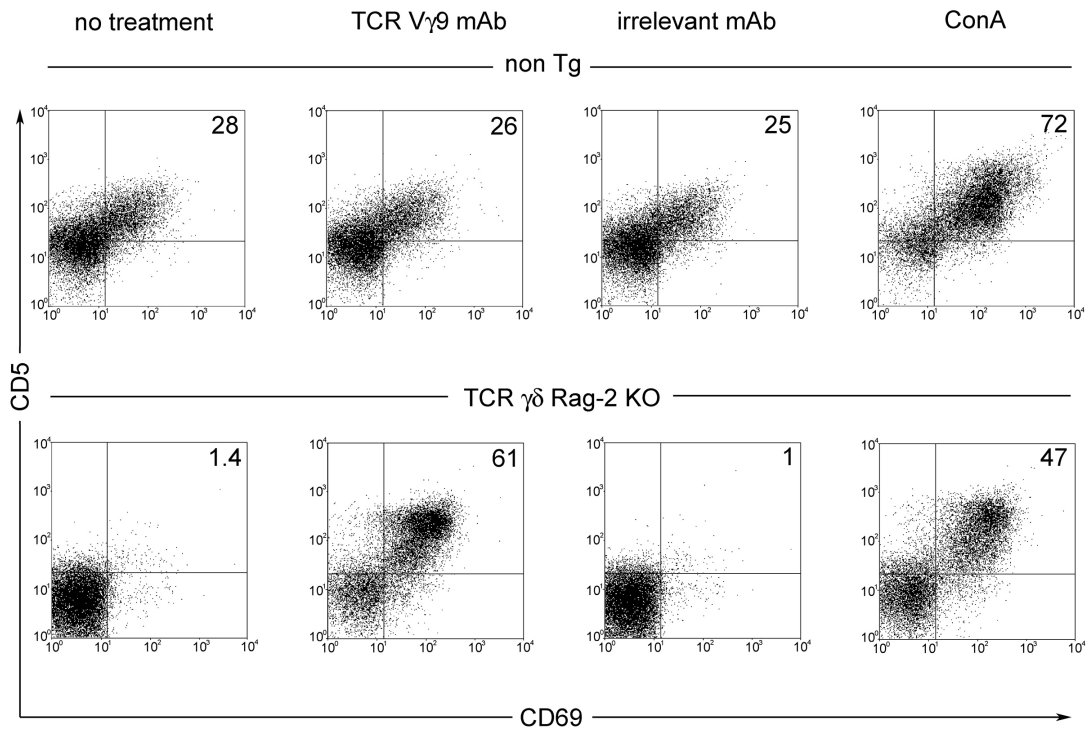

Figure 48. Tg thymocytes respond to an *in vitro* stimulation with anti-TCR Vγ9 mAb. Two color analysis of CD5 and CD69 expression after treatment with indicated stimuli. Numbers in upper left quadrant represent percentage of cells which were activated.

We further investigated whether Tg T cells were able to respond to the stimuli that activate human TCR Vγ9-Vδ2 cells. Various cell types of human or mouse origin, were treated with zoledronate (ZOL) and were used as APC. The stimulation of Tg T cells was evaluated by checking the expression of CD5 and CD69 activation markers. Tg thymocytes were activated by human DC and human thymic epithelial cells (TEC) but not by human thymocytes (Figure 49).

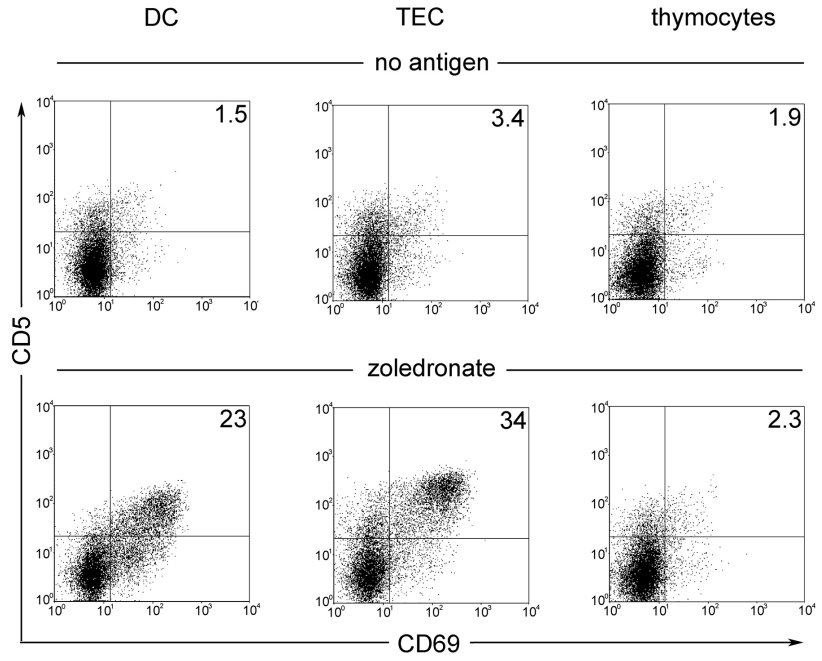

Figure 49. Human APC activate TRC γδ transgenic thymocytes *in vitro*.
Two color analysis of CD5 and CD69 expression on Tg thymocytes stimulated with ZOL-treated APC (DC, TEC and thymocytes).

Several other established tumor cell lines as well as freshly isolated cells of both human and mouse origin were also tested. Since only cells of human and

not of mouse origin stimulated Tg TCR Vγ9-Vδ2 cells (Table 8), we concluded that the lack of proper stimulation *in vivo* in the mouse was the reason why thymocytes do not fully mature and are unable to colonize peripheral lymphoid organs.

Responder cells:		Human TCR γδ clone		Tg TCR γδ thymocytes	
Cell line	**origin**	**no Ag**	**ZOL**	**no Ag**	**ZOL**
A375	human	-	+	-	+
A431	human	-	+	-	-
Daudi	human	+	+	+	+
Jurkat	human	-	+	-	-
MOLT-4	human	-	+	-	-
Raji	human	-	+	-	-
YMB-1	human	+	+	-	-
THP1	human	-	+	-	-
DC	human	-	+	-	+
thymocytes	human	-	-	-	-
TEC	human	-	+	-	+
DC	mouse	-	-	-	-
thymocytes	mouse	-	-	-	-
J558	mouse	-	-	-	-

Table 8. List of human and mouse APC tested in activation assays using either human TCR Vγ9-Vδ2 cell clone or Tg TCR Vγ9-Vδ2 thymocytes as responder cells.
In case of human TCR γδ clone the response was measured by IFNγ or TNFα release (+, statistically significant cytokine release; -, no cytokine release). In case of mouse thymocytes expressing human TCR γδ, activation was measured by analysis of CD5 and CD69 expression on the cell surface (+, upregulation of both markers as compared to control; - ,no change in expression as compared to the control).

DN TCR γδ thymocytes proliferate upon *in vitro* TCR stimulation

Further analysis of Tg thymocytes showed that upon *in vitro* stimulation they acquire CD4 CD8 DN phenotype (Figure 50). Accumulation of DN cells gradually increases with the time of stimulation. Surprisingly we also observed increased number of either CD4 SP or CD8 SP cells when we stimulated with anti TCR Vγ9 mAb or Daudi cells, respectively.

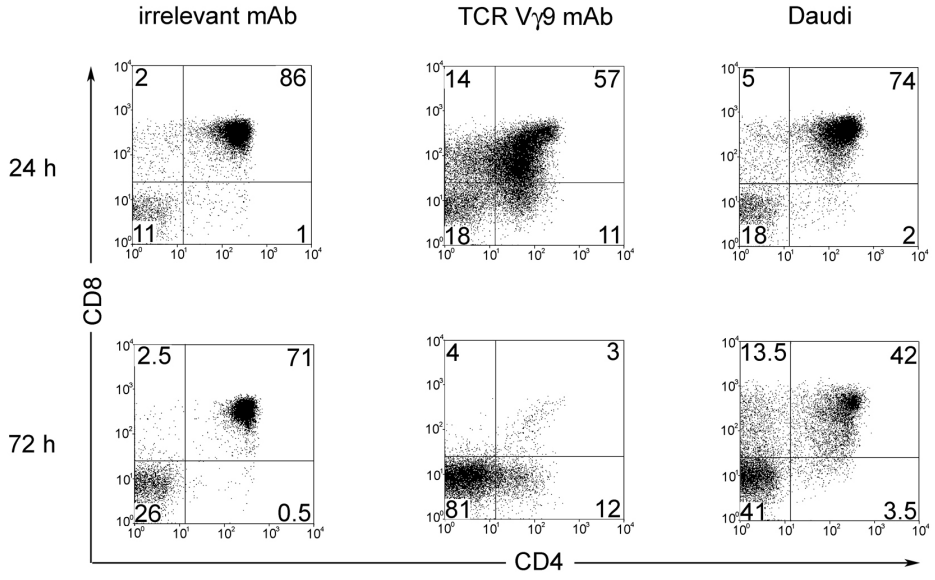

Figure 50. Enrichment of DN Tg thymocytes upon *in vitro* stimulation.
Analysis of CD4 and CD8 expression after 24h or 72 h treatment with indicated stimuli. Numbers in each quadrant represent percentage of cells.

The observed accumulation of DN Tg thymocytes after *in vitro* stimulation is most likely the result of their proliferation. In order to confirm this hypothesis we checked the capacity of DP and DN thymocytes to proliferate when

stimulated with anti-TCR Vγ9 mAb (Figure 51). We found that only DN thymocytes upon specific TCR stimulation were able to proliferate.

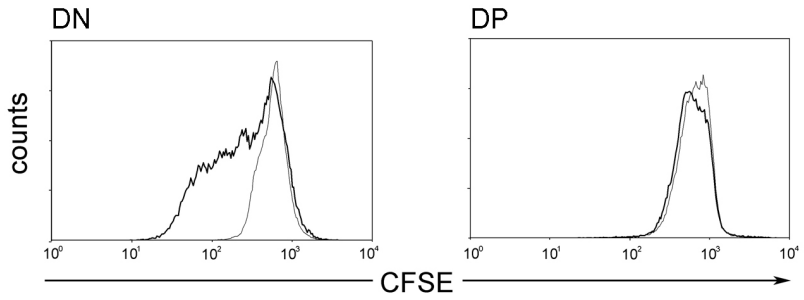

Figure 51. DN Tg thymocytes proliferate upon TCR stimulation.
Analysis of CFSE intensity in DN or DP sorted thymocytes treated for four days with anti-TCR Vγ9 mAb (bold line) or irrelevant mAb (thin line).

Triggering of Tg TCR *in vivo* induces maturation of Tg thymocytes

In order to bypass the observed maturation arrest and give a stimulus to these cells, we triggered the Tg TCR *in vivo*. Tg mice were injected intra-thymically (i.t.) with the anti-TCR Vγ9 mAb which was successfully used in the previous *in vitro* experiments. Already one week after injection, thymic accumulation of DN thymocytes was observed (Figure 52A). The number of DN cells in the thymus of treated animals increased approximately five times in comparison to the control animals injected with an irrelevant isotype-matched mAb. Direct TCR triggering resulted also in upregulation of CD62L and CD69. Approximately 23% of cells expressed high levels of CD62L and 6% of thymocytes increased their CD69 expression (Figure 52B). Further investigations

revealed that Tg thymocytes upon *in vivo* TCR γδ stimulation downmodulated CD24 and upregulated CD5 (Figure 52C).

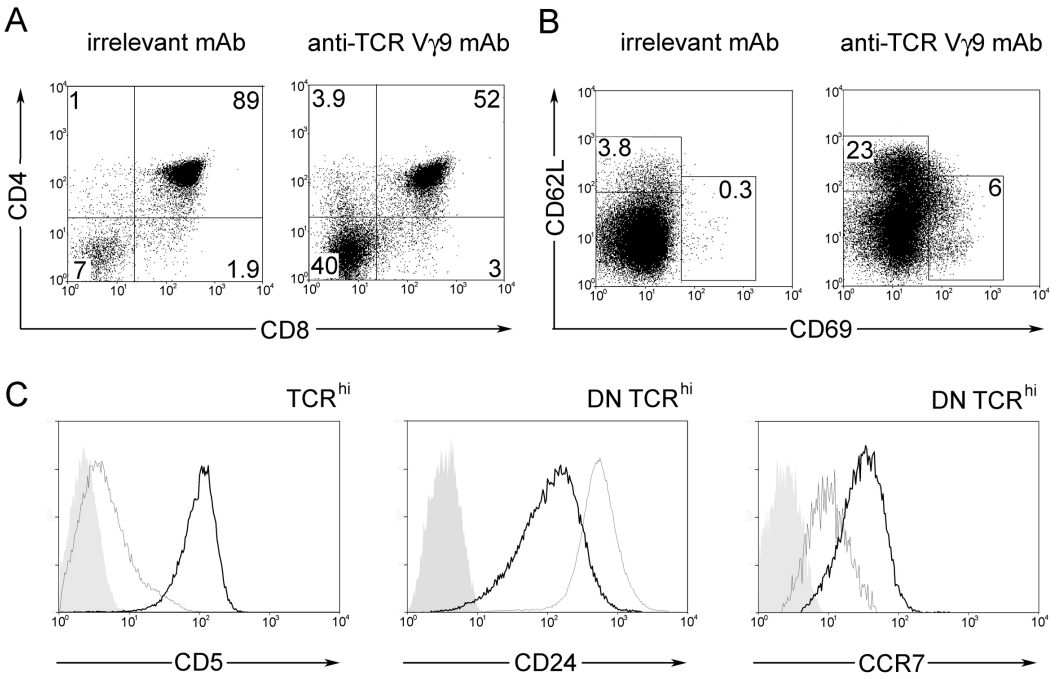

Figure 52. Analysis of thymocytes maturation stage after TCR triggering *in vivo*.
(A) CD4 versus CD8 analysis on total vital thymocytes isolated one week after i.t. injection of anti-TCR Vγ9 mAb or irrelevant mAb. Numbers in quadrants represent percentage of cells.
(B) CD62L and CD69 surface expression on thymocytes isolated from mice two weeks after i.t. injection of indicated antibodies.
(C) Surface expression of CD5, CD24 and CCR7 in thymocytes isolated one week after i.t. of anti-TCR Vγ9 mAb (bold line) or irrelevant mAb (thin line); grey histogram represents negative control (staining with an isotype-matched control antibody). The cell population analyzed in each panel is indicated above each histogram.

Moreover the levels of CC-chemokine receptor 7 (CCR7), whose expression is important for the cortico-medullary migration of positively selected thymocytes (Ueno et al., 2004) as well as for the exit from the thymus (Ueno et al., 2002), was increased following TCR triggering (Figure 53A). In order to test the functionality of CCR7 an *in vitro* chemotaxis assay was performed. Cells isolated from the thymi, after induction of T cell maturation, were specifically migrating towards secondary lymphoid-tissue chemokine (SLC), a selective CCR7 ligand (Figure 53B). As consequence of CCR7 upregulation the migration of thymocytes was enhanced, after induction of T cell maturation.

Taken together these results suggest that this Tg population needs a proper stimulation in the thymus for its normal development.

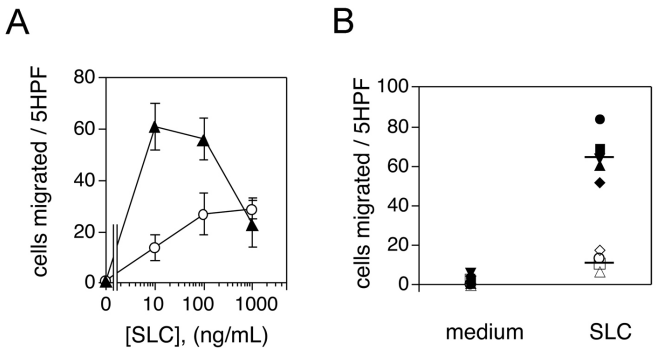

Figure 53. Functional analysis of CCR7.
(A and B) Migration of thymocytes isolated one week after i.t. injection of anti-TCR Vγ9 mAb (●) or irrelevant mAb (○) induced by the chemokine SLC, a selective CCR7 ligand. (A) Dose dependent migration of thymocytes from two representative animals. (B) Thymocytes migration in the presence or absence of the optimal dose (10 nM) of SLC is showed for several animals. Cell count was performed in 5 high-power fields (5HPF).

Upon TCR triggering TCR γδ T cells exit the thymus and colonize peripheral lymphoid organs

We also found that triggering of Tg TCR *in vivo* induces T cell colonization of peripheral lymphoid organs. Seven days post intrathymic injection of anti-TCR γδ mAb, Tg T cells were detected in the spleen (Figure 54A) and in the lymph nodes (data not shown). After the TCR triggering the percentage of Tg T cells was increased up to 6-10% in anti-TCR Vγ9 mAb injected animals compared to control mAb-injected animals where Tg T cells were 0.1-0.9%. This results in an increase of total number of Tg T cells from an average $0.14x10^5$ to $2.7x10^5$ cells per spleen (Figure 54B). Moreover after the egression from the thymus Tg T cells persist for a long time in the periphery as demonstrated by staining after 6 months post injection, which showed amounts of TCR Tg cells similar to the one observed after 1-2 weeks (data not shown).

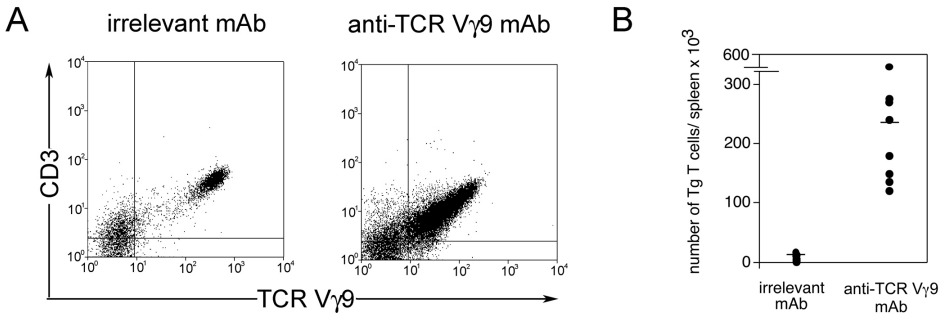

Figure 54. Analysis of Tg T cells in the periphery.
(A) Surface expression of CD3 and TCR Vγ9 chain on the Mac-1 negative cells from spleen. Cells were obtained from mice 2 weeks after i.t. injection of anti-TCR Vγ9 mAb or an irrelevant mAb.

(B) Total number of Tg T cells was calculated per each analyzed spleen of mice injected i.t. with either irrelevant mAb (n=8) or with anti-TCR Vγ9 mAb (n=8). Horizontal lines represent average number of cell in each group.

The vast majority of Tg T cells that colonize peripheral lymphoid organs is DN, expresses CD5 and has non activated $CD25^{-}$ $CD69^{low}$ phenotype (Figure 55).

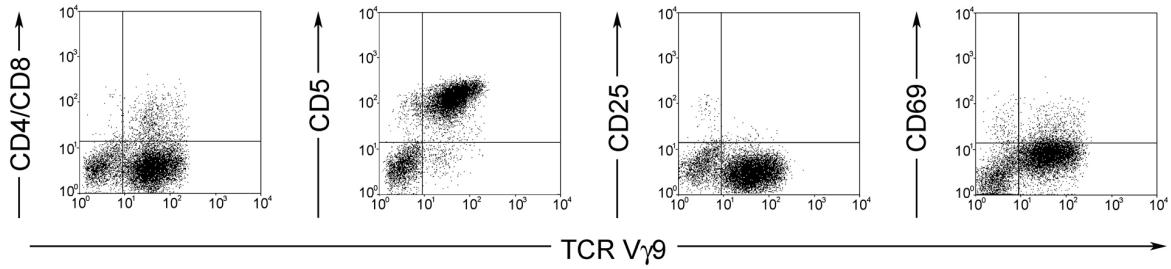

Figure 55. Phenotypic analysis of Tg T cells in the spleen.
Surface expression of CD4/CD8, CD5, CD25, CD69 and TCR Vγ9 two weeks after i.t. injection of anti-TCR Vγ9 mAb. Mac-1 positive cells were excluded from the analysis.

The functionality of the cells that colonize peripheral lymphoid organs was determined in an *in vitro* stimulation assay. Peripheral TCR γδ cells were activated in the presence of specific anti-TCR γδ mAb as well as in the presence of Daudi cells. Activated T cells upregulated CD25 and CD69 markers (Figure 56A) and also released IFNγ (Figure 56B). These data show that cells which colonize the periphery are functional.

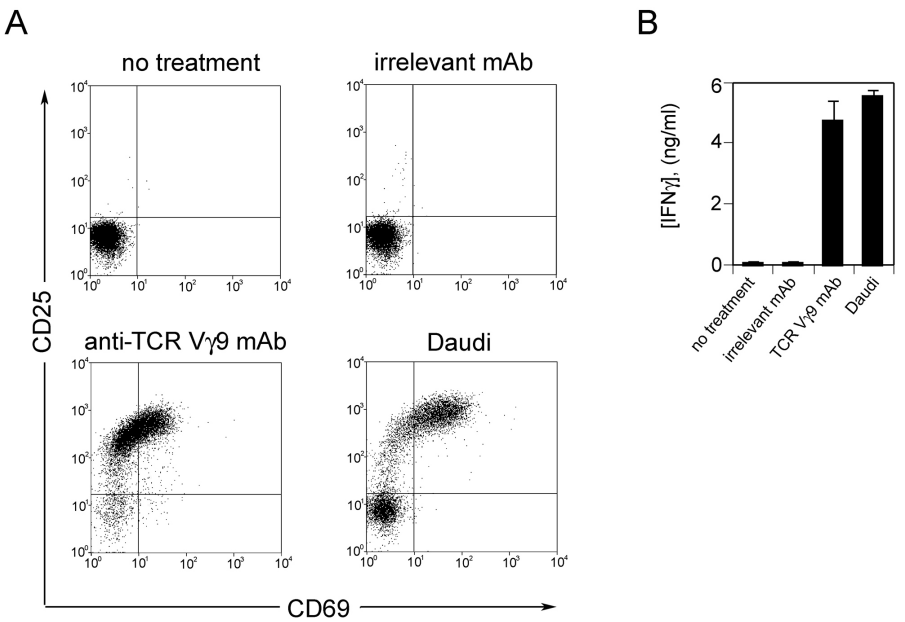

Figure 56. Tg splenocytes are functional *in vitro*.

(A) Splenocytes from mice injected i.t. with anti-TCR Vγ9 mAb were activated with indicated stimuli for 24 h and analyzed for expression of CD25 and CD69 activation markers. Mac-1 and propidium iodide positive cells were excluded.

(B) Splenocytes from mice injected i.t. with anti-TCR Vγ9 mAb were activated with indicated stimuli for 48 h and mean release of IFNγ ± SD was measured by ELISA.

Discussion

The mechanisms governing the development of human TCR γδ cells remain largely unknown. The TCR γδ cells development has been mainly studied using two mouse TCR γδ Tg models, G8 and KN6. Tg TCR γδ cells in G8 and KN6 mouse models carry the rearranged TCR γδ reactive to the MHC class Ib T22 molecule (Bluestone et al., 1988; Bonneville et al., 1989; Ito et al., 1990). So far it is not clear whether the selection process of these TCR γδ cells requires ligand engagement since contradictory results have been published (Wells et al., 1991; Pereira et al., 1992; Schweighoffer and Fowlkes, 1996; Haks et al., 2005). More recent reports provide evidence for ligand-mediated positive selection of murine DETC population (Xiong et al., 2004; Lewis et al., 2006).

In order to study TCR Vγ9-Vδ2 cells *in vivo* we have generated a transgenic mouse model in which human TCR γδ is expressed on mouse T cells. Tg TCR was cloned under the control of human CD2 promoter that assured early expression of transgene during ontogeny (Duplay et al., 1989; Sen et al., 1989; Yagita et al., 1989). Cells expressing the human Tg TCR are very abundant in the thymus of Tg mice and they represent the only T cell population present in the thymus of Tg mice with Rag-2-deficient background. However, in the peripheral lymphoid organs only few cells bearing Tg TCR are present.
The unusual tissue distribution of Tg T cells could be the result of impaired thymic maturation and/or selection. In the thymi of Tg mice we observed only a small increase in the percentage of DN cells (up to 10%), together with a reduced

number of CD4 SP and CD8 SP cells, as compared to non Tg littermates. This is in contrast with other Tg models namely the Tg G8 TCR γδ mice and Tg Vγ6-Vδ1 TCR γδ mice in which, DN cells reach approximately 50% of total thymocytes (Livak et al., 1997; Hayes et al., 2005). The DP cell compartment was comparable to the one observed in non Tg littermates. The presence of DP thymocytes in Tg mice is not completely surprising since also in the thymus of TCR β $^{-/-}$ mice, where TCR αβ development is arrested, DP cells are present in variable numbers (Mombaerts et al., 1993; Passoni et al., 1997). However, the DP cells in TCR β $^{-/-}$ mice derive from TCR γδ-expressing progenitor cells but are committed to the αβ T cell linage (Kang et al., 1998).

According to the signal-strength model, in which strong TCR signals promote γδ lineage development while weak TCR signals in synergy with Notch signaling promote αβ T cell differentiation through DP intermediates (Hayes et al., 2003; Haks et al., 2005; Hayes et al., 2005; Garbe and von Boehmer, 2007; Hayday and Pennington, 2007). The acquisition of DP phenotype might result from impaired stimulation of the Tg TCR γδ. This is also in agreement with the findings in Tg KN6 TCR γδ mice in which blocking of the TCR engagement is followed by expression of DP phenotype in 90% of thymocytes (Haks et al., 2005).

Our studies revealed that Tg thymocytes express a pattern of molecules characteristic for partially mature thymocytes (CD62L^{hi}, CD24^{hi,} CD5^{lo}, CCR7^{-}). TCR^{hi} DN cells express high levels of CD62L, a marker characteristic of mature thymocytes, whereas the CD24 molecule, which is low in mature cells, is not downmodulated. In Tg KN6 TCR γδ mice the absence of specific TCR γδ ligand

skewed DN thymocytes towards immature CD24^{hi} phenotype while the presence of the specific ligand lead to CD24 downmodulation and maturation of TCR γδ DN thymocytes (Haks et al., 2005). Additionally we found that, the expression CD5, which is upregulated after pre-TCR and TCR engagement (Azzam et al., 1998), is maintained at low levels on Tg TCR Vγ9-Vδ2 thymocytes.

During maturation processes T cells upregulate transiently CD69 very early after TCR triggering and decline the expression of this surface marker shortly after stimuli withdrawal (Testi et al., 1989). In the thymus DP thymocytes that are undergoing selection also transiently express CD69 (Swat et al., 1993; Brandle et al., 1994) and after the selection they lose the CD69 expression and gain expression of the peripheral homing marker CD62L (Gabor et al., 1997). Therefore, the absence in Tg TCR Vγ9-Vδ2 mice of cells expressing CD69 in another piece of evidence indicating that Tg thymocytes abort their maturation process.

Importantly, Tg thymocytes are able to respond *in vitro* to various stimuli capable of activating human TCR Vγ9-Vδ2 cells in a specific manner. Both anti-TCR Vγ9 mAb and human APC presenting endogenous antigens were able to activate Tg thymocytes indicating that the Tg human TCR associated with mouse CD3 complex is functional. Additional analysis of CD4 and CD8 expression showed that activated *in vitro* DN, but not DP, Tg thymocytes are able to proliferate. Thus DN Tg thymocytes in terms of phenotype and functionality reflect human TCR Vγ9-Vδ2.

Taken together these findings suggest that Tg TCR γδ thymocytes *in vivo* do not receive an appropriate TCR stimulation and therefore their thymic development is arrested. This is supported by our finding that shortly after *in vivo* TCR stimulation a significant change in the phenotype of Tg T cells is observed as compared with controls. Firstly, there was approximately five fold increase in the percentage of DN cells meaning that potentially there are more cells that can mature into TCR γδ cells. We also observed an increased percentage (from 3% to 23%) of cell expressing high levels of CD62L and appearance of CD69 positive cells. Moreover, the same DN cells which express high levels of Tg TCR and CD62L, also dowmodulated CD24 expression and upregulated CD5. Finally triggered Tg thymocytes upregulataed functional CCR7. This chemokine receptor is expressed in response to TCR engagement and is involved in the emigration of newly generated mature thymocytes to the peripheral lymphoid organs (Ueno et al., 2002).

The direct *in vivo* triggering of Tg TCR γδ induced change from immature ($CD62L^{hi}$, $CD24^{hi}$, $CD5^{lo}$, $CCR7^{-}$) to mature ($CD62L^{hi}$, $CD24^{lo}$, $CD5^{hi}$, $CCR7^{+}$) phenotype of Tg thymocytes, which become able of homing to peripheral lymphoid organs. The T cells that colonize the spleen have quiescent phenotype ($CD25^{lo}$ $CD69^{lo}$) and respond *in vitro* to human TCR γδ stimuli. Moreover, Tg T cells persist at least six months (data not shown) in the spleen after the i.t. injection of anti-TCR Vγ9 mAb despite the absence of stimulation.

In our studies we generated a mouse model in which human TCR Vγ9-Vδ2 cells are present in the thymus but do not exit to the periphery. We have shown

that inefficient maturation resulting in the thymic arrest of Tg thymocytes occurs due to the lack of appropriate stimulation. By providing specific TCR γδ stimulation, maturation and egression of Tg thymocytes was induced. However, we cannot conclude whether TCR signaling promotes proper γδ linage choice or drives positive selection of Tg TCR γδ cell or both. Importantly, results obtained in *in vitro* stimulation assays might suggest involvement of human DC and/or thymic epithelial cells, but not thymocytes, in these processes.

Conclusions

This work focuses on the mechanisms responsible for TCR Vγ9-Vδ2 cells activation and mechanisms required for the thymic maturation of TCR Vγ9-Vδ2 cells.

We have demonstrated that in tumor cells and bacteria infected cells alterations in the mevalonate pathway result in activation of TCR Vγ9-Vδ2 cells. Therefore, mevalonate dysregulation may serve as a novel type of danger signal (Seong and Matzinger, 2004) alerting the immune system of primates. Metabolites generated in mevalonate pathway are necessary for sterol synthesis, cell growth and membrane integrity (Edwards and Ericsson, 1999). As the mevalonate pathway is ubiquitous because it is essential to cell survival, mevalonate-deficient cells cannot be generated by selection-induced mechanisms. This makes detection of cells with mevalonate pathway alterations an optimal system for surveying the metabolic integrity of cells that may be altered, either upon tumor transformation or infection. This alert system is unique to primates, the only species so far known to have a population of T cells reacting to phosphorylated mevalonate metabolites. Although a selective advantage of this immune response remains obscure, it is tempting to speculate that it is an important protective mechanism during the initial phases of infection when antigen specific TCR αβ cells have not been recruited and expanded (De Libero, 1997). Therefore, this mechanism should not be inhibited and might be exploited for novel immunotherapy. An important consequence of these findings

is that the use of drugs that efficiently inhibit cholesterol synthesis by blocking HMGR enzymatic activity (Grundy, 1988; Greenwood et al., 2006) deserves careful evaluation in patients with infections

Up to date mechanisms of cellular trafficking and loading, on the putative antigen-presenting molecule, of TCR γδ stimulatory ligands has not been described.

Here we demonstrated that MRP5, an ATP-dependent transporter, participates in TCR γδ activation process by transporting mevalonate metabolites. Since MRP5 is known to transport cyclic nucleotides, it could contribute to the clinical resistance to nucleoside and nucleotide analogues which are used in anticancer and antiviral therapy (Kruh et al., 2001; Adachi et al., 2002). Therefore, targeting MRP5 has to be carefully planed since it might hamper the recognition of tumors by TCR Vγ9-Vδ2 cells.

Our results obtained with TCR γδ Tg mice show that the lack of unique restriction element in the mouse thymus results in maturation arrest of Tg TCR Vγ9-Vδ2 cells. We have shown that when the proper TCR γδ stimulation is provided, Tg T cells mature and colonize peripheral lymphoid organs. This suggests that TCR Vγ9-Vδ2 cells for the proper thymic development require selection process that might be of similar complexities as those required for the positive selection TCR αβ cells.

Materials and Methods

Bacteria

The following bacteria were used in infection experiments: *E. coli* DH5α and *S. aureus* Newman. Bacterial cultures in the logarithmic phase were obtained by growing *E. coli* in LB broth (Difco) and S. aureus in TSB broth (Difco) at 37℃. Series of 1/10 dilution was prepared and b acteria cell number was determined by CFU count after overnight cultures on LB-agar or MH-agar (Difco) plates.

Cell culture reagents

RPMI 1640 medium (BioWhittaker) was supplemented with 1 mM sodium-pyruvate (Gibco), 50 μM non-essential amino acid (Gibco), 2 mM UltraGlutamine II solution (Gibco) and 100 μg/ml Kanamycin (Gibco) and used as base cell culture medium. Base medium was supplemented with 10% fetal calf serum (FCS, BioWhittaker, Europe) for tumor cell line cultures or with 5% human serum (HS, from healthy, blood group AB+ donors, Blutspendezentrum Basel) and 100 U/ml human recombinant IL-2 for T cell clone cultures. For mouse cell culture the D-MEM medium (Gibco) was used to prepare the base medium.
All the cell culture media were filtered through Stericup GP Express PLUS 0.22 μM membrane filters (Millipore).

Cells

The following cell lines were used: A-431, an epidermoid cancer; Daudi, a Burkitt's lymphoma; THP-1, a monocytic leukaemia; CEM 1.3, a T cell lymphoma; HeLa, a cervix epithelial carcinoma; Colo-201, a coloncarcinoma line; HEP-G2, a hepatocarcinoma line, K562 an erythroleukemia line, Raji Burkitt's lymphoma, RJ 2.2.5 MHC class II-deficient mutant of Raji and J558, a mouse myeloma cell line all obtained from American Type Culture Collection (ATCC). YMB-1, HMC-1-8, and MRK-nu-1 mammary carcinomas were obtained from Health Science Research Resources Bank, Osaka, Japan. Primary human lung fibroblasts were obtained from M. Bihl; A-375 cells, a malignant melanoma from G. Spagnoli, HuH6 hepatoblastoma cell line from E. Köhler; glioblastomas U118 and BS125.3.2 from A. Merlo (all from University Hospital, Basel, Switzerland); astrocytoma A-243 was established from an anaplastic astrocytoma (WHO grade III). THP-1 cells transfected with CD1B gene were generated as described (Gilleron et al., 2004).

The following TCR Vγ9-Vδ2 cell clones were used: G2B2, G2B9, G2C19 obtained as described bellow.

Freezing and thawing of primary cells and cell lines

Cells were washed once with PBS (BioWhittaker), then resuspended in freezing medium containing 90% FCS and 10% dimethylsulfoxide (DMSO, Sigma-Aldrich). Samples were stored in liquid nitrogen containing tanks. In order to thaw cells, frozen vials were incubated at 37°C until they were thawed and

then cells were transferred to 15 ml tubes, slowly buffered with PBS and centrifuged.

Preparation of human monocytes and dendritic cells (DCs)

Monocytes and monocyte-derived DCs from healthy donors were obtained from peripheral blood mononuclear cells (PBMC). PBMC were purified from heparinized blood of healthy donors or buffy coat (Blutspendezentrum Basel) using a density gradient (Ficoll-Paque, Pharmacia). In brief, blood samples were diluted in PBS (two volumes) and slowly applied on a ficoll gradient (3 volumes blood: 1 volume ficoll). PBMC were separated by centrifugation at 1000 rpm, room temperature for 20 min with slow acceleration and slow breaking rate. Monocytes were sorted from PBMC using anti-CD14-labeled magnetic beads (MACS, Miltenyi) according to the product protocol. Isolated monocytes were either directly used in antigen presentation assays or cultured in 6 well plates (3 ml/well) for 5 days in base medium supplemented with 10% FCS, GM-CSF (50 ng/ml) and IL-4 (1000 U/ml) at a concentration of 4 x 10^5 cells/ml to induce their differentiation into DCs.
The DC differentiation was checked after 5 days of culture by surface staining evaluating expression of antigen-presenting molecules (CD1a, CD1b, MHC class I and MHC class II-DR) and costimulation molecules (CD80, CD86).

Expansion of human thymic epithelial cells (TEC)

TEC were expanded on a layer of irradiated feeder 3T3-J2 cells. Irradiated feeder cells (6000 RAD) 1.8 x 10^6 were plated in 75 cm^2 flasks in base D-MEM/F12

medium supplemented with 10% FCS, 5 μg/ml insulin (Sigma), 5 μg/ml human transferrin (Behring Diagnostic), 2 nM 3,3',5 triiodo-L-thyronine sodium salt (T3, Sigma-Aldrich), 0.4 μg/ml hydrocortisone (Behring Diagnostic), 100 nM cholera toxin (Scharz-Mann), 18 mM adenine (Boehringer Mannheim) ,10 ng/ml epithelial growth factor (human recombinant EGF, Chiron Corp.). After 3 h at 37°C TEC (2 x 10^5/flask) were added and grow until confluency.

Generation of human T cell clones

TCR γδ clones were established from PBMC of healthy donors. Cells expressing TCR γδ were sorted using pan anti-δ mAb (δ1) and cloned by limiting dilutions in Terasaki plates. Sorted cells were incubated in the presence of 5 x 10^5/ml irradiated PBMC (1500 RAD), in 20 μl of base medium supplied with 5% HS, 1000 U/ml IL-2 and 1 μg/ml phytohemaglutinin (PHA; Murex, Dartford). After 10-15 days growing cultures were transferred to 96 well-plates, expanded and tested for antigen specificity.

Maintenance of human T cell clones

Human T cell clones were cultured in base cell culture medium containing 5% HS and IL-2 at 100 U/ml and expanded by restimulation every 25-35 days. Restimulation was performed in the presence of irradiated (1500 RAD) PBMC (1:1 ratio) in the same cell culture medium supplied with 1 μg/ml PHA. For antigen dependent activation experiments, restimulated T cell clones were used 12-18 days after restimulation.

T cell stimulation assays

Experiments with bisphosphonates

APC were incubated with different doses of bisphosphonates for 3 h at 37℃ or at 4℃ (pulsing) followed by three washes i n PBS before plating (5 x 10^4 cells/well) and subsequent addition of T cells (5 x 10^4 cells/well). In some experiments 25 μM mevastatin (MEV, Sigma-Aldrich) was added 2 h before bisphosphonates and again after removal of bisphosphonates to maintain a constant concentration during incubation with T cells. Farnesol (Sigma-Aldrich) and 7-dehydrocholesterol (7-DHC, Sigma-Aldrich) were pulsed on APC for 12 h and rinsed out before addition of T cells. Stimulation with IPP (10 μM, Sigma-Aldrich) and PHA (1 μg/ml) served as positive controls to exclude unspecific or toxic effects of the tested drugs. To study the kinetics of activation induced by nitrogen-containing bisphosphonate drugs (nBP), Daudi cells were pulsed with 50 μM pamidronate (PAM), or zoledronate (ZOL), or etidronate (all supplied by Novartis) for selected periods (1-18 h), washed three times, and incubated with the TCR γδ clone G2B9 for 12 h. T cell activation was estimated by measuring cytokine release.

Bacterial infection experiments

APC were incubated for indicated time periods at 37℃ with different MOI (range from 0.003 to 3 bacteria: 1 APC), after 3-4 h gentamycin (15 μg/ml, Invitrogen) was applied 2 h later TCR γδ cells were added and incubated for 12-

46 h. In HMGR-blocking experiments, MEV (50 μM) was added 2 h before ZOL or bacterial infection and kept in culture during the whole T cell stimulation assay. Four different TCR Vγ9-Vδ2 cell clones were used with similar results. All experiments illustrated in the figures were carried out with the G2C19 TCR γδ cell clone and were repeated at least three times.
In control experiments the following TCR αβ clones were used: MHC class II-restricted clone G15A75 specific for a *M. tuberculosis* peptide antigen (*M. tuberculosis* H37Ra, 50 μg/ml, Difco) and CD1b-restricted clone Z4B27 specific for diacylsulphoglycolipids (Ac_2SGL, 10 μg/ml), (Gilleron et al., 2004).

Ligand transfer experiment

The mouse B cell lymphoma cell line M12.4.1 was pulsed with 100 μg/ml ZOL of for 2 h at 37°C, washed three times with PBS, and plated (25 x 10^4 cells/well) in 96 well plates. DC cells (5 x 10^4cells/well) and the TCR γδ clone G2B9 (5 x 10^4cells/well) were added. Activation of the TCR γδ clone was detected by measuring the TNFα release after 12 h culture.

Experiments with drugs inhibiting transport proteins

Series of drugs were tested to determine the type of transport protein involved in transport of endogenous phosphorylated TCR γδ antigens. In these experiments Daudi cells were used as APC. The treatment with ZOL (5 μg/ml) or sec-butylamine (SBA, 4 mM, Fluka) was used to induce accumulation of

endogenous ligands. Activation with exogenously added IPP or PHA was used to control the specificity of the inhibition. Additionally TCR αβ clone CD1a restricted and sulfatide specific was used to determined specificity of inhibition to TCR γδ cells.The transporter inhibitors were purchased from Sigma-Aldrich accept: PSC-883 and PKF-274-266 provided by Novartis; methotrexate and MK-571 purchased from Alexis.

APC were treaded with ZOL or SBA for 3h, then drugs were applied to the culture and 3 h later T cells were added. In parallel the pulsing experiments were performed. In case of pulsing experiments APC were treated with ZOL or SBA for 3 h then drugs were added for additional 6 h. Then APC were washed and T cells were added and incubated for 6 h. T cell activation was determined by measurement of TNFα release. When toxic effects where occurring by keeping the drugs overnight, only the data from the experiments with pulsed drugs were considered. A series of concentrations were tested for each drug, according to the literature. A positive inhibition of antigen transport was considered only at drug concentrations, inhibiting activation by SBA and ZOL, but not exogenous IPP, PHA activation, nor activation of the TCR αβ clone.

Some drugs showed at each concentration some toxic effects together with a strong inhibition of activation by ZOL or SBA. In these cases the highest value of inhibition detected with IPP, PHA or the TCR αβ clone was subtracted from the value of ZOL inhibition. Specific inhibition was expressed as percent of inhibition in relation to TCR γδ cell activation in the absence of drug (100% response).

Cytokine determination by Enzyme Linked Immunosorbent Assay (ELISA)

Released cytokines from T cell activation assays were measured by ELISA. ELISA plates (Maxisorb, Nunc) were coated with the capture antibodies for 12 h at 4℃, dissolved in PBS at a concentratio n from 1-10 μg/ml, containing 0.02% NaN_3 (Fluka). The incubation of the coating antibody was done using 70 μl of antibody solution per well. After coating, the ELISA plates were washed once with washing buffer containing PBS and 0.05% Tween 20 (Fluka) on a plate washer (ELX50 autostrip washer, Polygon Science) and wells were blocked with 200 μl of blocking buffer containing PBS, 0.05% Tween 20 and 1% bovine serum albumine (BSA, Sigma-Aldrich) for at least 1 h at room temperature. After blocking, plates were washed and incubated for 2 h with 70 μl of sample or the standard recombinant cytokines. Then plates were washed three times with washing buffer and incubated for at least 1 h with 70 μl of the detection antibody, diluted in blocking buffer at 1-5 μg/ml. After incubation plates were washed again three times and incubated with 70 μl of Streptavidin-HRP (Zymax Streptavidin-HRP conjugate, Zymed) diluted in blocking buffer 1:4000. After 1 h incubation, plates were washed five times and incubated with 90 μl of the substrate solution (fast o-phenylenediamine dihydrochloride tablet, Sigma-Aldrich). The substrate incubation was done for 5-10 min then stopped by addition of 10% H_2SO_4 (50 μl/well, Fluka). The absorption was read at 490 nm, with an ELISA reader (Spectra Max 190, Molecular Devices). The samples were expressed as duplicates or triplicates and concentration of the cytokine was calculated with a

standard curve made by serial dilutions of the appropriate recombinant cytokine (Pharmingen).

The following coating antibodies were used: MAb1 (BD, Pharmingen) for human TNFα; HB 8700 (ATCC) for human IFNγ; 8D48 antibody (BD Biosciences) for human IL-4 and JES6-1A12 (BD Biosciences) for mouse IL-2.

The following revealing biotinylated antibodies were used: MAb11 (BD Biosciences) for human TNFα; γ69-2GV for human IFNγ; MP4-25D2 (BD Biosciences) for human IL-4 and JES6-5H4 (BD Biosciences) for mouse IL-2.

Recombinant cytokines production

Human cytokines IL-4, IL-6, IL-2, GM-CSF and mouse GM-CSF were obtained by culturing J558 cells transfected with plasmid containing the appropriate human cytokine cDNA in base cell culture medium supplemented with 10% FCS. Cell-free culture supernatants were harvested from cell cultures containing 1-2 x 10^7 cells/ml. The concentration of hGM-CSF, hIL-4, hIL-6 and mGM-CSF were determined by ELISA. The active units of human IL-2 were measured using the standard IL-2-dependent cytotoxic T cell line (CTLL-2) proliferation assay.

Generation of stable transfectants

Hamster HMGR cDNA (pRed-227;ATCC), was amplified with the following primers:

5'HamHMG_XhoI_ ATCTCGAGGGACCGAGTGGCTACAATG;

3'HamHMG_HincII_ATGTCGACAGCTGACTTCTTGGTGCACG;

and subcloned (*Xho*I/ *Hinc*II) into pBluescript II KS (Stratagene). The gene was tagged at the 3' end with a sequence for 19 amino-acid long BirA peptide (KLGGGLNDIFEAQKIEWHE) and was further subcloned (*Xho*I/*Not*I) into BCMGSNeo expression vector (Karasuyama et al., 1990).
pABC11 (McAleer et al., 1999) plasmid construct containing MRP5 fused to GFP (N. Matthews Yamanouchi Research Institute, United Kingdom and P.Artursson Department of Pharmacy, Uppsala University, Sweden) was used to transfect different cell lines. A-375 cells were transfected with 200 ng DNA using Effectene (Qiagen) and Daudi cells were tansfected with 10 (g of DNA by electoporation. For both cell lines permanent transfectants were selected with 0.8 mg/ml of G418 (Calbiochem).

Genaration of MRP5 shRNA interference constructs

PSHAG-1 and pSHAG-Ff1 plasmids (Paddison et al., 2002) containing U6 promoter only and firefly luciferase gene specific small hairpine RNA (shRNA) interference, respectively were kindly provided by G.J. Hannon (Watson School of Biological Sicences, Cold Spring Harbor, USA). The following oligonucleotide pairs were annealed and inserted into pSHAG-1 plasmid (BseR I/BamH I) in order to generate MRP5 sequence specific shRNA interference constructs:

42_forward_TTCTCTCCCTCACACTTCTATACCCAGGGAAGCTTGCTTGGGTGTAGAAGTGTGAGGGGGAGAATCATTTTTT;
42_reverse_GATCAAAAAATGATTCTCCCCCTCACACTTCTACACCCAAGCAAGCTTCCCTGGGTATAGAAGTGTGAGGGAGAGAACG;

1501_forward_GGCGAGTTCTGGATACTGGAGTGGGAGGGAAGCTTGCTTCT
CATTCTAGTATCCAGAACTCGCCCAATTTTTT;
1501_reverse_GATCAAAAAATTGGGCGAGTTCTGGATACTAGAATGAGAAGC
AAGCTTCCCTCCCACTCCAGTATCCAGAACTCGCCCG;
2526_forward_CCTGGATGTAGACACCATATACTGACCAGAAGCTTGTGGTTA
GTATGTGGTGTCTGCATCCGGGCTGTTTTTT;
2526_reverse_GATCAAAAAACAGCCCGGATGCAGACACCACATACTAACCAC
AAGCTTCTGGTCAGTATATGGTGTCTACATCCAGGCG;
3344_forward_AATCTGCCCGTGCATAAGAACGATCATCGAAGCTTGGGTGGT
CGTTCTTGTGCACGGGCAGGTTCCCTTTTTT;
3344_reverse_GATCAAAAAAGGCAACCTGCCCGTGCACAAGAACGACCACC
CAAGCTTCGATGATCGTTCTTATGCACGGGCAGATTCG;
The name of each construct contains the number, which correspomds to the 5' binding position within the MRP5 mRNA sequence.

Further subcloning (*EcoR* V/*Not* I) into the pCMV/Zeo (Invitrogen) expression vector was performed. Obtained constructs were used to generate stable transfectants as before.

RT-PCR analysis of HMGR

Total RNA was extracted (RNeasy, Qiagen) from THP-1 cells infected with *E. coli* (MOI=1) for the time points indicated in Figure 29B (Part 2) and from A375 wt cells and A375 cells transfected with MRP5 shRNA interference constructs and control Ff1 construct. Two μg of RNA were used for cDNA synthesis (Superscript III, Invitrogen) and PCR was then performed on series of 1:3 cDNA dilutions using Taq Polymerase (NEB). The following primers were used:

HMGR: 5'for_TGGCTGAAACAGATACCCCAAAC;
3'rev_CACCTCCACCAAGACCTATTGC;

β-actin: 5'for_CACAGAGCCTCGCCTTTG;
3'rev_TGGATAGCAACGTACATG;

PCR conditions were adjusted according to the sequence of primers and the length of generated products. Bands were scanned, HMGR and MRP5 products were compared with β-actin level for all time points and dilutions.

Real-time quantitative PCR of MRP4 and MRP5

MRP4 and MRP5 mRNA expression levels were checked by real-time quantitative PCR as described (Pfrunder et al., 2003). Briefly, total RNA was isolated from different cell lines (RNeasy, Qiagen) and 1 μg of RNA was used for cDNA synthesis (Superscript III, Invitrogen) using random hexamers as primers. A total of 25 ng cDNA was used as a template for real-time quantitative PCR. To standardize the amount of sample cDNA added to the reaction the calculated amount of the gene of interest was compared with the amount of constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

The following primers and probes were used:

for MRP4:

Forward_ACCAGGAGGTGAAGCCCAAC;
Probe_CGCTGCAGGACGCGAACATCTG;
Reverse_AGGGATTGAGCCACCAGAAGA;

for MRP5:

Forward_CTGCAGTACAGCTTGTTGTTAGTGC;
Probe_CTGACGGAAATCGTGCGGTCTTGG;
Reverse_TCGGTAATTCAATGCCCAAGTC;

GAPDH:

Forward_GGTGAAGGTCGGAGTCAACG;
Probe_CGCCTGGTCACCAGGGCTGC;
Reverse_ACCATGTAGTTGAGGTCAATGAAGG;

Immunoprecipitation of HMGR

Daudi or THP-1 cells (5-2 x 10^7) either untreated or treated with: farnesol (50 μM) or 7-DHC (100 μg/ml) for 12 h or infected with bacteria (MOI=1) for different time points. Cells from each group were washed three times with PBS, counted and lysed on ice in lysis buffer containing 50 mM KH_2PO_4 (MERK), 150 mM NaCl, 5 mM EDTA (Sigma-Aldrich), 1% Triton X-100 (Fluka), 5 mM PMSF (Axon Lab AG), 10 μg/ml Leupeptin (Sigma-Aldrich), 50 μl Inhibitor mix (Boehringer Mannheim) at pH 7.4. Lysates were centrifuged at 15000 rpm for 15 min at 4℃ to remove insoluble material. Supernatan ts were transferred to new eppendorf tubes on ice.

In order to perform an immunoprecipitation lysates were firstly pre-cleared with 60 μl of protein G-sepharose (50% in lysis buffer, GE-Healthcare Biosciences AG) for 1 h on a turning wheel (Bender Hobein Scientific Instruments, Inc.) at 4℃. After the pre-clearing, lysates from each samp le were incubated with anti-HMG-CoA reductase rabbit polyclonal antiserum (R. Simoni, Stanford University, Palo Alto, CA and J. Roitelman, Sheba Medical Center, Tel Hashomer, Israel) for 12 h at 4℃ under rotation on a turning wheel. Then 60 μl of protein G-sepharose were added and incubated for 3 h on the turning wheel, to precipitate the antibody. After this step protein G-sepharose was centrifuged as above and

subsequently washed 2 times with 1 ml of lysis buffer and 2 times with 1 ml of PBS.

Electrophoresis, transfer and western blotting

Precipitated antigen was released from the antibody and protein G-sepharose pellet, by addition of 40 µl of 2x concentrated reducing sample buffer containing 60 mM Tris HCl pH 6.8 (Fluka), 30% glycerol (Fluka), 10% sodium dodecyl sulfatide (SDS, Sigma-Aldrich), 4 mM Na_2EDTA (Fluka), 4% 2-mercaptoethanol (Sigma-Aldrich) and 0.005% bromophenol blue (Fluka). Samples were incubated at 95°C for 5 min. Then the sepharose-residues were removed by centrifugation at 14000 rpm for 10 min at room temperature and supernatant applied on an electrophoresis gel.

20 µl from each sample were applied with Hamilton syringes (Hamilton Bonaduz) in the slots of a stacking polyacrylamide gel (5% polyacrylamide content, 0.75 mm thickness), overlaid above a separating acrylamide gel (8.5% polyacrylamide content, 0.75 mm thickness) in an electrophoresis aperture (Mini Protean II™). After application of the samples and a molecular weight marker (Rainbow, Biorad), the gel electrophoresis aparatus was connected with a power supply (Model 1000/500, Biorad) and voltage was applied at 20 Volt during run in the stacking gel and 40 Volt during the run in the separating gel.

Composition of running buffer for electrophoresis (pH 8.8): 3 g of Tris, 14.4 g of glycin (Fluka), 1 g of SDS per 1 l; 5% stacking gel: 2.3 ml of Tris (0.5 M in water, pH 6.8), 100 µl of SDS (10% in water), 1.5 ml acrylamide (30%, Biorad), 100 µl APS (10% in water, Fluka), 10 µl of TEMED (Fluka) and water up to 9 ml; 8.5%

separating gel: 2.5 ml of Tris (1.5 M in water, pH 8.8), 100 µl of SDS (10% in water), 2.9 ml acrylamide (30%), 50 µl APS (10% in water), 5 µl of TEMED and water up to 10 ml. After separation proteins were transferred to a nitrocellulose membrane. The separating gel was removed from the apparatus and preequilibrated in transfer buffer for 15 – 30 min. Then the gel was attached to a nitrocellulose membrane (0.2 µM Hybond-C extra, Amersham) of identical size and inserted in a transblot apparatus (Mini protean II, Biorad) supported by filter papers (GB 002 Gel-Blotting-Paper, Schleicher & Schuell) and sponges. The transfer apparatus was filled with transfer buffer, connected to a power supply (Model 1000/500, Biorad) and transferred under constant current conditions (200 mM) for 4 h at 4°C. Buffer was cooled by ice and stirred with a magnetic stirrer during transfer. When transfer was finished, nitrocellulose membrane was removed, washed in PBS and immersed in a solution of 5% gelatin (Top Block, Juro) dissolved in PBST (PBS, 0.05% Tween 20) at 4°C overnight for blocking. Composition of transfer buffer: 3 g of Tris, 14.4 g of glycin, 20 ml of CH_3OH (Fluka) per 1l.

After blocking, the membrane was washed three times for 5 min with an excess of PBST, then incubated with the mAb against HMGR (A9-biotinylated, ATCC) dissolved in PBST, 2.5% gelatin at a concentration of 5 µg/ml for 3 h under shaking at room temperature. Then the membrane was again washed three times in PBST and incubated with a Streptavidin-HRP (SBA), diluted 1:2500 in PBST + 2.5% gelatin for 1 hr. The membrane was washed three times with PBST, 2 times with PBS, finally the enhanced chemoluminescence reaction

(ECL-reaction) was performed. The ECL-reaction was done with a supersignal kit (Pierce). After 5 min incubation the excess of liquid was removed, membrane was wrapped and exposed to X-ray film (Biomax MR, Kodak) for various times. The film was developed using X-ray film developer (Curix 60, AGFA).

HMGR phosphorylation studies

A-375 HMGR-transfected cells (10^7/group) were lysed as before with addition of phosphatase inhibitor cocktail 1 (Sigma-Aldrich). Immunoprecipitation was performed as described above using the anti-BirA tag mAb Bir1.4 (Nowbakht et al., 2005). Blots were incubated with anti-phosphoserine mAb (7F12, Alexis), HRP-conjugated goat anti-mouse IgG1 (SBA) and chemiluminescence substrate (Supersignal,Pierce). As a control, membranes were stripped using the Restore^{TM} Western Blot Stripping Buffer (Pierce) and total HMGR Western blot was performed as described previously. In order to calculate % of HMGR phosphorylation, bands were scanned and the total HMGR protein levels were compared with corresponding levels of phosphorylated HMGR (time 0 was considered as 100%).

PP2A activity assay

A-375 HMGR-transfected cells were lysed (25 mM Tris HCl, pH 7.5, 2 mM EDTA, 10 mM 2-mercaptoethanol and protease inhibitors) and PP2A activity was determined using the Serine/Threonine Phosphatase Assay System (Promega) according to the manufacturer's protocol.

Calcium flux measurement

Daudi cells were labeled with CFSE (0.5 μM, Molecular Probes) 2 days before the assay. In the control samples a low dose of ZOL (5 μg/ml) was added to Daudi cells 1 day before the assay to induce accumulation of TCR γδ cells ligands. Cells were washed and incubated with phosphatase inhibitor: okadaic acid (OA, 100 nM, Alexis) or calyculin A (CA, 10 nM, Alexis) for 4 h. In control samples ZOL (50 μg/ml) was added together with inhibitors. Cells were washed and resuspended in 5% FCS RPMI 1640 without phenol red (fluxing medium) at 6 x 10^6/ml. TCR γδ cells were labeled with Indo-1 (Sigma-Aldrich) dye according to standard protocol and resuspended in fluxing medium at 6 x 10^6/ml. APC and T cells were mixed (1:1 ratio) and immediately analyzed with FACS Vantage™ (Becton Dickinson), to detect the base line. After 30 seconds cells were removed and centrifuged for 10 seconds (600 g) in order to facilitate formation of cell conjugates and reapplied to the cytometer. Acquisition was carried for 5 min. The ratio of fluorescence emission of Indo-1 Ca^{2+}-bound (405 nm) to unbound (485 nm) was plotted against time. Samples were analyzed using FlowJO software (Tree Star, Inc.) after gating on doublets containing Daudi cells ($CFSE^+$) and T cells (Indo-1^+).

HMGR activity assay

The assay is a slightly modified derivation of the method previously described (Park et al., 2001; Gerber et al., 2004). Cells (10^7/group) were lysed by seven freeze and thaw cycles using liquid nitrogen and a 37℃ water bath.

Homogenate cell pellets were resuspended in 500 µl of activation buffer containing 50 mM K_2HPO_4, 1 mM EDTA, 5 mM DTT (Fluka) at pH 7.5, and left for 20 min at 37°C to activate HMGR. To perform HMGR enzymatic assay, 250 µl of reaction buffer (125 mM K_2HPO_4, 12.5 mM DTT, pH 7.5) were added to 500 µl of homogenate. The reaction mix containing 15 mM NADPH (Sigma-Aldrich) and 0.4 mM HMG-CoA (Sigma-Aldrich) or water with 0.4 mM HMG-CoA were added to reach a final volume of 917 µl. The assay was performed at 37℃ and stopped after 60 min by addition of 83 µl of 37% fuming HCl (Fluka). Homogenates were incubated 15 h at room temperature to promote conversion of mevalonate into mevalonolactone (MVL) and to precipitate proteins. A calibration curve was performed on the homogenates treated with water. All samples were filtered through a Sep-Pak® Classic C18 Cartridges (Waters).

LC-MS analysis of HMGR products

A triple quadrupole TSQ Advantage Max (Finnigan) equipped with an electrospray source was coupled to a Rheos 2000 HPLC system (Flux). The HPLC separation was achieved on a reverse-phase Nucleosyl C18 column (Macherey-Nagel,125 x 2 mm i.d.). Ten µl were injected onto the column and eluted with 10% acetonitrile (Rathburn) and 0.1% formic acid (AppliChem) in water, at a flow rate of 0.2 ml/min during 10 min. The nitrogen nebulizing pressure was 45 psi with a heated capillary temperature at 300℃. The electrospray needle was set at 4.0 kV. Single ion monitoring (SIM) mode was selected (m/z 131 ± 10) for MVL in the third quadrupole. MVL detected in each

sample was quantified by creating a calibration curve by adding increasing amounts of MVL (0.1-5 μM) to samples containing cell homogenates with water. The concentration of MVL was determined from the SIR (m/z 131 ± 0.2) peak area at 2.7 min.

Mevalonate kinase, phosphomevalonate kinase and diphosphomevalonate decarboxylase activity assays

The assays are a slightly modified derivation of the method previously described (Biardi et al., 1994; Krisans et al., 1994). Cells were lysed by seven freeze and thaw cycles using liquid nitrogen and a 37℃ water bath. Homogenate cell pellets were resuspended in 100 μl of water and 300 μg of total protein from cell lysates were used in the enzymatic reactions. Radioactive precursors (50 mM, 1.22 x 10^5 dpm/nmol (RS) [2-^{14}C] mevalonate for MVK activity assay or 50 mM, 2.87 x 10^5 dpm/nmol (RS) [2-^{14}C] mevalonate-5-P for PMVK and MVD activity assay, GE Healthcare) were added to the reaction mix containing: 15 mM CHAPS (Sigma-Aldrich), 0.1 M KPO_4 (Fluka), 15 mM ATP (Sigma-Aldrich), 4 mM $MgCl_2$ (Fluka), 1 mM DTT, 1 mM EDTA at pH 7.4. Control reactions were performed in the absence of ATP.

Enzymatic reaction was carried out at 37℃ and stop ped after 60 min by boiling. Samples were separated on a 2 ml AG 1-X8 200-400 mesh formate resin column (Biorad) and products were eluted using: 2 N formic acid (for mevalonate elution), 4 N formic acid (for phosphomevalonate elution), 0.4 M ammonium formate in 4 N formic acid (for diphosphomevalonate elution) or 0.8 M

ammonium formate in 4 N formic acid (for IPP elution). Three ml fractions were collected for each elution step and each fraction was analyzed on β-counter.

Induction of mevalonate pathway products in cell lysates

Daudi cells (5 x 10^8) were lysed on ice in 4 ml detergent free, hypotonic lysis buffer containing 50 mM HEPES (BioWhittaker), 5 mM $MgCl_2$, 2 mM $MnCl_2$ (Merk) at pH7.5. The lysate was centrifuged at 10^4 g for 1 h, at 4°C on an ultracentrifuge (XL-70 K, Beckman-Coulter) using the rotor SW55TI (Beckman-Coulter), to remove organelles, membranes and insoluble material. Then the cell lysate was concentrated 4 times with a 10 kDa cutoff centrifugal filter (ultrafree centrifugal filter device, Millipore) on a centrifuge at 6000 rpm, until a volume of 1 ml was reached. 25 µl of the concentrated lysate was mixed with 75 µl of reaction buffer containing mevalonic acid (Anawa), (10 mM KH_2PO_4, 11 mM KF, 3 mM DTT, 7.5 mM $MgCl_2$, 7.5 mM ATP, 0.1 mM MVA (R, S-), 0.4 µCi MVA (R, S) [5-^{3}H], pH 7.5) and incubated for 60 min at 37°C. Reaction was stopped by addition of 100 µl methanol (Fluka) and putting on ice for several minutes. The sample was clarified from the precipitated proteins by centrifugation at 9000 rpm and stored at -20°C.

Twenty µl of the metabolite mixture were lyophilized in an eppendorf tube, resuspended in 45 µl water and treated with 6 units of alkaline phosphatase (Boehringer Mannheim) which cleaves organic pyrophosphate-esters. The reaction was performed for 3 h at 37°C. To test the biological activity of this supernatant, 25 µl of phosphatase-treated solution were added to 96 well plates,

lyophilized and resuspended in culture medium containing 10% FCS. THP-1 cells were used as APCs (5 x 10^4 cells/well) and the TCR γδ clone G2B9 (5 x 10^4 cells/well) as responder cells, in a final volume of 200 µl medium per well. After 12 h, activation was measured by the released of TNFα. Phosphatase-treated samples were compared with an equal amount of non-phosphatase treated samples. As control for toxicity, the glycolipid sulfatide was treated using the same conditions as above and used to activate a sulfatide specific T cell clone.

Separation of the mevalonate metabolites by HPLC

An aliquot of the filtered metabolite mixture was injected in a HPLC system (PU-980 HPLC pump, Jasco) and the organic components were separated on a Spherisorb SAX 5µ HPLC column (4.6 mm X 25 cm; VDS Optilab) using a gradient of two buffers A and B, composed of 78% 10 mM KH_2PO_4, 20% acetonitrile, 2% tetrahydrofuran (THF, Merk) at pH 4.5 and of 70% 10 mM KH_2PO_4, 25% acetonitrile, 5% THF at pH 4.5, respectively. This type of separation allowed separation of prenylated phosphorylated metabolites from other organic components in the mixture. The gradient formed by these two buffers was: 0-2 min 0% B, 2-13.5 min 57% B, 13- 14.5 min 99% B, 14.5-35 min 99% B. The metabolic products were detected by their radioactivity using a β-scintillation detector (Radiomatic Flow Scintillation Analyzer, Packard) connected with the HPLC system. As scintillation liquid an Ultima Flo M (Packard Instrument Co.) was used. In separate HPLC-runs, ^{3}H-IPP (ARC) and ^{3}H-MVA (Anawa Radiochemicals) were injected and used as reference to correlate the retention

times of radioactive peaks in the metabolite mixture, with the retention time of these standard substances. In other separate HPLC-runs, the β-scintillation detector was disconnected and instead the separated components were collected in sample tubes (Sarstedt) in intervals of 15 seconds, using a fraction collector (2211 Superrac, LKB Brommer). The acidic pH of the mobile phase was immediately neutralized by adding 20 µl of 2 N KOH (Fluka) in each fraction. From each fraction aliquots were transferred in a 96 well plate (5 µl/well), lyophilized, resuspended in cell culture medium and used to stimulate the TCR γδ clone G2B9 (5 x 10^4 cells/well) in the presence of THP1 cells as APC (1.5 x 10^4/well). After 12 h, activation was determined by measuring released TNFα by ELISA assay.

Structural identification of the antigenic fraction by mass-spectroscopy

The active fraction, identified by activation of TCR γδ cells was desalted before mass-spectroscopy. Desalting was done with an anion-exchange DEAE-sepharose filled in a cartridge (DEAE-sephacel, Pharmacia). The DEAE-sepharose (250 µl) was fixed with two filters in a cartridge, washed one time with 10 ml water, subsequently with 10 ml of a TEA-acetate solution (800 mM in water) and again with 10 ml of water. The active fraction was centrifuged under vacuum in a speedvac centrifuge (DNA 110, Savant) for 2 h, to remove the organic solvents THF and acetonitrile. The water/TEA-acetate pretreated cartridge was slowly loaded with the active fraction. Subsequently the cartridge was flushed with 20 ml of a 50 mM TEA-acetate solution to remove the inorganic

phosphate. The stronger bound organic pyrophosphorylated compounds were eluted with 800 µl of a 200 mM TEA-acetate solution and separated in this way from inorganic phosphate anions originating from the HPLC-buffer. The TEA-acetate was lyophilized and the solid substance solubilyzed in 10 µl of a 50% methanol solution. A few microliters were loaded into a nanospray tip (Protana Engineering A/S). The spray was initiated at a voltage of 1100 V. Spectra were recorded on a Finnigan TSQ7000 instrument (Finnigan) in negative ion mode set to 1 D resolution.

^{14}C-ZOL uptake

Daudi cells were pulsed with ^{14}C-ZOL (25 µg/ml, corresponding to 700 nCi/ml, Novartis) for 3 h at 37°C or at 4°C, and wa shed three times with PBS. Some experiments included monensin (20 µM, Sigma) and NaN_3 (0.05%) during the ZOL pulse. After the last wash, 10^6 Daudi cells from each group were resuspended in 1 ml PBS, transferred to scintillation tubes (Pico Pro Vial, Packard) and lysed by adding 2 ml of scintillation liquid (IRGA-SAFE PLUS, Packard Bioscience BV). Radioactivity was counted with a β-counter (TR 1900, Canberra Packard). Triplicate samples were evaluated.

Mice

Generation of human TCR γδ tg mice. Full length human cDNA containing TCR Vγ9-JP-Cγ1 (TCR Vγ9) or TCR Vδ2-Dδ3-Jδ1-Cδ (TCR Vδ2) regions, obtained by RT-PCR from the previously established TCR γδ clone D1C55, were cloned into VA expression vector under control of the human CD2 promoter (Zhumabekov et al., 1995).

Primers used for cloning of human TCRγ and TCRδ genes:

TCR Vγ9 chain: 5'gammaS2_GGGTCGACTGCGGTGATTTCA;

3'gammaN_GCATGCGGCCGCCACCGTC;

TCR Vδ2 chain: 5'deltaS_GGGTCGACGTGGTTGAGAGGC;

3'deltaN_GCATGCGGCCGCTTCCTCATG;

Each construct, devoid of vector sequences, was used for microinjection into (C57BL/10 x CBA) F_2 fertilized oocytes. Mice transgenic for human TCR Vγ9 (B6;CBA-Tg(CD2-TRGV9/JP/C1)7Dfim) or Vδ2 (B6;CBA-Tg(CD2-TRDV2/D3/J1/C)15Dfim) chains were obtained and inter-bred in order to generate TCR Vγ9-Vδ2 double transgenic animals. TCR γδ transgenic mice having RAG-2 deficient background were also obtained by breeding with B6.129S6-Rag2tm1FwaN12 (Shinkai et al., 1992).

MyD88$^{-/-}$ (Adachi et al., 1998) and C57BL/6 mice were maintained and bred in the animal facility at our Institute. All animals used for experiments were sacrificed at 4 -10 weeks of age. This study was approved by the "Kantonales Veterinaeramt Basel-Stadt".

Screening of transgenic mice

Mice were marked for long time identification and tail biopsy was performed according to the rules of “Kantonales Veterinaeramt Basel-Stadt “. Tail samples were placed in Eppendorf tube (1.5 ml) and incubated over night at 56℃ in 500 μl of lysis buffer containing 100 mM Tris HCl, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 0.4 mg/ml Proteinase K (Axon Lab). Lysates were centrifuged for 10 min at maximal speed and transferred to fresh tubes. DNA was precipitated with one volume of isopropanol (Fluka) and recovered using glass capillaries. After placing the capillaries for 5 min in 70% ethanol, DNA was dried and resuspened in 400 μl of TE (10 mM Tris HCl, 1 mM EDTA, pH 7.5). Obtained DNA samples were diluted in TE buffer to reach a concentration of 30 μg/ml and 1 μl was used for PCR.

PCR was preformed according to the protocol using Taq Polimerase (NEB).

Primers used for identification of TCR γδ tg mice:

for TCR Vγ9 chain and TCR Vδ2 chain as above;

mRag 2: 5’mRAG2for_TCTAAAGATTCCTGCTACCTCCCAC;
3’mRAG2rev_TGAAAAGACAGCCCATCCTGAAG;

neomycin cassette: 5’NEOfor_TGGGCACAACAGACAATCGG;
3’NEOrev_TGGATACTTTCTCGGCAGGAGC;

PCR conditions were adjusted according to primer sequences and size of the generated products.

Intrathymic injections

Animals were anesthetized by i.p. injection of 30 μg/10 g body weight of ketamine (Ketalar, 50 mg/ml, Parke-Davis): xylazine (Xylasol, 20 mg/ml, Graeub) mixed 2:1 in sterile 0.9% NaCl solution (Fluka). The upper part of the thoracic cavity was opened to expose thymic lobes. Lobes were injected with 40 μg of indicated mAb in sterile PBS solution. Mice were administered s.c. with the analgesic, 1 μg/10 g body weight Temgesic (Reckitt Benckiser Healthcare, UK). Mice were sacrificed between 1 and 3 weeks after injection.

Preparation of mouse lymphoid cells

The organs of interest were isolated from sacrificed mice under sterile conditions and placed in D-MEM (Gibco) medium supplied with 10% FCS, 100 μg/ml Kanamycin and 10 mM HEPES buffer. Cells were isolated by mashing the organs on metal sieves using 2 ml syringe plunges. Isolated cells were transferred to 15 ml conical bottom tubes and incubated for 5 min at room temperature. Clear suspensions were transferred to new tubes avoiding tissue debris. In case of cell preparation from spleen, red blood cells were lysed by adding 5 ml of ice cold Guy's solution to the cell pellet. Cells were incubated on ice for 2-3 min then lysis was stopped by addition of 10 ml medium containing 10% FCS. Additional debris were removed and cells were washed once with fresh medium. Isolated cells were either directly used for staining or were cultivated in base D-MEM medium with 10% FCS.

Preparation of mouse bone marrow derived dendritic cells

Bone marrows were isolated from mouse femurs and tibias. After the removal of muscles both ends of bones were cut and marrows were flushed out with 2 ml of base culture medium using a syringe. Cells were resuspened by pipetting and then washed. In order to generate the DCs were cultivated for 8 days in base cell culture medium supplied with 5% FCS and 1000 U/ml of mGM-SCF.

The DC differentiation was checked by surface staining evaluating expression of CD11c, CD1d and MHC class II.

Activation assays with Tg T cells

Human and mouse APC were incubated with 50 μg/ml of ZOL for 2 h at 37°C then washed twice with PBS. APC (1-1.5 x 10^6/well) were incubated with freshly isolated mouse thymocytes or splenocytes (3 x 10^6/well) for 24 h in 24 well plates and then samples were analyzed by surface staining. In some experiments anti-TCR mAbs were bound to the plastic at 5 μg/ml for 12 h at 4°C then wells were washed and thymocytes or splenocytes were added and incubated as before. As a positive control thymocytes were activated in the presence of 5 μg/ml of Concanavalin A (ConA, Calbiochem).

Cell surface markers staining

Cells were washed with FACS-buffer (0.5% human albumin, 0.02% NaN_3 in PBS) and incubated with the primary mAbs or with the appropriate isotype-matched

irrelevant mAb for background determination. After washing with FACS buffer cells were stained with fluorochrome conjugated secondary reagents. All incubations and washings were done at 4°C in FACS-buffer.

Intracellular staining

Cells were washed three times with PBS, fixed with 2% paraformaldehyde (PFA, Merk) solution in PBS for 15 min at room temperature and washed three times with FACS-buffer. In order to permabilized cell membrane, cells were incubated in 0.1% saponin (Sigma-Aldrich) FACS-buffer for 5 min at room temperature. Cells were stained with primary antibodies or isotype matching control antibodies, then washed three times and incubated with of fluorochrome conjugated secondary reagents. All incubation steps for staining were done at room temperature for 30 min and all the reagents were prepared in FACS-buffer containing 0.1% saponin.

Flow cytometry

The following antibodies specific for human determinants were used: anti-HMGR (A9) from ATCC, anti-hTCR Vγ9 (B3) and anti-hTCR Vγ2 (4G6) antibodies as previously described (Carena et al., 1997).

The following antibodies specific for mouse determinants were used: anti-CD3ε (145-2C11), anti-CD4 (GK1.5), anti-CD8α (53-6.7), anti-CD11c (HL-3) and anti-CD24 (M1/69) from BD Biosciences; anti-CD5 (CG16), anti-CD69 (H1.2F3), anti-CD62L (MEL-14) and anti-CD25 (PC61 5.3) from Caltag; anti-CCR7 (4B12),

(kindly provided by Professor Zwirner, University of Göttingen, Germany), (Ritter et al., 2004). Anti-CD1d (HB322) and anti-MHC class II (m5 114) purified from hybrydoma culture supernatants.

BirA-tagged HMGR was followed with Bir1.4 mAb (Nowbakht et al., 2005).

The following secondary reagents were used: Goat Anti-Mouse IgG Alexa 647 (Molecular Probes), Goat Anti-Mouse IgG FITC (SBA), Goat Anti-Mouse IgG PE (SBA), Streptavidin RPE (SBA), Streptavidin APC and Streptavidin PE-TR (Caltag) were used as revealing secondary reagents when the mAbs were not directly labeled to fluorocromes.

Samples were analyzed using CyAn ADP flow cytometer (DakoCytomation, Baar, Switzerland). Nonviable cells were excluded from the analysis using light scatter and incorporation of propidium iodide (Fluka). Data were analyzed using Summit 4.2 software (DakoCytomation).

Chemotaxis assay

Chemotaxis was assyed in 48-well Boyden microchambers (Neuro Probe, Cobin John, MD) by using polyvinylpryrrolidone-free polycarbonate (Nucleopore) membranes as previously described (Paoletti et al., 2005). In brief, thymocytes after selection were cultured for an 18 h before the chemotaxis assay. 1 x 10^5 cells diluted in chemotaxis buffer (RPMI 1640, 20 mM HEPES, 1% BSA) were added to the upper wells (30 μl/well). The attractive chemokine SLC (R&D) diluted in chemotaxis buffer was placed in the lower well at different concentrations. After 60 min of incubation, the membrane was removed, washed

in the upper side with PBS, fixed (50% methanol) and stained (Diff-Quick, DADE). Migrated cells were counted at 1000-folds magnification in 5 randomly selected fields. All the experiments were performed in triplicate.

Production of monoclonal antibodies from hybrydoma

Hybridoma cells producing monoclonal antibodies (mAb) were cultured and expanded in medium containing 10% FCS and then adapted to 5% FCS medium. For final antibody production cells were cultivated in flasks (175 cm^2, BD Falcon) in medium containing 3% low IgG serum (Gibco). mAb were purified from collected supernatants using 5 ml protein G column (HiTrap ProteinG, Pharmacia). The column was connected with a peristaltic pump (2132 Mikroperpex peristaltic Pump, LKB Bromma) and equilibrated with PBS, before the hybridoma supernatant was loaded at a flow rate of approximately 1 ml/min at 4℃. Each time 500 to 1000 ml of supernatant wer e used for antibody purifcation. After supernatant loading, the column was connected with an UV-spectrophotometer (2238 UVICORD SII, LKB Bromma) set at 280 nm and washed with PBS until all contaminating serum components were removed. Then the antibody was eluted with a solution of 100 mM glycine dissolved in water at pH 3.0. The antibody was collected in 15 ml tubes and immediately neutralized with 500 µl of a Tris solution (1M, pH 7.4). The antibody was filtered, NaN_3 was added at a final concentration of 0.02% and the concentration was determined with an UV-spectrophotometer (V-530, Jasco) at 280 nm wavelength. An OD of 1.3 was estimated to correspond to 1 mg/ml of antibody concentration.

Biotinylation of purified antibodies

The purified antibody solution was dialyzed against borate buffer (pH 8.5) containing 0.2 M H_3BO_3 (Fluka), 0.1 M KCl (Fluka), in order to remove all small organic molecules containing free amino groups. Dialysis was performed in dialysis tubes (3.5 kDa cutoff, Spectra/Por CE Float A Lyzer, Spectrum). Antibody concentration was adjusted to at least 1 mg/ml. Biotinylation was performed in the borate buffer pH 8.5 in small glass vials containing a magnetic stirrer. During stirring at room temperature, biotin-NHS ester (Sigma-Aldrich) was added stepwise (in 4 steps) at 5 min interval between each addition step. The amount of biotin was calculated as 20 times molar excess to antibody molecules, so that ratio between biotin-NHS ester:antibody was 20:1.
Biotin-NHS ester was dissolved in DMSO under nitrogen atmosphere at a concentration of 10 mg/ml. After the last addition of biotin-NHS ester, the solution was further stirred for 30 min then dialyzed against PBS. The concentration of the biotinylated antibody was determined by UV-absorption as described and success of biotinylation was tested by either FACS staining or by ELISA.

Statistical analysis

The Dunnett Multiple Comparison Test was used to compare groups in the semi-quantitative RT-PCR experiments, HMGR phosphorylation studies and HMGR, MVK, PMVK and MVD activity assays. One-tailed P value was calculated for comparison of PP2A activity between experimental groups.

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Appendix

Eur. J. Immunol. 2006. 36: 1083–1092

European Journal of Immunology Antigen processing

Functional CD1a is stabilized by exogenous lipids

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Self-glycosphingolipids bind to surface CD1 molecules and are readily displaced by other CD1 ligands. This capacity to exchange antigens at the cell surface is not common to other antigen-presenting molecules and its physiological importance is unclear. Here we show that a large pool of cell-surface CD1a, but not CD1b molecules, is stabilized by exogenous lipids present in serum. Under serum deprivation CD1a molecules are altered and functionally inactive, as they are unable to present lipid antigens to T cells. Glycosphingolipids and phospholipids bind to, and restore functionality to CD1a without the contribution of newly synthesized and recycling CD1a molecules. The dependence of CD1a stability on exogenous lipids is not related to its intracellular traffic and rather to its antigen-binding pockets. These results indicate a functional dichotomy between CD1a and CD1b molecules and provide new information on how the lipid antigenic repertoire is immunologically sampled.

Received 28/9/05
Revised 25/1/06
Accepted 20/2/06

[DOI 10.1002/eji.200535544]

Key words: Antigen loading · Antigen presentation · CD1 · Lipid recognition

Supporting information for this article is available at http://www.wiley-vch.de/contents/jc_2040/2006/35544_s.pdf

Introduction

The immune system has evolved different pathways to present antigens to T cells, including presentation of peptides by MHC molecules and presentation of lipids by CD1 molecules [1, 2]. The two pathways complement each other and contribute to elimination of infectious agents and immunoregulation.

CD1a, b, c and d molecules present a variety of microbial and self-lipids to T cells [3]. CD1 ligands are made of a polar head group, interacting with the TCR and of a hydrophobic tail, responsible for CD1 binding as documented by the analysis of the crystal structure of CD1a, CD1b and CD1d [4].

Several studies have addressed the function of lipid-reactive cells, the structure of lipid antigens and the intracellular trafficking of CD1 molecules. However, little is known about the maturation of CD1 molecules, their mode of interaction with antigenic lipids, and the mechanisms of antigen loading [3].

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Abbreviations: **β_2m:** β2-microglobulin · **BFA:** brefeldin A · **CHD:** cytochalasin D · **GSL:** glycosphingolipids: **HS:** high serum · **LS:** low serum · **LC:** Langerhans cells · **PA:** phosphatidic acid · **PC:** phosphatidylcholine · **PEA:** phospatidylethanolamine · **PI:** phosphatidylinositol · **PS:** phosphatidylserine · **Sulfatide:** sulfo-galactosylceramide

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In a previous study, we demonstrated that CD1a and CD1b molecules reach the cell surface and are immunologically competent in the absence of *de novo* synthesis of self-glycosphingolipids (GSL) [5]. However, CD1 molecules may associate during their assembly with endogenous lipids different from GSL such as phospholipid phosphatidylinositol [6]. A second possibility is that newly synthesized CD1 molecules leave the ER in association with stabilizing chaperones that guide CD1 traffic to other cellular compartments in which the glycolipid antigens are loaded. One example is the association of CD1d with the invariant chain, which is not absolutely required for refolding and maturation of this CD1 molecule, but nevertheless contributes to trafficking in a late endosomal compartment, likely a main station of CD1d loading [7, 8].

A third possible scenario is that mature CD1 molecules bind to, and are stabilized by, exogenous lipids on the cell membrane. An important distinction from MHC class I molecules is that recombinant soluble CD1a and CD1b molecules successfully refold *in vitro* in the absence of added ligands and remain available for ligand binding [9, 10], suggesting that exogenous ligands might bind to CD1 molecules on the cell surface. In this case, serum lipids capable of binding to CD1 molecules would be the most abundant source of CD1 ligands.

Here, we show that CD1a, but not CD1b, requires binding to exogenous lipids to remain properly folded and immunologically functional. Thus, the repertoire of CD1a-bound ligands is continuously shaped by the repertoire of ligands present in the extracellular environment.

Results

CD1a expression on the cell surface is affected by serum deprivation

To investigate whether exogenous ligands present in the serum participate in CD1 maturation and stabilization on the cell surface we cultivated MOLT-4 cells, which express both CD1a and CD1b molecules, in medium containing very low amounts (0.2%) of FCS (LS) for 24 h and then performed immunofluorescence analysis. Under these LS conditions, CD1a expression was detectable at about 50% of that found under control (10% serum, HS) culture conditions. CD1b, MHC class I molecules (Fig. 1A), CD71 (transferrin receptor) and MHC class II molecules (data not shown) remained unchanged, thus ruling out the possibility that culture in LS induces a general reduction of surface protein levels. Comparable results were obtained with human DC and B cells transfected with CD1A or CD1B genes (data not shown), thus also excluding an effect specific to the MOLT-4 cells. CD1a surface expression in LS was analyzed with nine CD1a-specific mAb (Fig. 1C). Reduction of expression levels ranged from 3 to 56%. The differences observed with various CD1a-specific mAb infers that CD1a loss in LS is not due to its internalization and suggest that different CD1a epitopes are no longer available for mAb recognition, likely due to conformational changes. These findings show that CD1a, but not CD1b, requires serum components to maintain an unaltered expression on the cell surface.

To asses whether the serum dependence of CD1a high expression is also observed *ex vivo*, freshly isolated human $CD4^+CD8^+$ thymocytes were analyzed. CD1a and CD1b expression on fresh thymocytes was investigated either immediately or after 24 h of culture in HS or LS. Incubation in LS and not in HS induced a 50% reduction of surface CD1a, without decreasing expression levels of CD1b or MHC class I (Fig. 1B). Thus, CD1a molecules complexed with ligands captured *in vivo* in the thymus also undergo alterations in LS.

Surface CD1a molecules are the ones affected by serum deprivation

To investigate whether surface, recycling, or *de novo* synthesized CD1a molecules are altered in LS conditions, MOLT-4 cells were cultivated in LS in the presence of brefeldin A (BFA) or cytochalasin D (CHD). Amongst its effects, BFA blocks protein egression from the trans-Golgi network, thus affecting surface expression of *de novo* synthesized proteins. CHD inhibits fluid-phase, receptor- or caveolae-mediated uptake of extracellular and transmembrane molecules [11–13]. In the presence of BFA or CHD, CD1a continued to be altered after 24 h of culture in LS (Table 1). These results show that CD1a alteration occurs in the absence of *de novo* synthesized proteins and protein internalization, and suggest that CD1a molecules already present on the cell surface are affected.

Surface CD1a molecules unfold during serum deprivation and become immunologically inactive

To examine whether unfolded CD1a molecules remain on the cell surface in LS, we generated transfectants expressing CD1a fused with GFP at the cytoplasmic tail. The addition of GFP tail did not modify CD1a intracellular trafficking as detected by confocal analysis (supplementary online information). These hybrid molecules were studied with specific mAb to follow the extracellular CD1a domains and with GFP to localize the intracellular tail. A disappearance of surface staining with anti-CD1a mAb and concomitant detection of

membrane-associated GFP would provide evidence for presence of altered CD1a on the cell surface. CD1a-transfected cells were incubated 24 h under HS or LS conditions, and then capping of surface CD1a was induced using anti-CD1a mAb. Confocal microscopy of cells cultivated in HS showed capping of all surface CD1a and colocalization of the anti-CD1a mAb and GFP signals (Fig. 2). In contrast, cells cultured in LS showed diffused distribution of membrane-associated GFP staining, capping of the CD1a molecules still detectable with mAb, and only partial colocalization of the mAb and GFP signals (Fig. 2).

These findings confirm that part of the CD1a molecules unfolds in the absence of serum and remains anchored to the cell surface. The fact that specific mAb do not detect the altered molecules, raises the question whether CD1a also becomes functionally inactive. Therefore, we studied whether antigen presentation to CD1a-restricted T cells is also affected. MOLT-4 cells expressing 50% of CD1a after 24 h cultivation in LS were incubated with sulfatide (sulfo-galactosylceramide) or GM1, and used to stimulate CD1a- or CD1b-restricted T cell clones, respectively. MOLT-4 cells cultivated in HS were used as control APC. The response of a CD1a-restricted T cell clone was significantly reduced after antigen presentation with MOLT-4 kept in LS (Fig. 3A), whereas antigen presentation to a CD1b-restricted T cell clone was not affected, thus excluding a

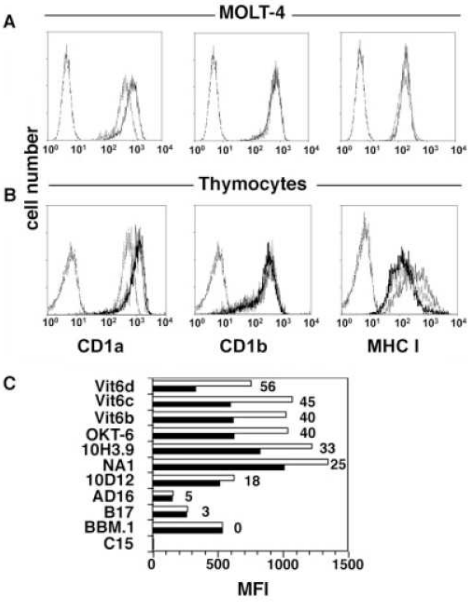

Figure 1. CD1a surface expression is altered in LS. (A) MOLT-4 or (B) freshly isolated human $CD4^+CD8^+$ thymocytes were cultured in LS (A and B, dotted lines), in HS (A and B, thin lines) for 24 h or analyzed immediately after isolation (B, bold lines) for surface expression of CD1a, CD1b, MHC class I (OKT-6, WM-25, W6/32 mAb, respectively). Background staining is indicated by broken lines. (C) MOLT-4 cultured for 24 h in HS (white bars) or LS (black bars) were stained with various anti-CD1a mAb. Numbers indicate percentage of CD1a modulation as compared to HS conditions. BBM.1 is specific for human β_2m, C15 is a negative control mAb.

Table 1. Effect of BFA and CHD on the expression of CD1a, CD1b and MHC class I[a)]

Surface molecule (mAb)	No drug		BFA		CHD	
	HS	LS	HS	LS	HS	LS
CD1a (OKT-6)	100	48	58	29	109	50
CD1b (WM-25)	100	98	67	71	97	93
MHC class I (W6/32)	100	103	73	67	162	119

[a)] MOLT-4 were cultured in HS or LS, without or with inclusion of BFA (1 μg/mL) or CHD (10 μg/mL). After 24 h cells were labeled with the indicated mAb and analyzed by FCM. Results are expressed as percentage of MFI of the positive control (cells cultivated in HS without drugs).

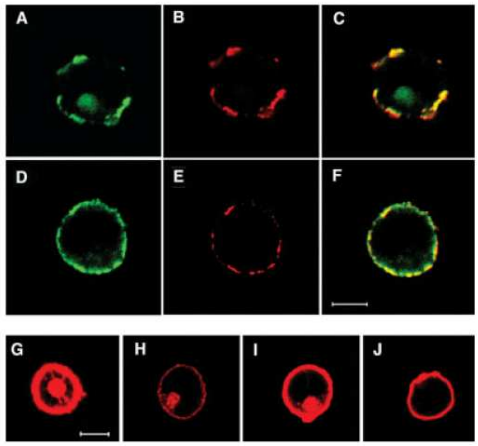

Figure 2. Cell surface CD1a molecules are unfolded in LS (A–F). J558 transfected with CD1a-GFP were cultured for 24 h with HS (A–C) or LS (D–F), and then capping of surface CD1a molecules was induced by OKT-6. Confocal microscopy detected GFP as a green signal and CD1a in red. Scale bar: 5 μm. Restoration of CD1a expression by sulfatide (G–J). MOLT-4 were cultured in HS (G) or LS (H, I, and J) for 24 h. Then cells were further cultured in LS alone (H), or with inclusion of sulfatide (I), or sulfatide and BFA+CHD (J). CD1a was detected with OKT-6 and Cy3-conjugated anti-mouse IgG and analyzed by confocal microscopy. Scale bar: 5 μm.

generic reduced capacity of antigen presentation after LS culture (Fig. 3B). These findings show that the altered CD1a molecules that remain on the cell surface are functionally inactive, and that serum components are required to maintain their antigen-presenting function.

The extracellular CD1a domain and not CD1a recycling pathway confers susceptibility to serum deprivation

CD1a is internalized in early endosomes, whereas the other CD1 molecules reach mature late endosomes [14–17]. This difference could expose CD1a to a set of ligands distinct from those for the other CD1 molecules, thus providing an explanation for the dependence of CD1a on exogenous ligands.

We studied transfectants expressing either hybrid CD1a molecules fused with the cytoplasmic tail of CD1b (CD1ab) that recycle in late endosomes, or hybrid CD1b molecules expressing the cytoplasmic tail of CD1a (CD1ba) and recycling to early endosomes (manuscript in preparation). When transfectants were cultured in LS, hybrid molecules with extracellular CD1a domain continued to be altered, similarly to control transfectants expressing wild type CD1a (Table 2). In contrast, hybrid molecules with extracellular CD1b domain were not altered.

These findings show that the extracellular domain of CD1a and not recycling to early endosomes confers susceptibility to deprivation of exogenous ligands.

Sulfatide stabilizes CD1a on the cell surface

With respect to the nature of active serum components, we speculated that ligands binding to CD1a could be important for stabilization. Initially we studied sulfatide, which is very abundant in serum. MOLT-4 cells were

Table 2. The extracellular CD1a domain is responsible for dependence on exogenous lipids[a)]

	mAb anti-					
	CD1a		CD1b		MHC class I	
CD1	HS	LS	HS	LS	HS	LS
CD1a	729	272 (-63)	9	11	291	266
CD1ab	232	119 (-49)	11	15	307	296
CD1b	9	10	368	341	55	55
CD1ba	17	16	126	135	326	310

a) Numbers show MFI of staining of T2 cells expressing CD1a or CD1b or CD1 hybrid molecules. The percentage of CD1a alteration detected by staining with OKT-6 is indicated in parentheses as negative value.

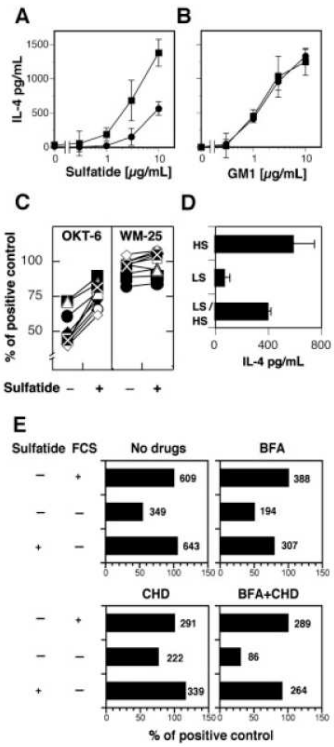

Figure 3. Altered CD1a molecules are functionally inactive and HS or sulfatide restore their functional expression. (A, B) MOLT-4 were cultured for 24 h in LS (circles), or in HS (squares) and then used as APC to stimulate CD1a-restricted and sulfatide-specific (A) or CD1b-restricted and GM1-specific (B) T cell clones. IL-4 released in the supernatants was measured by ELISA and expressed in pg/mL ± SD. (C) MOLT-4 cells were cultured in LS for 24 h before inclusion of sulfatide and further cultured for 24 h. Surface expression of CD1a (OKT-6) and CD1b (WM-25) was measured by FCM. MFI of cells cultured throughout in HS (positive controls) was considered as 100%. Each line represents a separate experiment. (D) MOLT-4 cultured for 24 h in LS and then further 24 h in HS (LS/HS) were used to present sulfatide (10 μg/mL) to the CD1a-restricted sulfatide-specific T cell clone. MOLT-4 always kept in HS or in LS served as controls. (E) Sulfatide efficiently stabilizes CD1a molecules present on the cell surface. MOLT-4 were kept in LS for 24 h, and then additional 24 h in the presence of BFA or CHD alone, or both together and without or with inclusion of sulfatide as indicated. Cells were stained with OKT-6 and analyzed by FCM. Results are expressed as percentage of MFI of the positive control (untreated cells in HS). MFI values are indicated next to the respective bar. The results represent one of three independent experiments.

cultivated first for 24 h in LS and then for a further 24 h in LS supplemented with sulfatide. Sulfatide as well as HS restored up to 90% of CD1a, whereas CD1b (Fig. 3C) and MHC class I (data not shown) molecules were not affected. Moreover, restored CD1a molecules also re-acquired their immunological activity. MOLT-4 cells incubated in LS and then in HS for additional 24 h presented sulfatide to CD1a-restricted T cells very efficiently (Fig. 3D), showing the functional flexibility of surface CD1a molecules.

Further experiments were performed to investigate whether sulfatide stabilizes newly synthesized CD1a molecules when they reach the cell surface and/or restores the conformation detectable with mAb of the molecules already present on the cell surface. MOLT-4 cells maintained for 24 h in LS were cultured for a further 24 h in the presence of BFA, CHD or both together, and with or without inclusion of sulfatide (Fig. 3E). Cells maintained first in LS but then further cultured in HS served as controls. In the presence of BFA, sulfatide restored CD1a to 80% of that on control cells (MFI: 307 *vs.* 388), indicating that cell surface and recycling CD1a molecules, not blocked by BFA [18], are stabilized. When CHD was used, sulfatide restored CD1a expression to level higher than on control cells (MFI: 339 *vs.* 291), indicating that the recycling and the *de novo* expressed CD1a molecules, blocked at the cell surface by CHD, are stabilized very efficiently. In the combined presence of BFA and CHD there was a more profound reduction of CD1a, which, however, was efficiently restored by sulfatide (91%, MFI: 264 *vs.* 289). This latter result suggests that surface CD1a molecules are rescued without a requirement for internalization, even when already altered.

Restoration of CD1a expression was also investigated by confocal microscopy (Fig. 2, G–J). MOLT-4 cells cultured for 24 h in LS conditions exhibited a marked reduction of both surface and intracellular CD1a (Fig. 2G and H). Inclusion of sulfatide during the additional 24 h period of culture in LS resulted in a restoration of CD1a to levels almost comparable to those of cells cultured in HS (Fig. 2G and I). Both surface and intracellular molecules were restored by culture in the presence of sulfatide. Furthermore, when sulfatide was added simultaneously with CHD, a strong CD1a staining was detected on the cell surface but not within intracellular compartments (Fig. 2J). Since CHD has a variety of effects including blocking of internalization of surface proteins, this finding indicates that CD1a internalization and recycling are not required for its stabilization.

Table 3. Restoration capacity of different types of lipids[a)]

Exogenous lipid	CD1a (MFI)	% Restoration
LS	461	0
HS	749	100
Sulfatide	716	89
GM1	572	39
Sphingomyelin	577	40
PS	573	39
PC	562	35
PI	548	30
PA	462	0
PEA	445	-5
Cholesterol	406	-19
Distearin	352	-38

[a)] MOLT-4 were first cultured in LS for 24 h and then for additional 24 h in the presence of 10 μg/mL of indicated lipid. CD1a expression is reported as MFI of OKT-6 staining and % restoration calculated according to the Materials and methods section.

Glycosphingolipids and phospholipids restore surface CD1a

We tested the ability of several lipids to restore CD1a conformation under LS conditions (Table 3). Sulfatide, GM1, sphingomyelin, phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylinositol (PI) restored CD1a expression albeit with different efficiencies. In contrast, cholesterol, distearin, phosphatidylethanolamine (PEA) and phosphatidic acid (PA) were not able to restore surface CD1a expression, thus ruling out that the alterations observed in LS can be rescued by a generalized increase in cellular lipid content. These findings support the hypothesis that different ligands stabilize CD1a.

Stabilization of CD1a on the cell surface by sulfatide and β2-microglobulin

In addition to antigen binding to CD1a, we tested whether exogenous β2-microglobulin (β_2m) stabilizes CD1a in a similar manner. Purified human β_2m at a concentration of 0.2 or 1 μg/mL, equivalent to the concentration range present in HS medium, restored up to 80% of CD1a expression in LS-pretreated MOLT-4 cells, as detected by surface staining with OKT-6 mAb, similar to what was observed with sulfatide (Fig. 4A). The addition of both sulfatide and β_2m restored CD1a expression to an even higher extent (96% of control), suggesting that the two molecules act independently.

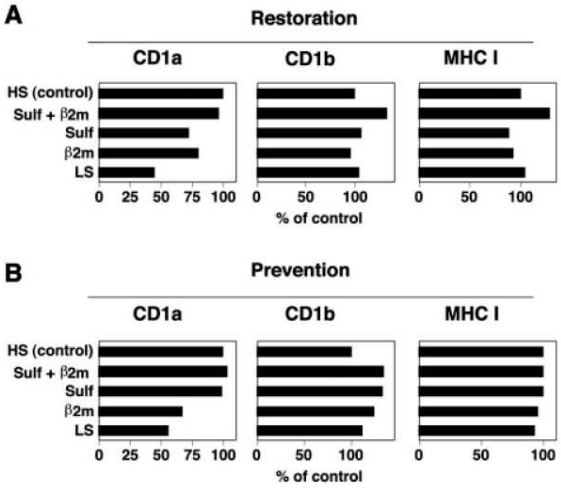

Figure 4. Distinct role of sulfatide and β_2m in CD1a cell surface stabilization. (A) Restoration of CD1a expression after 24 h cultivation of MOLT-4 in LS was tested by adding sulfatide (10 μg/mL) or β_2m (1 μg/mL) or both or HS as control and culturing cells for additional 24 h. (B) Prevention of CD1a alteration in MOLT-4 cultured 24 h in LS supplemented with sulfatide (10 μg/mL) or β_2m (1 μg/mL) or both or HS as control. Results in (A) and (B) are expressed as percentage of control MFI of staining for the indicated molecules.

Stabilization of the surface CD1a in its immunologically active conformation was also observed in experiments aimed at preventing the alteration observed in LS. In fact, by culturing cells with LS in the presence of sulfatide alone or sulfatide and β_2m, CD1a was not altered (Fig. 4B). On the contrary, addition of β_2m alone did not completely prevent alteration. These experiments further confirm that exogenous GSL stabilize CD1a and that they exert this effect with a mechanism different from that of β_2m.

CD1a expression in human epidermal Langerhans cells

We next quantified the levels of CD1a expression on the surface of freshly isolated Langerhans cells (LC) as short as 1 h after incubation with FCS or sulfatide (Table 4). LC incubated in LS supplemented with sulfatide up-regulated CD1a expression (MFI average 37%) similarly to what observed with HS (MFI average 43%). The levels of MHC class I surface expression did not significantly change in any of these conditions. These findings show that also with freshly isolated LC, surface CD1a can be up-regulated in the presence of serum or of an appropriate lipid, thus further supporting the conclusion that CD1a is stabilized by exogenous ligands.

Discussion

In this study we show that plasma membrane CD1a, but not CD1b, is kept in a functional conformation by exogenous ligands. CD1a efficiently presents lipid antigens to T cells only when present in this conformation. Surface levels of CD1b, MHC class I, MHC class II and CD71 are not affected by cell culture in LS, indicating that this phenomenon is specific to CD1a and not associated with a general reduced expression of surface molecules. CD1a functional alteration occurs independently of cellular internalization and mostly affects molecules residing on the cell surface. This is shown by differential staining with different CD1a-

Table 4. Basal levels of surface CD1a in freshly isolated LC are up-regulated by serum and by sulfatide[a)]

	CD1a			MHC class I		
	LS	HS	Sulfatide	LS	HS	Sulfatide
Exp. 1	978	1777 (+82)	1130 (+56)	ND	ND	ND
Exp. 2	954	1275 (+34)	1369 (+44)	1567	1484 (-5)	1152 (-26)
Exp. 3	2218	2515 (+13)	2455 (+11)	1244	876 (-30)	1376 (+11)

[a)] LC were incubated 1 h at 37°C with LS, HS, or LS supplemented with 10 μg/mL of sulfatide. Numbers show MFI. The percentage of modulation with respect to LS is indicated in parentheses as positive or negative values. ND: not done.

specific mAb using cytofluorimetry and confocal evidence of sustained surface GFP-tagged CD1a expression after LS-induced modulation.

CD1a functional alteration also occurs when freshly isolated thymocytes are incubated in LS, also showing that CD1a molecules that are likely to be associated with *in vivo*-bound ligands are stabilized by exogenous lipids. These findings demonstrate that surface CD1a becomes unstable and unfolds in the absence of exogenous ligands.

Previous studies have shown the unique capacity of CD1a to exchange associated β_2m with that present in the serum [19, 20]. Our results confirm that β_2m participates in CD1a stability, however, they also add the novel finding that lipids are necessary for CD1a stabilization. The observation that sulfatide, but not β_2m, efficiently prevents CD1a alteration occurring in LS further outlines the importance of lipids in stabilizing CD1a.

Glycosphingolipids such as sulfatide, ganglioside GM1 and sphingomyelin as well as phospholipids such as PS, PC and PI restore the expression of previously altered CD1a molecules with different efficiencies. The three GSL ligands share a ceramide tail, which anchors them to CD1a. The crystal analysis of the CD1a-sulfatide complex has outlined the importance of sphingosine which is inserted into the A1′ pocket and is likely of major importance for binding and stabilizing the entire complex [21]. In contrast, PA, PEA, distearin and cholesterol do not restore the CD1a conformation. This may reflect a low binding to CD1a, although we cannot exclude other factors such as low availability of free molecules in aqueous media that could also be relevant.

CD1a functional alteration shows important and unique features. Altered CD1a molecules become immunologically incompetent as they lose their antigen presentation capacity. Thus, there is a direct relationship between surface CD1a levels and exogenous ligands availability, a relationship not observed for other antigen-presenting molecules.

Surface CD1a molecules are restored on the cell surface without absolute requirement for internalization, or *de novo* synthesis, as demonstrated by flow cytometry and confocal microscopy in the presence of CHD and BFA.

Stabilization by exogenous ligands is a feature of CD1a and does not apply to CD1b. This difference is not imposed by the intracellular distribution and traffic capacities, which differ between the two molecules, as shown by the behavior of CD1ab and CD1ba hybrid molecules. Therefore, it is likely that the distinct manner of lipid binding accounts for the observed different stability requirements. CD1a is characterized by a F′ pocket, which is relatively large, is exposed to the surface [21] and allows binding of exogenous lipids in the absence of acidification [10]. The structure of this pocket might facilitate ready exchange of bound lipids, and when empty might induce collapse of the molecule. CD1b is instead characterized by a more intricate net of pockets [22], which allow binding of glycolipids with long acyl tails and may be stabilized by a variety of self ligands [10, 23]. CD1a structural characteristics render therefore this molecule unique for its property of becoming more available for antigen-presentation when increased ligand concentration is available.

Surface binding and stabilization by exogenous ligands also differentiates CD1a from MHC molecules. Important features of MHC class I molecules are that they do not readily exchange peptide ligands on the plasma membrane [24], in the absence of peptides they become unstable, dissociate from β_2m [25] and after this denaturation step they lose the capacity to bind exogenous peptides [26]. This is a key mechanism to retain immunological identity and prevent killing of cells inappropriately presenting exogenous peptides. In the case of CD1a, bound ligands may be readily exchanged on the cell surface and perhaps the role of CD1a molecules is not to confer immunological identity in surveillance for virus-infected cells.

An important difference is also evident with respect to MHC class II molecules. Physiological antigen presentation by MHC class II molecules occurs after antigen internalization, processing and loading in specialized endosomal compartments in which the class II-associated invariant chain is exchanged with antigenic peptides. On the contrary, most immunogenic GSL may bind to CD1a on the cell surface and are immunogenic in the absence of processing.

Our results also show that CD1a expression on the surface of LC freshly isolated from the epidermis is up-regulated by serum or sulfatide. This up-regulation is very efficient and fast, as up to 43% of CD1a increase is observed already after 1 h of incubation. Thus, normal LC in the skin present a reservoir of CD1a molecules that upon ligand binding are recognized by specific mAb and likely become immunologically competent.

According to these results, we propose the following physiological role for CD1a. Newly synthesized CD1a molecules assemble in the ER and reach the cell surface in association with lipid ligands which are not GSL [5], with unknown chaperones or, alternatively, with an empty antigen-binding groove. CD1a molecules reaching the plasma membrane become available for lipid exchange or, if empty, are susceptible to unfolding and after cellular internalization undergo subsequent degradation. Loading of exogenous lipids stabilizes CD1a and might prolong its surface permanence in an immunologically competent form. According to this model, the repertoire of CD1a-bound antigens is represented by exogenous ligands, and thus CD1a

may continuously sample the extracellular space for the presence of abundant lipid antigens. These ligands may be self-lipids, which are integral components of lipoproteins present in serum [27] and may be exchanged with other ligands bound to CD1a [10], according to their affinity and concentration. A second source of CD1a ligands is represented by foreign lipids of microbial origin. One microbial antigen that binds to CD1a is the lipopeptide didehydromycobactin, which is secreted by *Mycobacterium tuberculosis* [28]. This ligand, as well as other still unknown microbial ones [29] released by intracellular pathogens might traffic from phagosomes to early endosomes [30], or be released in the serum. If CD1a loading of soluble bacterial antigens is as efficient as that of self-GSL, it might also ensure immediate and adequate presentation when microbial antigens are generated in CD1a-negative cells. Thus, CD1a may sense lipid variations occurring within the extracellular milieu and, accordingly, readily present the repertoire of available antigens to T cells.

Materials and methods

Cells

MOLT-4 and T2 cells are from American Type Culture Collection (ATCC, Manassas, VA). C1R-CD1a and C1R-CD1b were obtained from S. Porcelli, J558 from K. Karjalainen. Cells were grown in RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, 2 mM UltraGlutamine II, MEM nonessential amino acids, 1 mM Na-pyruvate (all from Cambrex, Brussels, Belgium) and 100 μg/mL kanamycin (Invitrogen, Basel, Switzerland). DC and T cell clones were generated as described [23]. Fresh thymocytes, isolated from thymi of children subjected to heart surgery, were kindly provided by M. Heberer. Fresh LC were obtained from surgical specimens of human skin [31].

Cell culture in the presence of exogenous compounds and drugs

Cells were washed twice with PBS and 5×10^5 cells/well were plated in HS or LS. The optimal serum concentration (0.2%) used in LS has been found by titrating FCS from 0.05 to 0.5% and evaluating cell viability and change in surface levels of CD1a, b and MHC class I expression over a 48-h period by FCM. The capacity of different compounds to restore CD1a expression altered after 24 h in LS was tested by incubating cells further 24 h with 10 μg/mL of the following compounds: sulfatide, PEA, sphingomyelin (all from Fluka Chemie AG, Buchs, Switzerland or from L. Panza), GM1, PA (Matreya, Pleasant Gap, PA), PC, PS, PI (Avanti Polar Lipids, Alabaster, AL), cholesterol or distearin (Sigma). Purified human β_2m (Sigma) was used at 0.2 or 1 μg/mL. The restoration of CD1a expression was calculated as follows: % Restoration = $(MFI_{compound} - MFI_{LS})/(MFI_{HS} - MFI_{LS}) \times 100$, where MFI is the CD1a median fluorescence intensity in the presence of the compound, in LS, or in HS. This latter value was taken as control for 100% restoration. In some experiments, BFA (1 μg/mL, Sigma) and CHD (10 μg/mL, Sigma) were added alone or together on MOLT-4 for 24 h. Cell viability after these treatments was always above 98%. To control the activity of BFA, monocytes were treated with 1 μg/mL BFA for 24 h in the presence of GM-CSF and IL-4 in order to induce differentiation of DC and *de novo* expression of CD1 molecules. The absence of CD1a and CD1b on the surface of BFA-treated cells confirmed the activity of the drug (not shown). The control for CHD activity was done by confocal microscopy, demonstrating CD1a expression increased at cell surface and decreased intracellularly (not shown and Fig. 2J).

Antigen-presentation assay

MOLT-4 were washed twice with PBS, incubated in HS or LS, as described above and after 24 h, washed twice and incubated (5×10^4 cells/well) with HS or sulfatide (0.3–10 μg/mL) for 2 h before adding T cells (6×10^4/well) in medium containing 0.5% FCS. CD1a alteration after incubation in 0.5% FCS is the same as that observed in LS (data not shown). Supernatants were harvested after 24 h and cytokine measured by ELISA [23].

Antibodies, FCM and confocal imaging

Cells were stained with 10 μg/mL of the following CD1a-specific mAb: Vit6b, Vit6c, Vit6d, B17, 10D12.2, 10H3.9.3, NA1/34 (W. Knapp and A. Woolfson), OKT-6 (Instrumentation Laboratory, Schlieren, Switzerland) and AD16 (obtained in our laboratory). Evidence that OKT-6, AD16 and B17 recognize different epitopes was obtained by competition assays (data not shown). Other mAb used were WM-25 (anti-CD1b, Immunokontakt, Switzerland), BCD1b3.1 (anti-CD1b, S. Porcelli), W6/32 (anti-MHC class I, ATCC), L243 (anti-MHC class II, ATCC), anti-CD71 (anti-transferrin receptor, PharMingen, Basel, Switzerland), BBM.1 (anti-β_2m, ATCC-HB28). The mAb were revealed with FITC-conjugated goat anti-mouse Ig (Southern Biotechnology Associates, Birmingham, AL) and cells analyzed on a FACScan flow cytometer (Becton Dickinson, Allschwil, Switzerland). Background fluorescence was evaluated using isotype-matched irrelevant mAb. For confocal microscopy, cells were fixed permeabilized and stained as previously described [5]. For CD1a detection OKT-6 was used followed by Cy3-conjugated sheep anti-mouse IgG (Sigma). Capping of surface CD1a molecules was induced in J558 cells transfected with CD1a-GFP by incubating 30 min at 37°C with OKT-6, followed by cell fixation and staining with sheep anti-mouse IgG. Cells were examined by confocal laser scanning microscope (Zeiss, Jena, Germany). The 0.5-μm Z-sections were analyzed by LSM software (Zeiss).

Generation of stable CD1a- and CD1b-expressing cell lines

Cell lines expressing WT CD1A and CD1B cDNA were obtained as described [5]. CD1A-GFP fusion construct was prepared in the pEGFP-N3 expression vector (Clontech, Basel, Switzerland) after amplification of pCD1A-Bluescript II with the following primers: 5′XhoCD1A 5′TTCTCGAGATGCTGTTTT-

TGCTA and 3′CD1ABam 5′GGATCCACAGAAACAGCGTTTCC, lacking the stop codon. Hybrid CD1A/B and CD1B/A cDNA constructs were obtained using as templates pCD1A- and pCD1B-Bluescript II and the *Advantage*-HF 2 PCR kit (Clontech). CD1A/B contains extracellular (XC) and transmembrane (TM) gene segments of CD1A and cytoplasmic (CY) CD1B gene segment. CD1B/A contains XC and TM of CD1B and CY of CD1A. The following primers were used: 5′XhoXC1A_for 5′ATGCCTCGAGATGCTGTTTTTGCTACTTC, 3′CY1BTM-1A_rev 5′ACCGGCGCCTGAACCAAAGCGCAAGA-CC, 5′TM1ACY1B_for 5′GCGCTTTGGTTCAGGCGCCGGTCA-TATC, 3′HincCY1B_rev 5′ATCTGTCGACTCATGGGATATTC-TGATATG, 5′SalXC1B_for 5′TATAGTCGACATGCTGCTGCT-GCCATTTCAAC, 3′CY1ATM1B_rev 5′AGCGTTTCCTCATATA-CCATAATGC, 5′TM1BCY1A_for 5′TTATGGTATATGAGGAA-ACGCTGTTTC, 3′NotCY1A_rev 5′ATAAGAATGCGGCCGCTT-AACAGAAACAGCGTTTCC.

The amplified CD1A/B and CD1B/A hybrid constructs were sequenced after cloning into pBluescript II KS^+ (Stratagene, La Jolla, CA), subcloned into the vector BCMGSNeo and transfected by electroporation into T2 cells. Stable transfectants expressing CD1ab or CD1ba hybrid molecules were selected with G418 sulfate (0.8 mg/mL, Calbiochem, La Jolla, CA) and analyzed at FACS for surface expression of CD1a or CD1b or at confocal microscope for intracellular colocalization studies. For CD1a modulation studies cells were incubated with BFA (1 μg/mL) for 24 h before staining.

Acknowledgements: We acknowledge A. Donda and A. Shamshiev for helpful discussions, Q. Schefer for preliminary experiments, L. Angman for excellent technical assistance, M. Heberer, W. Knapp, M. Kronenberg, L. Panza, S. Porcelli, and A. Woolfson for providing us with precious reagents, and B. Erne for assistance in confocal imaging. We also thank P. Dellabona, T. Resink, H. de la Salle and C. Watts for reading the manuscript and G. van Meer for stimulating discussions. This project was supported by grants to GDL from Swiss National Found (3100–66769.01 and 3100A0–109918) and Human Frontier Science Program (RG0168/2000-M). The work on LC was funded by ARMESA and by the Scientific Council of EFS (CS/2002/018). VM received an EMBO fellowship.

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Education

1992 to 1996	Karol Marcinkowski 1st High School of General Education, Poznań, Poland
Oct 1996 to Mar 2001	Adam Mickiewicz University, Faculty of Biology, specialization: Molecular Biology, MSc, graduated: Mar 2001 (grade: very good). Thesis title: Biological and biochemical properties of milky sap from *Chelidonium maius* L.
April 2002 to December 2007	Post-graduate study in Medical and Biological Research at the Faculty of Science. University of Basel, under the supervision of Prof.G. De Libero. Thesis title: Antigen recognition and thymic maturation of human TCR Vgamma9-Vdelta2 cells.

Language skills

English: fluent
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Computer skills

Microsoft Office, Internet, Delta Graph, Photoshop, MacVector, Chem Draw, SoftMax Pro, Instat, Flow-Jo, Summit, Cell Quest Pro

Professional experience

Oct 1998 to Jun 2000	Young Scientist Society, Molecular Biology Division, at Adam Mickiewicz University, Poznań, Poland
Oct 1999 to Mar 2001	MSc student, Molecular Virology Group, Dept. of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznań, Poland
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Training courses

Mar 2003	LTK Module 1E: Introductory course in Laboratory Animal Science
September 2004	FEBS, International Summer School for Immunology, Ionian Village, Greece
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Practical experience

Molecular biology and biochemistry techniques: PCR, RT-PCR, cloning, expression of recombinant proteins in mammalian system, protein immunoprecipitation, purification and quantification, SDS-PAGE, SSCP and TGGE analysis, immunobloting; Cellular

biology and immunology techniques: immunofluorescence, FACS (up to seven colors), ELISA, cell culture (tumor and primary cells), isolation of primary cells from body fluids, gene transfection, T cell activation assays, T cell clones generation, cell cloning, collection of mouse organs and cell culture preparation, generation of cell hybrydomas. *In vivo* experiments (in mice): anesthesia, intra peritoneal, intra venous, subcutaneous and intra thymic injections, immunization.

Publications

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Conferences presentations

1. Swiss Society for Allergology and Immunology (SGAI) Annual Meeting March 30th-31st 2006, Zurich; Title: Requirements for maturation and peripheral expansion of TCR Vgamma9 /Vdelta2 cells in a mouse model. Selected for an oral presentation

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