The University of Milano Bicocca

THE PhD PROGRAM DIMET

The PhD Program in Translational and Molecular Medicine (DIMET) is an inter-departmental project between the School of Medicine and the Faculty of Science, organized by the University of Milano-Bicocca.

DIMET - Dr. Simone Caielli - A.A. 2008-09

PhD

PROGRAM IN TRANSLATIONAL AND MOLECULAR MEDICINE

DIMET

UNIVERSITY OF MILANO-BICOCCA
SCHOOL OF MEDICINE AND FACULTY OF SCIENCE

On/off TLR signaling decides immunogenic or tolerogenic dendritic cell maturation upon NKT cell contact

Coordinator: Prof. Andrea Biondi
Tutors: Prof. Andrea Biondi/ Dr. Marika Falcone

Dr. Simone Caielli

Matr. No. 708295

XXII CYCLE
ACADEMIC YEAR
2008-2009

1 In agreement with Italian law 22 april 1941 n. 633, Art. 70

2 In agreement with Italian law 22 april 1941 n. 633, Art. 70

3 In agreement with Italian law 22 april 1941 n. 633, Art. 70

4 In agreement with Italian law 22 april 1941 n. 633, Art. 70

TABLE OF CONTENTS

Chapter 3 : Summary, Conclusions and Future

8 In agreement with Italian law 22 april 1941 n. 633, Art. 70

9 In agreement with Italian law 22 april 1941 n. 633, Art. 70

CHAPTER 1 : General Introduction

10 In agreement with Italian law 22 april 1941 n. 633, Art. 70

iNKT CELLS

There are several remarkable features of natural killer T cells (iNKT cells) that have emerged from studies carried out during the previous decade. First, although they constitute less than 1% of mouse T lymphocytes, they exert a critical influence on a variety of immune responses and pathologic conditions.

Second, to a surprising extent they function differently from conventional T cells. A salient feature that distinguishes NKT cells from conventional T cells is their ability to rapidly secrete a large amount of different cytokines within a few hours after their activations both in vitro than in vivo.

Therefore, not surprisingly, this small lymphocyte subpopulation has captured the attention of many immunologist.

WHAT NKT CELLS ARE?

NKT cells were first described in C57BL/6 mice as lymphocytes that co-express a T-cell receptor (TCR) and the NK-cell receptor NK1.1 (also known as NKR-P1C or CD161c). However, this definition is confusing because many commonly used mouse strain (such as BALB/c, 129 and NOD mouse) do not express NK1.1 on their surface [1].

In addition, NKT cells are heterogeneous population of lymphocytes with different specificities and functions. Prototypical mouse NKT cells, often referred as iNKT cells, express a semi-invariant TCR, with an alpha-chain composed

of Va14-Ja18 paired with a beta-chain biased towards Vb8.2, Vb7 or Vb2 **(Figure 1)**. A similar population of cells has been identified in humans, other primates and rats.

Human NKT cells express TCR a-chains (Va24-Ja18) and b-chains (Vb11) that are homologous to the TCR-a and TCR-b expressed by mouse NKT cells.

The main distinguishing feature of NKT cells, compared with other cell types often referred to as NKT cells, is their capacity to react with a-GalCer **(Figure 2)** in the context of the MHC class-I related molecule CD1d [2].

However, despite the clearly defined identity of this exogenous ligand, the physiological antigen that mediate the immunological functions of NKT cells remain to be determined although several candidates have been identified.

NKT cells are abundant in the thymus, liver and bone marrow and they are found in considerable numbers in the spleen and peripheral blood but are rare in lymph nodes.

Most mouse NKT cells express CD4 and the remaining NKT cells lack expression of CD4 and CD8. Whereas few NKT cells in mice express CD8 a significant proportion of NKT cells in humans, macaques and rats express CD8 on their surface [3, 4]. *[Adapted from: Van Kaer; Nat. Rev. Imm. 5, 31-42 (2005)]*

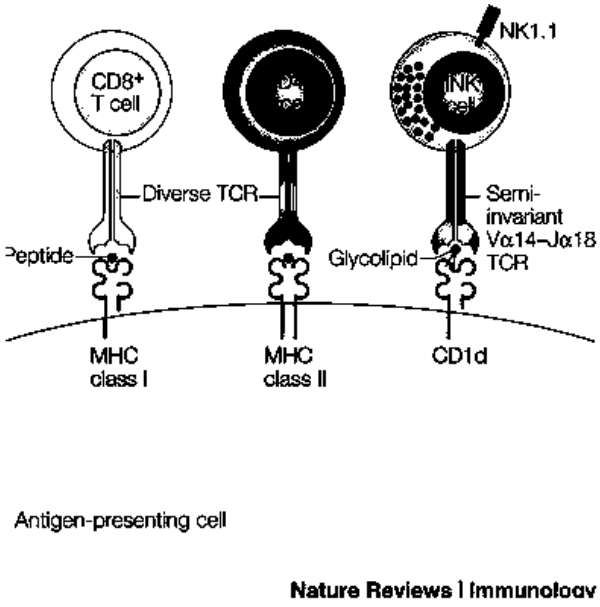

Figure 1: Schematic view of the ligand-specificity of NKT cells compared with conventional T cells. CD8 T cells express diverse T-cell receptors (TCR) that recognize peptide antigens presented by MHC class I molecules. CD4 T cells express diverse T-cell receptors (TCR) that recognize peptide antigens presented by MHC class II molecules. By contrast NKT cells express a semi-invariant TCR that recognize glycolipid antigens presented by CD1d molecules at the surface of antigen-presentig cells. NKT cells also express some NK cell markers such as NK1.1. *[Adapted from: Van Kaer; Nat. Rev. Imm. 5, 31-42 (2005)]*

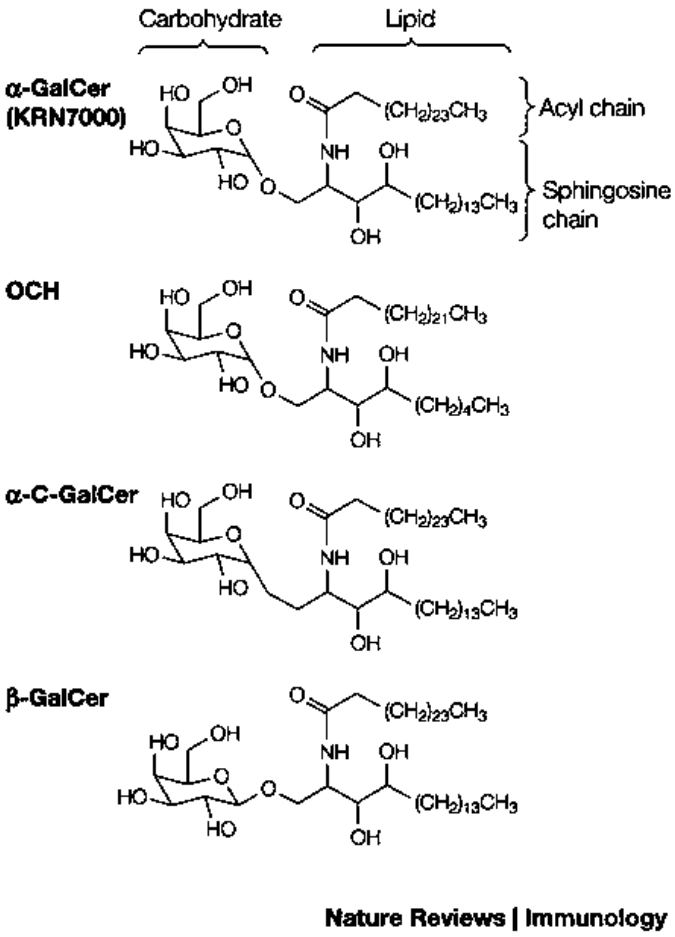

Figure 2: Structure of a-GalCer and related glycolipids. a-GalCer is the general designation for glycosphingolipids that contain a galactose carbohydrate attached by an a-linkage to a ceramide lipid. KRN7000 is a synthetic a-galcer that is most frequently used in experimental studies. OCH differs from KRN7000 by truncations in the acyl chain and the sphingosine chain. a-C-galcer is a C-glycoside analogue to a-galcer. *[Adapted from: Van Kaer; Nat. Rev. Imm. 5, 31-42 (2005)]*

Various cell types are commonly referred as natural killer T cells in the scientific literature which has led to considerable confusion.

Prototypical NKT cells, usually referred to as invariant or NKT cells, express a semi-invariant T cell receptor and recognize glycolipid antigens that are presented by CD1d.

NKT cells can be detected most reliably in mouse and human cell populations by using CD1d tetramers or dimers loaded with a-galactosylceramide (a-GalCer) **(Figure 3)**.

CD1d dependent T cells that do not express the NKT cell TCR and fail to react with a-GalCer have also been identified and might best be referred to as non-classical NKT cells. In addition, various other cell types, which include CD1d independent NK1.1 positive T cells and other T cell subsets that express semi-invariant TCR, share cell-surface markers and functional properties with NKT cells [5]. Although the latter cell types are often referred to as NKT cells in the scientific literature, this has resulted in considerable confusion. Therefore, it would be better to restrict the term of NKT cells to CD1d dependent T cells and to refer to other T cells subsets, that resemble NKT cells, as NKT like cells.

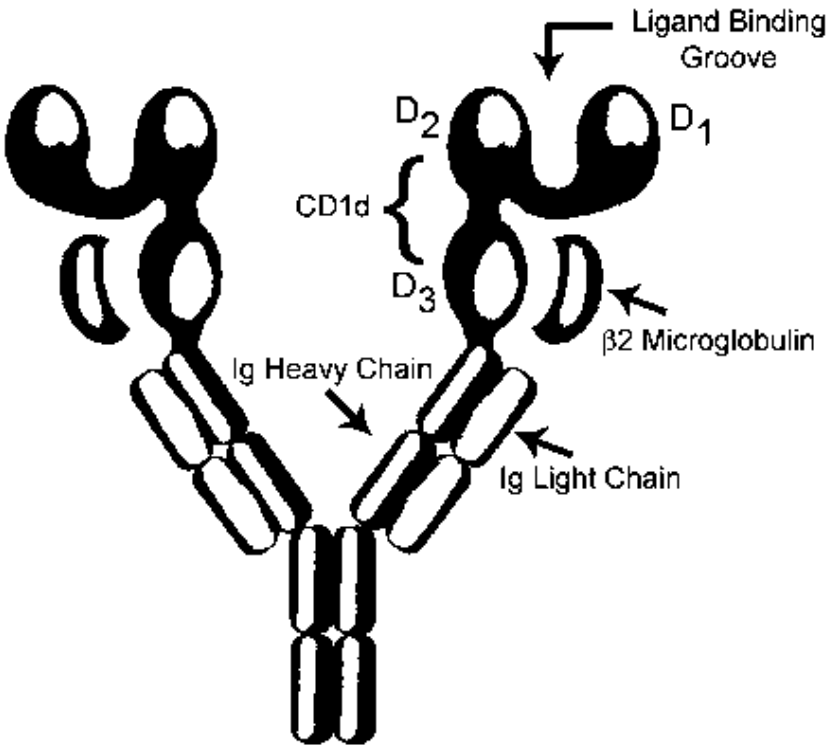

Figure 3: DimerX recombinant soluble dimeric mouse CD1d:Ig fusion protein. The CD1d:Ig fusion protein consist of the extracellular major histocompatibility complex (MHC) class I like domains of the mouse CD1d molecule fused with the VH regions of mouse IgG1. Like MHC class I molecules, the CD1d noncovalently associates with b2 microglobulin (b2M). For this reason, BD Pharmingen DimerX consists of recombinant CD1d:Ig and b2M. Recombinant CD1d molecules, like the DimerX fusion protein, are useful for studying NKT cells functions by immunofluorescent staining and flow cytometric analysis of antigen specific NKT cells. *[Adapted form BD Biosciences]*

DEVELOPMENT OF NKT CELLS

Using CD1d dimers or tetramers permits investigators to detect developing NKT cells in a normal physiologic setting without the potentially distorting effects on the repertoire of TCR transgenes. Investigators now agree that NKT cells arise in the thymus in the perinatal period and do not reach significant levels until at least three weeks after birth [6]. Their positive selection in mediated by CD1d-expressing bone marrow-derived cells rather than by cortical epithelial cells, a finding that helped investigators establish the distinctive nature of this T lymphocyte subset [7].

Among the bone-marrow derived cells, CD1d double positive thymocytes are almost certainly the critical cell type for positive selection. In addition to this requirement by NKT cells for an unusual positively selecting cell type, NKT cell development and maturation are differentially effected by a number of mutations that have relatively little effect on conventional cells.

Several recent studies have focused on members of the NF-kB family. NKT cell development requires the expression of NF-kB1 (p50) in a cell-autonomous manner [8].

NKT cell development also requires the expression of the gene for the inhibitor of NF-kB kinase ikk2. Moreover, RelB (p65) expression in an irradiation-resistant cell is required in NKT cell development [9].

Recently, it has been shown that, for their differentiation, NKT cells require the transcription factor T-bet (T-box expression in

T cells), a factor originally identified as important for the induction of IFN-gamma synthesis and Th1 immunity in several cell types [10].

Why are the genetic requirements for NKT cell development unique and complex? A few of the mutations that specifically diminish the NKT cell population, such as those affecting AP-3 subunit and prosaposin, probably act by affecting the pathway required for the loading of endogenous glycolipids into the CD1d groove. The adaptor protein AP-3 binds to the CD1d cytoplasmic tail and is required for CD1d trafficking to lysosomes where endogenous glycolipids may be processed and loaded into CD1d.

Sphingolipid activator proteins, four of which are encoded by the prosaposin precursor, are lysosomal proteins that interact with CD1d molecules. They make lipids available for CD1 loading and they apparently perform an editing or quality control function for the lipids bound to CD1d [11].

In contrast to conventional thymocytes, most of the NKT cells in the thymus are part of mature, immune-competent population capable of producing IL-4 and IFN-gamma immediately after TCR stimulation.

Therefore the set of genes required for NKT cell differentiation may reflect not only those required for their early maturation but also those that are required for lymphocyte expansion and differentiation to effector cells. This could explain, for example, the requirement for expression of NF-kB transcription factors in developing NKT cells as these transcription factor seem to

affect the survival of the NKT cells after they have expressed the TCR undergone some expansion and reached an intermediate stage of their differentiation.

The use of *α*GalCer-loaded CD1d tetramers has permitted several research groups to analyze the phenotype of NKT cells during these later stages of their differentiation, after they have acquired a TCR. Expression of several molecules, including the IL-7 receptor, CD24, DX5, NK1.1, and Ly49 family NK receptors, occurs during later maturation steps. Induction of NK1.1 expression probably can occur in the thymus, although most of the recent thymus emigrants are NK1.1 negative , suggesting that the final maturation stages for NKT cells also occur in the periphery [12,13]. An unresolved issue concerns the elements that direct developing thymocytes into this sublineage. Two alternative models are illustrated in **Figure 4**. Investigators have presented evidence suggesting that NKT cells have a double-positive precursor. The existence of a double-positive precursor is consistent with an instructional model in which the expression of the V*α*14*i* TCR and recognition of endogenous ligands presented by CD1d commit the developing precursor to become *i*NKTcell. A relatively high-affinity recognition of endogenous ligands presented by CD1d and/or the consequences of selection by other double-positive thymocytes may be responsible for this commitment [14]. Other researchers have suggested that a subset of thymocytes is precommitted to become NKT cells before antigen receptor

rearrangement. For example, the requirement for the SRC family kinase Fyn in NKT cell development is cell autonomous [15]. In contrast, conventional T lymphocytes undergo relatively normal thymus differentiation in the absence of Fyn. This special requirement exhibited by of NKT cells can be overcome by expression of a V*α*14*i* transgene. In mixed bone marrow chimera experiments, in which *fyn*+ and *fyn−/−* V*α*14*i* transgenic precursors were cotransferred to irradiated recipients, the *fyn−/−* precursors contributed to the V*α*14*i* NKT cell compartment as effectively as those that express Fyn. On the basis of these results, investigators have suggested that V*α*14*i* NKT cell deficiency in *fyn−/−* mice is not simply due to a failure to properly signal through the V*α*14*i* TCR or to expand V*α*14*i* NKT cell precursors. They suggest instead that *fyn* acts upstream of TCR expression, and that it in some way aids in the TCR-independent commitment of thymocytes to the V*α*14*i* NKT cell sublineage, with expression of the transgene acting downstream to overcome this defect [16, 17]. V*α*14*i* NKT cells do not have a preferential rearrangement of V*α*14*i* on the unexpressed allele, and therefore even if a precommitment occurred, the cells precommitted to this sublineage would then also have to undergo selection on the basis of TCR specificity.

A growing body of evidence favors the concept that the thymus does not discard all self-reactive T cells and that some potentially self-reactive T lymphocytes are preserved to carry out specialized or regulatory functions. Consistent with this

theory, evidence suggests that TCR*αβ*+ CD8*αα* intraepithelial lymphocytes and CD4+ CD25+ regulatory T cells are inherently self-reactive. The CD1d autoreactivity of NKT cells has led to the suggestion that these T lymphocytes also are positively selected by self-agonist ligands. The forced expression of CD8*α* and CD8*β* transgenes in T cells leads to a decrease in NKT cells, suggesting that CD8-mediated enhancement of the intrinsically high affinity of the V*α*14*i* TCR for self-antigens presented by CD1d pushes the developing NKT cell over the threshold of negative selection. Direct evidence is lacking, however, indicating that CD8 can serve as a coreceptor for CD1d-mediated antigen recognition, and the CD8 transgenic mice have other abnormalities, including a decrease in total thymocyte numbers [18].

An alternative model for the lack of CD8 expression by NKT cells is the finding that increased or prolonged Lck signaling preferentially directs thymocytes to be CD4 positive. Therefore, although the reason for the lack of CD8 expression by NKT cells remains to be determined, the concept that they are positively selected by self-agonists or relatively high-avidity interactions remains an appealing one [19]. Unlike in mice, in humans a minority of NKT cells expresses CD8*α*, and some express CD8*β* as well [20].

Although NKT cells may require self-agonist for their development, there must be an upper limit to the avidity window

that permits their positive selection, as the results from several experiments support the concept that these cells can be negatively selected in the thymus.

Addition of *α*GalCer to fetal thymic organ cultures causes NKT cell negative selection, and increased expression of CD1d in transgenic mice causes a decrease in the number of NKT cells [21]. Moreover, the remaining NKT cells in these CD1d transgenic mice were hyporesponsive. Mouse NKT cells tend to coexpress V*β*8.2, constituting more than 50% of the total, with V*β*7 and V*β*2 also highly represented. Evidence suggests that the V*β*8.2-containing V*α*14*i* TCRs tend to have the highest affinity for *α*GalCer presented by CD1d, and in the CD1d transgenic mice, V*β*8.2 NKT cells were underrepresented, which is consistent with the elimination of cells expressing the highest affinity TCRs [22]. Chronic exposure of young adult mice to *α*GalCer also led to NKT cell deletion. When treatment was halted, newly exported cells from the thymus reconstituted the NKT cell population. The recovered cells were hyporesponsive, however, and they had increased expression of inhibitory NK receptors of the Ly49 family [23, 24]. This is consistent with other data indicating that the balance of signals between activating and inhibitory NK receptors, as well as TCR avidity, sets the affinity threshold and regulates the development of NKT cells. *[Adapted from: Kronenberg; Ann. Rev. Imm. 26, 877-900 (2005)]*

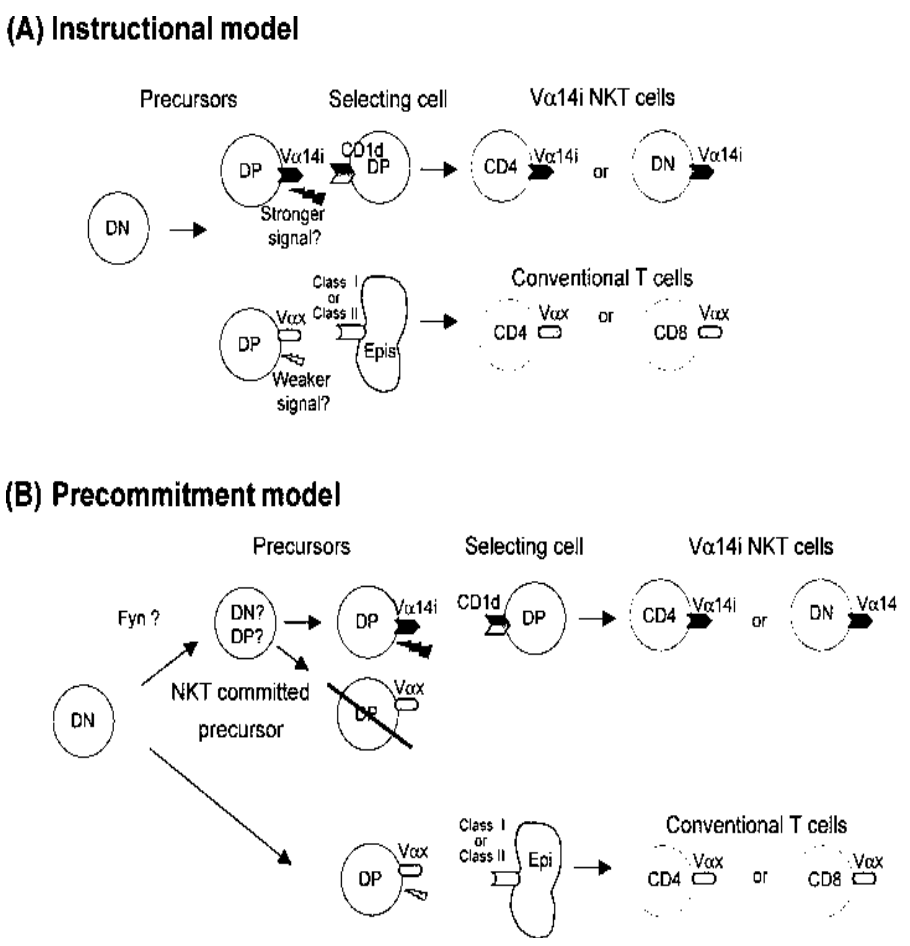

Figure 4: Models for the development of NKT cells. A) In the instructional model, expression of the V*α*14*i* TCR instructs thymocytes to become NKT cells, perhaps because of high-affinity interaction with CD1d presented ligands or interaction with double positive thymocytes. Expression of other TCR (Vax) leads to the positive selection of conventional T cells my MHC class I and II expressing epithelial cells (Epsi). B) According to the precommitment model, a precursor decides to become a NKT cell before TCR rearrangement as depicted. *[Adapted from: Kronenberg; Ann. Rev. Imm. 26, 877-900 (2005)]*

NKT CELLS LIGANDS

Although disputed initially, there is now a general consensus that CD1d, like other CD1 family members, evolved to present lipids to T cells. However, the nature and the source of the various lipids that bind naturally to CD1d remain poorly elucidated. Early studies of CD1d immunoprecipitates obtained from cell detergent lysates suggested a predominance of phospholipids— particularly glycosylphosphatidylinositols, an anchor for various surface proteins, and phosphatidylinositols [25].

However, because these early studies used detergents that could potentially displace natural lipids bound to CD1d, or soluble forms of CD1d that did not traffic through the endosome and might have acquired irrelevant lipids from membrane compartments or culture medium, their interpretation is uncertain. Future studies of CD1d molecules engineered to express an enzymatic cleavage site at the membraneproximal portion of their extracellular domain constitute an attractive approach to reexamining this fundamental issue [26]. Despite a lack of direct biochemical studies of CD1-bound lipids, combinations of genetic, cell biological, and chemical approaches have nevertheless uncovered some key NKT ligands discussed below.

The first NKT ligand emerged from studies initiated at Kirin Pharmaceuticals to identify natural anticancer medicines. Extracts from *Agelas mauritianus*, a marine sponge collected in the Okinawan sea, prolonged survival of mice bearing B16 melanoma. The structure of the active principle was identified as an α-branched galactosylceramide and slightly modified for optimal efficacy to produce a compound termed KRN7000, also commonly referred to as αGalCer. The lipid nature of this compound, its strong effect on liver metastasis, and its activation of dendritic cells (DCs) independent of MHC class I or class II led to the identification of Vα14 NKT cells as their target [27]. As a surrogate ligand of very high activity in vitro and in vivo, in the picomolar range αGalCer has been used broadly in various functional assays and to generate the first CD1d tetramers specific for mouse and human NKT cells. The affinity of interaction between CD1d-αGalCer and mouse TCRs is one of the highest ever recorded for natural TCR/ligand pairs with a Kd ~100 nM, owing to a slow off rate, for several Vα14-Jα18/Vβ8 combinations examined and may be significantly lower in the human system (~7 μM) [28]. Although the

expression of this ligand in marine sponges could not be linked with any physiologically relevant function, the striking properties of αGalCer have provided early support for the hypothesis that the conserved TCRs of NKT cells evolved to recognize conserved lipids. More than 95% of cloned mouse and human NKT cells recognize αGalCer, irrespective of their variable CDR3 β sequence, and the mouse CD1d- αGalCer tetramers stain human and nonhuman primate NKT cells as well, attesting to the high degree of conservation of this recognition system [29].

The lack of physiological relevance of αGalCer should be revisited with the recent discovery that closely related structures that substitute for lipopolysaccharide (LPS) are found in the cell wall of *Sphingomonas*, a Gram-negative, LPS-negative member of the class of α-proteobacteria [30]. These glycosphingolipids are responsible for the strong stimulation of NKT cells and their role in clearing infection.

The most abundant glycosphingolipids have only one sugar, galacturonyl or glucuronyl, α-anomerically branched to the ceramide backbone (GSL-1). Thus, they differ from the stimulating αGalCer or αGlcCer mainly by the carboxyl group in C6, a position permissive to NKT cell recognition [30]. Other more complex but less abundant glycosphingolipids include GSL-2, -3, and -4. Because in general it is known that extracts from *A. mauritianus* have different properties depending on season and location and because these sponges are often

colonized by α-proteobacterial symbionts, particularly by *Sphingomonas* the marine spongeαGalCer may in fact have originated from bacterial symbionts [31].

Although the discovery of bacterial NKT ligands provides a fascinating new perspective on the evolutionarily relevant functions of NKT cells, considerable attention has also focused on self ligands. Indeed, mouse and human NKT cells exhibit conspicuous low-level autoreactivity to various CD1d-expressing cell types. This autoreactivity and the presence of IL-12, triggered by Toll like receptor (TLR) signaling, are required for the commonly observed IFN-γ secretion by NKT cells during immune responses against Gram-negative, LPS-positive bacteria. Autoreactivity may also underlie the thymic development of NKT cells, which includes an expansion phase after positive selection and the acquisition of a memory phenotype independent of microbial exposure or TLR signaling [32, 33].

Recent findings demonstrate that the glycosphingolipid iGb3, both natural and synthetic, could activate a majority of mouse Vα14 and human Vα24 NKT cells, irrespective of their Vβ chain, upon presentation by DCs or plastic-bound CD1d/iGb3 preformed complexes. iGb3 appears to be a weaker agonist

than αGalCer, requiring ~30- to 100-fold higher concentrations

to achieve the same level of stimulation [34]. This may explain the failure to stain NKT cells using CD1d/iGb3 tetramers. However, solubility issues and more stringent requirements for professional antigenpresenting cells (APCs) may contribute to its lower apparent activity, and the affinity of CD1d/iGb3-TCR interactions remains to be measured directly, particularly to dissect the contribution of on and off rates.

Different lines of experiments suggest that iGb3 is an important physiological NKT ligand. β-hexosaminidase-B-deficient mice, which lack the ability to degrade iGb4 into iGb3 in the lysosome, exhibited a 95% decrease in thymic NKT cell production, and β-hexosaminidase-B-deficient thymocytes could not stimulate autoreactive Vα14 NKT cell hybridomas [35]. Notably, unlike other mutations of enzymes or transporters involved in lipid metabolism and associated with lipid storage, the defect in β-hexosaminidase-B-deficient cells appeared to be specific in that β-hexosaminidase-B-deficient bone marrow–derived DCs normally presented several complex derivatives of αGalCer that required lysosomal processing prior to NKT cell recognition, but lost their ability to process and present iGb4 the precursor to iGb3 or GalNAcβ1,4GalαCer, both of which require removal of the outer, β-branched hexosamine for NKT cell recognition. In addition, the *Griffonia simplicifolia* isolectin B4 (IB4) specific for the terminal Galα1,3Gal blocked CD1d-mediated presentation of both exogenous iGb3 and endogenous ligand (natural autoreactivity), but not αGalCer [36,

37]. These studies suggest that iGb3 is an important physiological ligand of NKT cells. Additional findings reviewed below suggest that iGb3 may also be the natural ligand activating NKT cells during Gram-negative, LPS-positive infections. These results are therefore consistent with the requirement for endosomal trafficking of CD1d and the role of lysosomal saposins functioning as glycosphingolipid exchange proteins in the presentation of the NKT ligand in vivo [38]. It should be noted, however, that the presence of iGb3 among CD1d-bound lipids remains to be demonstrated and that iGb3 itself has not yet been directly identified in human or mouse tissue, a task complicated by the rarity of iGb3 and the dominance of the regioisomer Gb3 [39]. Furthermore, other than the enzymatic pathways of synthesis and degradation, little is known about the general biology of iGb3, its subcellular location, or its function. *[Adapted from: Bendelac; Ann. Rev. Imm. 25, 297-336 (2007)]*

CELL BIOLOGY OF LIPID PRESENTATION BY CD1d

CD1d is prominently and constitutively expressed by APCs such as DCs, macrophages, and B cells, particularly marginal zone B cells, with relatively modest changes associated with TLR activation and inflammatory cytokines. CD1d is also strikingly expressed on cortical thymocytes, where it is essential for NKT cell development, and on Kupffer cells and endothelial cells lining liver sinusoids, where the highest frequencies of NKT cells are found in mice [40].

Hepatocytes express CD1d constitutively in mouse and upon disease induction in human, for example, in the context of hepatitis C. CD1d expression in the liver is not required, however, for NKT cell homing, and neither is CXCR6 expression by NKT cells, although CXCR6/CXCL16 interactions are essential for survival in this organ. CD1d is upregulated on microglial cells during inflammation [41]. Similar to the MHC class II system, most other solid tissue cells and non-antigen-presenting hematopoietic cells express low or undetectable levels of CD1d.

The intracellular trafficking of CD1d has been studied thoroughly (**Figure 5**). Biosynthesis of the heavy chain associated with β2-microglobulin involves the endoplasmic reticulum chaperones calnexin and calreticulin and the thiol oxidoreductase ERp57 [42].

It is logical to assume that endogenous lipids in the endoplasmic reticulum would fill the groove of CD1d, and one study suggested the presence of phosphatidylinositol, with the caveat that contamination by membrane phospholipids could not be formally excluded. CD1d rapidly reaches the plasma membrane within 30 min after biosynthesis and undergoes extensive internalization and recycling between the plasma membrane and endosomal/lysosomal compartments in a manner dependent upon a tyrosine motif encoded in the CD1d cytoplasmic tail. The tyrosine motif in the cytoplasmic tail primarily binds adaptor protein (AP)-2 and AP-3 in mouse, where the bulk of CD1d accumulates in the lysosome, and AP-2 in humans, where CD1d tends to reside in the late endosome [43]. Additional but largely redundant contributions by the invariant chain or invariant chain/MHC class II complexes that bind weakly to CD1d have been documented in mouse and human. The CD1d intracytoplasmic tail also expresses a lysine targeted for ubiquitination by the MIR proteins of the Kaposi sarcoma–associated herpes virus, causing downregulation from the cell surface without degradation. Interestingly, another herpes virus, herpes simplex virus-1 (HSV-1), induces CD1d downregulation from the cell surface, but the mechanism appears to be distinct, involving lysosomal retention through impaired recycling to the plasma membrane [44].

Tail-truncated CD1d molecules fail to access the late endosome and lysosome, causing a profound disruption of CD1d-mediated

antigen presentation in vitro in cell lines and in vivo in knockin mice. Particularly affected are the presentation of the NKT endogenous ligand and, consequently, the thymic generation of Vα14 NKT cells [45]. The presentation of diglycosylated αGalCer variants requiring processing prior to NKT cell recognition, an important tool for research, or of iGb4, which requires processing into iGb3 prior to recognition, is also abolished. However, other lipids that do not require processing still exhibit variable requirements for the late endosome and lysosome trafficking of CD1d, either partial in the case αGalCer (three- to fivefold shift in dose response) or substantial in the case of iGb3 (>10-fold shift). Recent studies of lipid uptake, trafficking, and loading have begun to shed some light on these observations [46].

Lipids in the circulating blood or in culture medium are bound to lipoproteins, and a dominant role for VLDL in the serum and its receptor, the LDL receptor, at the cell surface has been proposed for the clathrin-mediated uptake of some lipids into endosomal compartments (**Figure 5**). Other extracellular lipids can be captured by the mannose receptor langerin or can insert themselves directly in the outer leaflet of the plasma membrane and undergo endocytosis through clathrin-dependent or –independent pathways [47].

Glycosphingolipids tagged with a fluorochrome, BODIPY, on the acyl chain reached the late endosome and were rapidly

sorted to the endoplasmic reticulum and the Golgi. In contrast, a prodan-conjugated (on carbohydrate C6) αGalCer accumulated selectively in the lysosome. These pathways overlap only partially with those governing the trafficking of endogenous glycosphingolipids, which are synthesized in the lumenal part of the Golgi and thought to reach the plasma membrane first, then the endosome, through clathrin-dependent and-independent endocytosis until they are degraded in the lysosome. How exogenously administered or endogenous intracellular lipids choose between these pathways and the consequence for antigen presentation are questions that are just beginning to be addressed and may depend on intrinsic properties such as length or insaturation of alkyl chains, composition of the polar head, and solubility in aqueous environments, as well as extrinsic variations in the mode of administration such as use of detergents, liposomes, or lipid-protein complexes [48]. The development of new methodologies, genetic manipulation, and reagents will be required to address these essential questions. In addition, recognition of microbial lipids in the context of infection most likely involves different pathways because the uptake of bacteria is governed by different sets of cell surface receptors and the release of cell wall lipids would occur through degradation of the microorganism in the lysosome before processing and loading onto CD1d [49].

Although an intrinsic, pH-dependent mechanism appears to favor the acquisition of some lipids by CD1 proteins, perhaps through a conformational change, lipid exchange now appears to be regulated by specialized lipid transfer proteins. By using various detergents, early studies of lipid binding to CD1 molecules tacitly dealt with the fact that in general lipids are insoluble in water, forming micelles that cannot transfer monomeric lipids onto CD1. These detergents, however, also tended to dislodge lipids bound to CD1, as shown directly in the crystal structure of CD1b complexed with phosphatidylinositol, where two molecules of detergent cohabited with the lipid in the groove. In contrast, during biological processes, membrane lipids are extracted and transported by lipid exchange proteins. Prosaposin is a protein precursor to four individual saposins, A, B, C, and D, released by proteolytic cleavage in the lysosome [50]. Prosaposin-deficient mice provided the first genetic link between NKT cells and lipid metabolism, as they lacked NKT cells and exhibited greatly impaired ability to present various endogenous and exogenous NKT ligands. In cell-free assays, recombinant saposins readily mediated lipid exchange between liposomes and CD1d in a nonenzymatic process requiring equimolar concentrations of CD1d and saposins [51].

Although they exhibited some overlap in lipid specificity, individual saposins differed in their ability to load particular lipids. More detailed studies of the effects of these and other lipid exchange proteins such as NPC2 and the GM2 activator

are required to understand their function individually or cooperatively at different phases of lipid processing and loading [52]. In addition, the structural basis of the lipid exchange mechanism and its relative specificity for lipid subsets remain to be elucidated.

Another lipid transfer protein expressed in the endoplasmic reticulum, microsomal triglyceride transfer protein (MTP), assists in the folding of apolipoprotein B by loading lipids during biosynthesis. Coprecipitation of MTP with CD1d suggested that MTP might play a similar role for CD1 molecules. Indeed, genetic or drug-induced inhibition of MTP was associated with defects in lipid antigen presentation. MTP was suggested to transfer phosphatidylethanolamine onto CD1d in a cell-free assay, but the efficiency of this process remains to be established, and cell biological studies are required in vivo to fully understand the role of MTP in CD1d-mediated lipid presentation. CD1e is a member of the humanCD1family that is not expressed at the plasma membrane but is instead found as a cleaved soluble protein in the lysosome [53]. Recent experiments have shown that CD1e could assist the enzymatic degradation of phosphatidylinositolmannoside, suggesting that this protein may have diverged from other CD1 molecules to perform ancillary functions rather than to carry out direct antigen presentation.

NPC1 is a complex membrane multispan protein present in the late endosome that is mutated in Niemann-Pick type C1 disease and associated with a lipid storage phenotype similar to NPC2, a soluble lipid transfer protein present in the lysosome. *NPC1*-mutant mice exhibited broad defects of NKT cell development and CD1d-mediated lipid presentation, which could be attributed in part to an arrest of lipid transport from late endosome to lysosome. The precise function of NPC1 remains unknown, and it is unclear how this putative flippase translocating lipid between leaflets of the membrane bilayer could induce general alterations of lipid trafficking [54].

Mutations of several proteins involved in glycosphingolipid degradation or transport are accompanied by lipid storage within distended lysosomal vesicles, the impact of which depends on the enzyme, the cell type, the mouse strain, and the age at which cells are examined. This lipid accumulation may disrupt rate-limiting steps of lipid metabolism and indirectly alter CD1- mediated lipid antigen presentation through defective lipid trafficking or lipid competition for loading CD1d. For example, while *NPC1*-mutant cells showed a block in lipid transport from late endosome to lysosome, this block could be partially reversed by inhibitors of glycosphingolipid synthesis such as N-butyldeoxygalactonojirimycin, presumably through alleviation of the lipid overload. Bone marrow–derived DCs from mice lacking β-hexosaminidase B, α-galactosidase A, or galactosylceramidase did not show much alteration of general

lipid functions because they conserved their ability to process several complex diglycosylate derivatives of αGalCer for presentation to NKT cells, although a divergent report was recently published [55]. In contrast, β-galactosidase-deficient cells exhibited more general defects than expected from the specificity of the mutated enzyme.

Paradoxically, studies of cathepsin-mutant mice led to the first reports of defects inNKT cell development and CD1d-mediated lipid antigen presentation. This is particularly well established for cathepsin L because mutant thymocytes, but not DCs (perhaps owing to the redundancy of other cathepsins), failed to stimulate Vα14NKT hybridomas in vitro and consequently failed to select NKTcells in vivo. Although its target remains to be identified, cathepsin L may be directly or indirectly required for thymocytes to process prosaposin into saposins [56]. *[Adapted from: Bendelac; Ann. Rev. Imm. 25, 297-336 (2007)]*

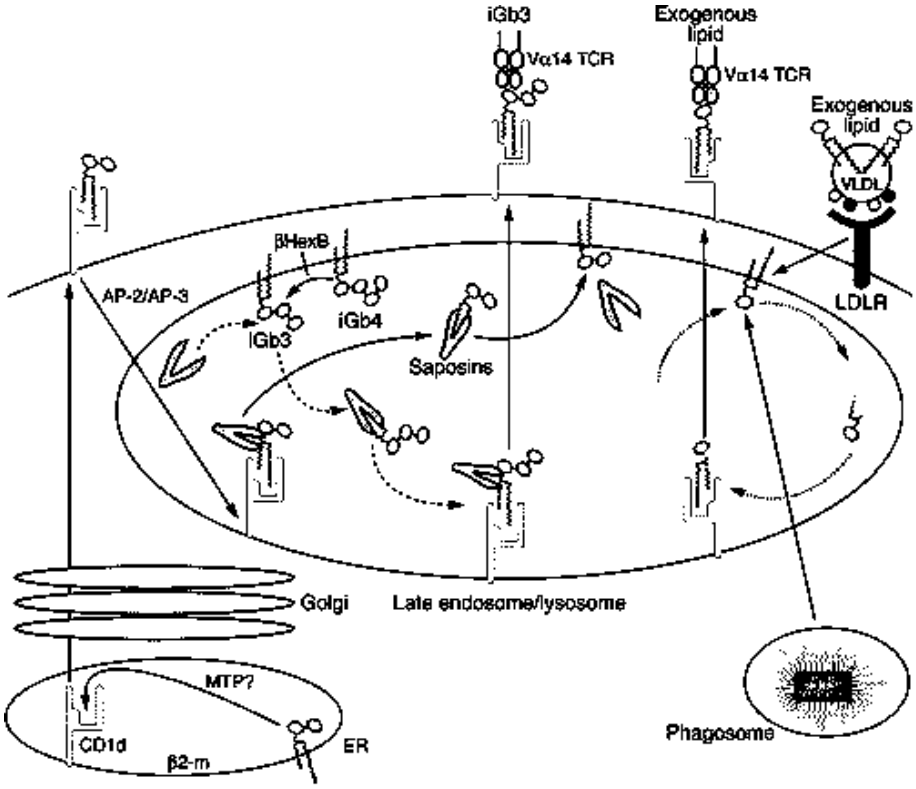

Figure 5: Intracellular trafficking and lipid loading of CD1d. CD1d molecules reach the plasma membrane and are internalized through an AP2/AP3 pathway to late endosomal/lysosomal compartments where lipid exchange is performed by saposin. CD1d recycles between lysosome and plasma membrane allowing further lipid exchange. Additional lipid exchange proteins may be involved in these process particularly during biosynthesis when a role for MTP has been proposed. *[Adapted from: Bendelac; Ann. Rev. Imm. 25, 297-336 (2007)]*

IMMUNOLOGICAL FUNCTIONS OF NKT CELLS

One hallmark of iNKT cells is their capacity to produce, within hours of activation, large amounts of cytokines, including the characteristic TH1 cytokine interferon-γ (IFN-γ) and the characteristic TH2 cytokine interleukin-4 (IL-4). iNKT cells constitutively express mRNA encoding IFN-γ and IL-4, which allows these cells to rapidly dispatch their effector functions, a property that they share with cells of the innate immune system. In addition, TCR-mediated signals received by iNKT cells might be modified or amplified by a variety of other receptors that are expressed by these cells. For example, IL-12, OESTRADIOL and stimulatory NK1.1-specific antibodies favour IFN-γ production by iNKT cells, whereas IL-7, IL-18, and CD86-specific and CD154 (CD40 ligand)-specific blocking antibodies favour IL-4 production by iNKT cells [57, 58, 59].

Furthermore, signalling through the MHC-class-I-specific receptors of the LY49 family dampens iNKT-cell responses. However, although various reagents can modify the cytokine-production profile of iNKT cells, so far, attempts to permanently polarize iNKT cells for selective TH1- or TH2-cytokine production have failed. Consistent with their capacity to produce copious amounts of cytokines, iNKT cells have been implicated in a myriad of immune responses, including responses to pathogens, tumours, tissue grafts, allergens and autoantigens.

In most cases, however, the mechanisms responsible for iNKT-cell activation and the downstream events that result in immune

modulation remain elusive. In some systems, the capacity of iNKT cells to produce TH1 cytokines seems to be important, whereas in others TH2-cytokine production seems to be crucial. Although much remains to be learned about the immunological functions of iNKT cells, the available evidence indicates that iNKT cells can sense endogenous ‘DANGER’ SIGNALS to help initiate adaptive immune responses and to regulate ongoing immune responses [60].

As such, iNKT cells straddle the innate and adaptive immune systems, a property that they share with other cell types that have been referred to as innate B and T cells. A potential link between iNKT cells and autoimmunity was revealed by the finding that, compared with non-autoimmune mouse strains, various mouse strains — including NON-OBESE DIABETIC (NOD) MICE, MRL *lpr*/ *lpr* mice and SJL/J mice — that are genetically susceptible to autoimmunity have reduced numbers of iNKT cells, which have defective functions [61]. Defective iNKT-cell function in these mouse strains seems to be genetically determined, and at least in the case of NOD mice, iNKT-cell defects are associated with increased susceptibility to autoimmunity. In addition, in some experimental models of autoimmunity, depletion of iNKT cells by breeding with CD1d- or Jα18-TCRdeficient mice resulted in disease exacerbation.

Conversely, restoration of iNKT-cell numbers by adoptive transfer of iNKT cells or by transgenic overexpression of the

iNKT-cell TCR ameliorated disease. Consistent with these findings, defects in iNKT-cell numbers and functions have been observed in patients with a wide variety of autoimmune diseases. However, whether these defects in human iNKT cells are genetically determined or are a consequence of disease remains controversial. Nevertheless, the connection between iNKT cells and autoimmunity raised the possibility of exploiting the immunomodulatory properties of these cells for therapeutic intervention in autoimmune diseases. *[Adapted from: Van Kaer; Nat. Rev. Imm. 5, 31-42 (2005)]*

IMMUNOMODULATORY ACTIVITIES OF α-GALCER

α-GalCer was originally discovered by the Pharmaceutical Division of the Kirin Brewery Company during a screen for reagents derived from the marine sponge *Agelas mauritianus* that prevent tumour metastases in mice. In most studies, a synthetic analogue, KRN7000 , of this natural product has been used and is usually still referred to as α-GalCer. Interestingly, reactivity with α-GalCer is not restricted to iNKT cells from mice but also includes iNKT cells from humans macaques and rats [62].

The response of iNKT cells to stimulation with α-GalCer is characterized by the rapid production of various cytokines, including IFN-γ, tumour-necrosis factor, OSTEOPONTIN, IL-2, IL-4, IL-10, IL-13, granulocyte/macrophage colony-stimulating factor and transforming growth factor-β (TGF-β) [63,64]. The *in vivo* fate of iNKT cells after the administration of α-GalCer to mice has been an issue of considerable contention. Until recently, it was thought that, soon after α-GalCer administration to mice, most iNKT cells succumb to ACTIVATION-INDUCED CELL DEATH (AICD).However, it is now clear that, shortly after α-GalCer administration, iNKT cells downregulate crucial cell-surface receptors (that is, TCR and NK1.1), rendering them invisible to flow-cytometric detection with reagents such as TCR-specific antibodies, NK1.1- specific antibodies and α-GALCER–CD1d TETRAMERS [65]. Downregulation of TCR

expression by iNKT cells was observed within 2 hours of α-GalCer injection; however, it was only transient, and relatively normal levels were restored after 24 hours. Downregulation of NK1.1 expression became evident in 8–12 hours after α-GalCer injection and was maintained for at least 2 weeks (**Figure 6**).

Indeed, rather than rapidly undergoing AICD, it is now clear that iNKT cells that are activated in this manner rapidly proliferate and clonally expand 10- to 15-fold in the spleen and 2- to 3-fold in the liver at about day 3 after α-GalCer injection. After this period of marked population expansion, most iNKT cells die, so homeostatic levels are restored in 1–2 weeks after α-GalCer injection. Although α-GalCer-activated iNKT cells produce multiple cytokines in a period of several days, the initial response has been shown to be dominated by IL-4 production and then to shift towards dominant IFN-γ production at 24 hours, with little cytokine secretion after day 3 [66]. So, in mice, the response of iNKT cells to stimulation with α-GalCer is characterized by transient TCR downregulation and sustained NK1.1 downregulation, a specific pattern of cytokine secretion, clonal expansion and homeostatic contraction (**Figure 6**). Recently, similar observations have been made in humans injected with α-GalCer or α-GalCer-pulsed dendritic cells (DCs), indicating that the *in vivo* response of human iNKT cells to α-GalCer resembles that of their mouse counterparts.

In vivo activation of iNKT cells with α-GalCer results in the subsequent activation of several cell types, including NK cells, neutrophils, macrophages, DCs, B cells and conventional T cells (**Figure 7**). In turn, many of these cells secrete cytokines that can further modulate immune responses. In this way, the administration of α-GalCer results in a complex array of immune reactions. Collectively, these interactions explain the waves of cytokines that are detected in the serum of mice after administration of α-GalCer: IL-4, mainly produced by iNKT cells themselves, peaks at 2 hours; IL-12, mainly produced by antigen-presenting cells (APCs), such as DCs, peaks at 6 hours; and IFN-γ, mainly produced by NK cells, peaks at 24 hours [67, 68]. There is considerable evidence for crosstalk between iNKT cells and other cell types (**Figure 7**).

For example, the interaction of iNKT cells with α-GalCer–CD1d complexes at the cell surface of DCs results in the ligation of CD40 molecules on DCs with their ligand, CD154, on iNKT cells. These interactions stimulate the production of IL-12 by DCs, which in turn increases the production of cytokines by iNKT cells. Immunization with α-GalCer also influences the TH1/TH2 balance of ongoing immune responses. Most studies have provided evidence that α-GalCer promotes TH2 responses, at least in mouse strains such as C57BL/6, BALB/c and NOD. In many cases, deviation to a TH2 response required repeated injections with α-GalCer, but in some studies, even a single injection of α-GalCer was effective in inducing a

significant increase in serum levels of IgE7. Little is known about the response of humans to α-GalCer. However, results from recent phase I trials have shown that *in vivo* activation of human iNKT cells with α-GalCer results in the subsequent activation of NK, B and T cells, indicating that responses are similar to those observed in mice [69].

Although most evidence indicates that iNKT cells lack immunological memory, one report has provided evidence that immunization of mice with the ganglioside GD3 clonally expands a subset of iNKT cells that are reactive to both GD3 and α-GalCer. By contrast, administration of a single dose of α-GalCer to mice fails to induce a clonally expanded pool of longlived α-GalCer-reactive iNKT cells (**Figure 6**).

Interestingly, α-GalCer restimulation of splenocytes from mice that were injected 1–2 weeks earlier with α-GalCer typically results in a blunted response, particularly with regard to IFN-γ production, compared with the response of splenocytes from naive mice. We have recently found that this blunted recall response results from the induction of long-term hyporesponsiveness or anergy in iNKT cells, with respect to the capacity of these cells to proliferate, produce cytokines and transactivate other cell types. Because these anergic iNKT cells have increased susceptibility to AICD, it is perhaps not surprising that chronic administration of α-GalCer to mice results in substantial depletion of peripheral iNKT cells. A CD95

(FAS)–CD95-liganddependent mechanism is probably responsible for this iNKT-cell depletion, because repeated administration of α GalCer to CD95-deficient MRL-*lpr/lpr* mice leads to clonal expansion of iNKT-cell populations rather than to depletion. A recent study has further shown that depletion of iNKT cells after chronic treatment with α-GalCer is followed by thymus-dependent repopulation of iNKT cells with increased expression of inhibitory MHC-class-I-specific receptors of the LY49 family. These reconstituted iNKT cells were hyporesponsive to challenge with α-GalCer [70].

KRN7000, the synthetic α-GalCer analogue that has been used in most experimental studies, is a high-affinity ligand for the iNKT-cell TCR.The immunomodulatory properties of several other α-GalCer analogues have been investigated.
OCH differs from KRN7000 by having a substantially shortened sphingosine chain. OCH is a less potent stimulator of iNKT-cell responses than KRN7000: it fails to induce significant clonal expansion of iNKT cells *in vivo*, favours the production of IL-4 by iNKT cells and is more effective than KRN7000 in promoting TH2-cell responses in mice [71].

Deviation of the iNKT-cell response to a TH2 response seems to be a general consequence of making truncations in the acyl or sphingosine chains of KRN7000.Conversely,α-C-GalCer, a C-glycoside (carbon glycoside) analogue of α-GalCer, activates iNKT cells at very low concentrations and promotes TH1

responses *in vivo*. Although β-anomeric GalCer molecules cannot stimulate iNKT-cell hybridomas *in vitro*, when β-GalCer is administered at high doses to mice, it can activate iNKT cells *in vivo* with the one caveat that potential contamination with α-anomeric GalCer has not been excluded [72].

Because of their marked effects on the immune response, KRN7000 and related α-GalCer compounds have received considerable interest for development as vaccine adjuvants and for treatment of various pathologies, including cancer, infections, and autoimmune and inflammatory conditions. In light of the many situations that have been studied, here, I focus on the preclinical studies that have evaluated the impact of treatment with α-GalCer on autoimmune diseases. *[Adapted from: Van Kaer; Nat. Rev. Imm. 5, 31-42 (2005)]*

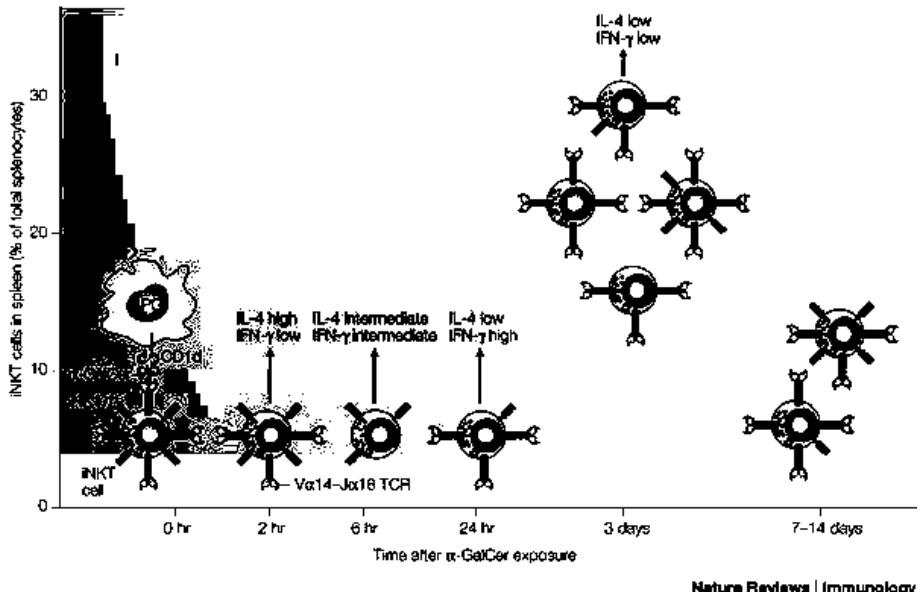

Figure 6: The *in vivo* response of iNKT cells to stimulation with α-GalCer. Soon after administration of α-GalCer to mice, this reagent binds CD1d at the cell surface of antigen-presenting cells and is presented to iNKT cells. Within hours, iNKT cells become activated and start to secrete IL-4 and IFN-γ. α-GalCer-activated iNKT cells also rapidly downregulate cell-surface-expressed T-cell receptor (TCR) and NK1.1, rendering these cells undetectable with flow-cytometric reagents that bind these markers. TCR expression levels return to almost normal at 24 hours after injection of α-GalCer. iNKT cells then rapidly proliferate, clonally expanding 10- to 15-fold in the spleen and less extensively in other organs. Most iNKT cells subsequently die, to maintain homeostatic numbers. Early during the response to α-GalCer, iNKT cells mainly produce IL-4. At 24 hr, these cells mainly produce IFN-γ, and at 3 days after injection of α-GalCer, when iNKT-cell numbers are maximal, these cells produce few cytokines. *[Adapted from: Van Kaer; Nat. Rev. Imm. 5, 31-42 (2005)]*

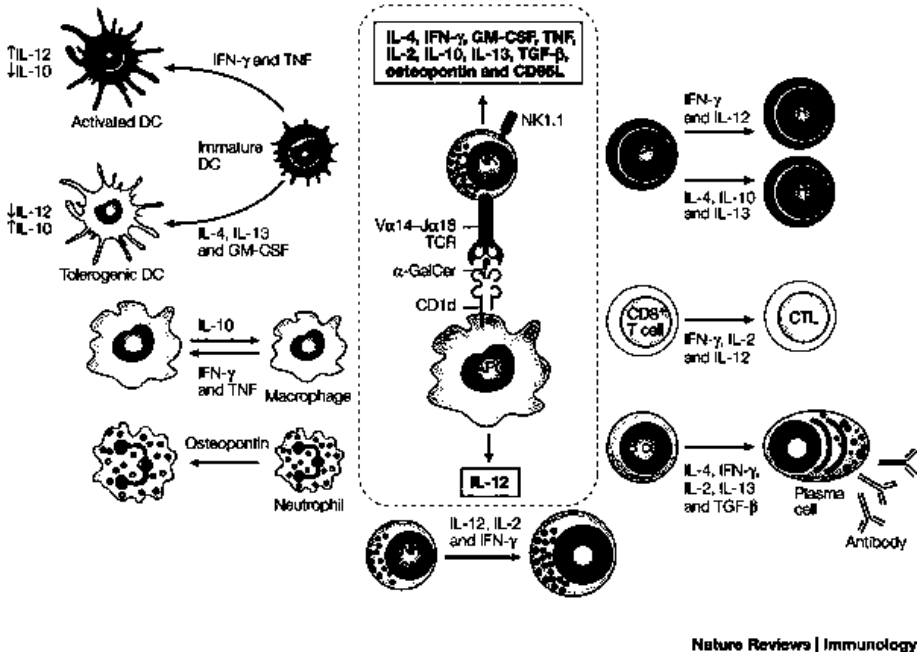

Figure 7: Capacity of α-GalCer-activated iNKT cells to modulate innate and adaptive immunity. Injection of mice with α-GalCer results in the CD1d-restricted presentation of α-GalCer by antigen-presenting cells (APCs) to iNKT cells. Reciprocal interactions between iNKT cells and APCs lead to the production of various immunomodulatory cytokines, including the production of IL-12 by APCs and the production of IL-4, IFN-γ, GM-CSF, TNF, IL-2, IL-10, IL-13, TGF-β and osteopontin by iNKT cells. These cytokines influence both innate and adaptive immunity, including the differentiation of CD4+ T cells into TH1 and TH2 cells, the differentiation of CD8+ T cells into cytotoxic T lymphocytes (CTLs), the differentiation of B cells into immunoglobulin-secreting plasma cells, the activity of NK cells, the function of macrophages and neutrophils, and the differentiation of immature dendritic cells (DCs) into activated or tolerogenic DCs, which produce high levels of IL-12 and IL-10, respectively. *[Adapted from: Van Kaer; Nat. Rev. Imm. 5, 31-42 (2005)]*

MODULATION OF AUTOIMMUNITY WITH α-GALCER

The capacity of α-GalCer to modulate autoimmunity has been tested in several experimental models of autoimmunity in mice .

Type 1 diabetes.

Type 1 diabetes is a form of diabetes that is caused by selective, immune-mediated destruction of pancreatic β-cells in the islets of Langerhans. Diabetes in NOD mice is the most tractable experimental model for studies of type 1 diabetes [73]. In this animal model, disease incidence is more prevalent in female than in male animals. The development of diabetes in NOD mice is initiated by infiltration of lymphocyte into the islets (that is, INSULITIS), at 4–5 weeks of age, and most (80–90%) female animals develop overt diabetes by 30 weeks of age.
Several studies have evaluated the capacity of α-GalCer to modulate the progression of diabetes in NOD mice. Repeated treatment of young female NOD mice with α-GalCer (KRN7000) prevents the development of spontaneous diabetes in 50–90% of the treated animals, depending on the precise treatment protocol used.

Disease protection seemed to be most effective when mice were treated chronically (once or twice per week, throughout the experiment) with relatively high doses of α-GalCer (100 micrograms per kilogram of body weight). Although treatment

with α-GalCer showed some efficacy when initiated after the onset of insulitis, it was most effective when initiated in young animals, before the development of marked insulitis [74].
This indicates that α-GalCer either inhibits the generation of pathogenic T cells or induces the generation of regulatory T cells that keep the pathogenic T cells 'at bay'. Consistent with this possibility, α-GalCer treatment of young NOD animals had relatively little effect on the development of insulitis, and splenocytes from protected mice could not induce disease when adoptively transferred to naive NOD–SCID (severe combined immunodeficient) mice. Treatment with α-GalCer also protected mice against the recurrence of diabetes after transplantation of syngeneic islets from pre-diabetic animals to recently diabetic mice [75].

Treatment with α-Galcer was also effective in the cyclophosphamide-enhanced model of diabetes in NOD mice. Although the precise mechanism by which cyclophosphamide accelerates diabetes remains unclear, it has been suggested that this compound depletes regulatory T cells. Interestingly, in this model, the efficacy of treatment with α-GalCer was increased by co-injection of IL-7, which favours IL-4 production by iNKT cells, indicating a role for deviation to a TH2 response. Consistent with these findings, two studies provided evidence that prevention of diabetes in the spontaneous model correlated with a relative shift of the islet-antigen-specific T-cell response from TH1- to TH2- cytokine production and with suppressed

islet-antigenspecific antibody responses. However, two other studies could not find evidence for deviation to a TH2 response. In addition, conflicting findings have been obtained regarding the role of IL-4 and IL-10 in disease protection. In support of the idea of deviation to a TH2 response, one study reported that α-GalCer cannot prevent spontaneous diabetes in IL-4-deficient NOD mice, and another study reported that neutralization of IL-10 abrogates the protective effect of α-GalCer in cyclophosphamide-induced diabetes [76]. However other studies reported that α-GalCer can prevent cyclophosphamide-induced disease in IL-10-deficient NOD mice, as well as spontaneous disease in IL-4- deficient mice and mice deficient in both IL-4 and IL-10. Although the reasons for these conflicting findings are unclear, results from the latter studies imply that the requirement for IL-4 and IL-10 is not absolute and indicate that mechanisms other than deviation to a TH2 response are involved. An attractive possibility is that α-GalCer promotes the differentiation of regulatory T cells that are distinct from TH2 cells. However, treatment with α-GalCer could not induce clonal expansion of CD4+CD25+ cells in the spleen or enhance the capacity of splenic CD62L+ cells to inhibit diabetogenic T cells in co-transfer models, indicating that regulatory T-cell subsets that are identified by these surrogate markers are not involved. Nevertheless, because treatment with α-GalCer results in the recruitment and accumulation of iNKT cells in pancreatic islets and lymph nodes, it is probable that regulatory mechanisms that are involved in diabetes

suppression are active at the site of autoimmune attack. This possibility has been supported by another study, which showed that treatment with α-GalCer promotes the emergence of tolerogenic DCs in the pancreatic lymph nodes [77]. These tolerogenic DCs had reduced capacity to produce IL-12 and could prevent disease when adoptively transferred to pre-diabetic NOD mice. Collectively, these findings indicate a role for deviation to a TH2 response and induction or recruitment of tolerogenic DCs in the prevention of diabetes that is mediated by α-GalCer.

Experimental allergic encephalomyelitis.

The capacity of α-GalCer to modulate EXPERIMENTAL ALLERGIC (AUTOIMMUNE) ENCEPHALOMYELITIS (EAE) has been evaluated in both monophasic and relapsing–remitting models of this disease. α-GalCer could prevent disease induced with central nervous system (CNS) proteins or peptides in B10.PL, PL/J and C57BL/6 mice, but it had little effect, or even exacerbated disease, in a relapsing remitting model of EAE in SJL/J mice. In addition, the dose, timing, route and frequency of α-GalCer injections all influenced treatment efficacy [78].

For example, one group of investigators showed that giving a single intraperitoneal injection of α-GalCer before induction of EAE with a myelin basic protein (MBP)-derived peptide was effective in preventing disease in B10.PL mice, but α-GalCer

increased disease when injected at the time of immunization with MBP-derived peptide. However, other studies showed that injection of α-GalCer at the time of EAE induction was equally effective, as long as α-GalCer was administered subcutaneously and mixed together with the immunizing CNS antigen. OCH, which favors IL-4 production by iNKT cells, was more effective than α-GalCer in preventing EAE in C57BL/6 mice, and OCH showed some efficacy even when treatment was initiated several days after EAE induction. OCH was also effective when administered orally, which is the favoured treatment route for humans. In most studies, disease prevention afforded by α-GalCer or OCH correlated with a blunted TH1 and/or an augmented TH2 response to the immunizing CNS antigen, and vice versa, disease exacerbation correlated with increased TH1 and/or reduced TH2 responses. These findings were supported by studies using IFN-γ-, IL-4- or IL-10-deficient mice, IL-4-neutralizing antibodies or CD86-specific blocking antibodies, indicating a crucial role in disease prevention for TH2 cytokines produced by iNKT cells and/or regulatory T cells. This crucial role of TH2 cytokines in protection against disease also provides a potential explanation for the effects of α-GalCer on MBP-induced EAE in SJL/J mice (that is, increased disease), as this mouse strain has low numbers of iNKT cells, which have a reduced capacity to produce IL-4. One study, however, found that neutralization of IFN-γ but not IL-4 abrogated the disease protection that is conferred by α-GalCer in C57BL/6 mice, indicating that IFN-γ production by iNKT cells

and/or other cell types might also have a role [79, 80]. Taken together, these studies, with one notable exception, indicate that deviation to a TH2 response has a crucial role in the ability of α-GalCer to prevent EAE.

Arthritis.

Repeated (twice per week) administration of α-GalCer to C57BL/6 mice that have been immunized with collagen type II moderately protected these animals against COLLAGEN-INDUCED ARTHRITIS (CIA). However, similar treatment of collagen-immunized SJL/J mice had no impact on the development of arthritis. By contrast, OCH was effective in protecting both C57BL/6 and SJL/J mice against the development of CIA. Interestingly, OCH had some efficacy even when treatment was initiated after the onset of CIA [81]. Disease protection correlated with the capacity of OCH to promote the production of TH2 cytokines by iNKT cells and to bias collagen-specific T cells for production of TH2 cytokines. Consistent with these findings, neutralization of IL-4 and IL-10 abolished disease prevention by OCH.

Systemic lupus erythematosus.

The effects of α-GalCer on various manifestations of SYSTEMIC LUPUS ERYTHEMATOSUS (SLE) have been investigated in both hereditary and induced models of this

disease. Repeated administration of α-GalCer to MRL-*lpr/lpr* mice, a hereditary model of SLE, alleviated inflammatory dermatitis but did not influence kidney disease. In these animals, which have reduced baseline levels of iNKT cells, repeated injection of α-GalCer resulted in marked clonal expansion of iNKT-cell populations and increased levels of serum IgE, indicating the involvement of deviation to a TH2 response as a mechanism of α-GalCer-induced suppression of SLE dermatitis. Dichotomous results of treatment with α-GalCer have been observed in the NZB/W mouse hereditary model of SLE. Treatment of young NZB/W mice resulted in amelioration of SLE symptoms, but treatment of older animals resulted in disease exacerbation. Disease exacerbation correlated with induction of TH1 responses in the old NZB/W mice, which is in contrast to the TH2 responses that were induced in the young NZB/W mice and in most other mouse strains. Furthermore, α-GalCer protects BALB/c mice against SLE that is induced by the hydrocarbon oil pristane but exacerbates pristane-induced disease in SJL/J mice [82]. Disease protection in BALB/c mice correlated with a mixed TH1 and TH2 cytokine-production profile of iNKT cells and the tendency of α-GalCer to promote TH2 responses in this mouse strain. Conversely, disease exacerbation in SJL/J mice correlated with a TH1 bias in the cytokine profile of iNKT cells and the tendency of α-GalCer to promote TH1 responses in these animals [83]. So, consistent with the findings obtained using the disease models discussed here, the capacity of α-GalCer to prevent or promote SLE-like

autoimmunity might be associated with its effects on the TH1/TH2 balance of autoantigen-specific immune responses.

Inflammatory colitis.

Repeated injection of α-GalCer moderately protected C57BL/6 mice against the development of experimental COLITIS induced by dextran sodium sulphate [84]. Although the mechanism for this protection was not investigated, the authors speculated, on the basis of the pathogenesis of dextran-sodium-sulphate-induced colitis, that regulatory cytokines such as IL-10 might be involved.

Graves' thyroiditis.

The capacity of α-GalCer to modulate the development of GRAVES'THYROIDITIS was evaluated in BALB/c mice treated with a non-replicative adenoviral vector encoding the thyroid-stimulating-hormone receptor (TSHR) [85]. Simultaneous administration of α-GalCer and the TSHR-encoding adenovirus suppressed TSHR specific IFN-γ production and protected mice against Graves' disease, with a concomitant reduction in TSHR specific antibodies. However, in situations in which an anti-TSHR immune response had already developed, α-GalCer was ineffective at preventing disease. *[Adapted from: Van Kaer; Nat. Rev. Imm. 5, 31-42 (2005)]*

MODEL OF α-GALCER ACTION

With notable exceptions, most of the studies that have investigated the mechanism of action of α-GalCer on the progression of autoimmunity have provided evidence for a crucial role of deviation to a TH2 response in disease protection. Furthermore, in cases in which treatment with α-GalCer resulted in disease exacerbation, deviation to a TH1 response was usually implicated. Deviation to a TH2 response is a well-known mechanism for protection against TH1-dominant autoimmunity. In addition to suppressing pathogenic TH1 cells, regulatory TH2 cells might also suppress the development and function of pathogenic CD8+ T cells and suppress the production of pathogenic antibodies by B cells. In the case of α-GalCer treatment, deviation to a TH2 response might result from several mechanisms (**Figure 8**). First, IL-4, which is rapidly produced after administration of α-GalCer and is maintained at considerable levels during chronic treatment with α-GalCer, is well known for its capacity to promote TH2 responses [86].

Second, chronic production of IL-4, as a result of repeated injections with α-GalCer, might cause anergy or apoptosis of self-reactive TH1 cells. Third, IL-13, which is also produced by iNKT cells after activation by α-GalCer, also promotes TH2 responses. Fourth, chronic production of IL-10 by iNKT cells might contribute to suppression of TH1 responses. Fifth,

although the role of IFN-γ in the protective effects of α-GalCer on autoimmunity remains controversial, it has recently been shown that IFN-γ, at least in some cases, also contributes to the priming of TH2 responses, which might provide an explanation for some of the apparently conflicting reports about the mode of action of α-GalCer. Sixth, there is considerable evidence that α-GalCer promotes the generation of tolerogenic DCs. Such tolerogenic DCs might promote the priming of nondestructive TH2 responses and suppress the development of pathogenic TH1 responses [87, 88]. So, various mechanisms might contribute to the deviation to a TH2 response of autoantigen-specific T cells during treatment with α-GalCer.

Although deviation to a TH2 response is often observed during experimental manipulations that protect against autoimmune diseases, it is sometimes an outcome, rather than the cause, of disease protection. Because there is currently no direct evidence that deviation to a TH2 response is responsible for the protective effects of α-GalCer against TH1-dominant autoimmunity, additional mechanisms might be involved. In many of the preclinical studies that have been carried out, treatment with α-GalCer did not completely abolish the development of autoantigen specific TH1 cells, indicating that pathogenic T cells might be kept at bay by active mechanisms of suppression. Recent studies have shown an important role for regulatory T-cell subsets — such as TGF-β- producing TH3 cells, IL-10-producing T regulatory 1 (TR1) cells, CD8+

regulatory T cells, CD4+CD25+ T cells and CD4+CD62L+ T cells in controlling self tolerance. Although no evidence was found for a role for splenic CD4+CD25+ and CD62L+ cells in the capacity of α-GalCer to prevent diabetes in NOD mice, it remains possible that these and other regulatory T cells are enriched in the organs targeted by the autoimmune-disease process and/or the lymph nodes that drain these organs. Evidence in the diabetes model has further shown that iNKT cells are recruited to the site of autoimmune attack, possibly in response to locally produced inflammatory chemokines. In turn, cytokines and chemokines that are produced by locally activated iNKT cells might promote the generation and recruitment of regulatory T cells to target organs [89]. Furthermore, tolerogenic DCs, which accumulate at the site of autoimmune attack, might further promote the development of regulatory T cells. Consequently, the prevention of autoimmunity afforded by α-GalCer probably involves various TH2-dependent and -independent mechanisms (**Figure 8**).

The precise contribution of each of these mechanisms to disease protection might depend on the particular disease model, the genetic background of the mice and the precise treatment protocol (for example, single dose, multiple dose or chronic administration). *[Adapted from: Van Kaer; Nat. Rev. Imm. 5, 31-42 (2005)]*

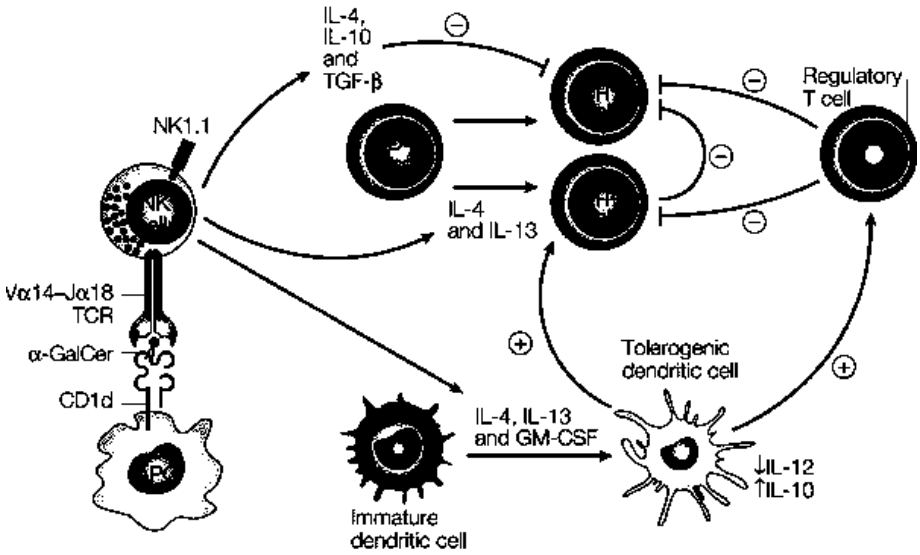

Figure 8: Proposed mechanism for the capacity of α-GalCer to modulate TH1-dominant autoimmunity. Soon after the injection of mice with α-GalCer, this reagent is presented at the cell surface of APCs in the context of CD1d molecules to iNKT cells. Primary injection of α-GalCer results in the production of various TH1 and TH2 cytokines by iNKT cells and other cell types. Repeated or chronic administration of α-GalCer, however, results in markedly suppressed TH1-cytokine production but sustained TH2-cytokine production. The TH2 cytokines IL-4 and IL-13 directly promote the differentiation of autoantigen-specific T cells into TH2 cells, whereas IL-4, IL-10 and TGF-β suppress pathogenic TH1 cells. IL-4, IL-13 and

granulocyte/macrophage GM-CSF promote the differentiation of immature DCs into tolerogenic DCs that produce IL-10 but little IL-12. In turn, tolerogenic DCs promote the differentiation of CD4+ T cells into TH2 cells and the generation of regulatory T cells that suppress autoantigen-specific immune responses. Some of these interactions might occur in local lymphoid organs or at the site of autoimmune attack, with the net result being suppression of pathogenic TH1 cells and deviation to a TH2 response. The

precise contribution of each of these proposed mechanisms to disease protection probably depends on a variety of parameters, including the α-GalCer-treatment protocol, the autoimmune disease model and the genetic background of the mice. TCR, T-cell receptor. *[Adapted from: Van Kaer; Nat. Rev. Imm. 5, 31-42 (2005)]*

DENDRITIC CELLS

Dendritic cells (DCs) constitute a heterogenous population of professional, bone-marrow-derived antigen presenting cells (APCs). They are derived from multiple lineages, have distinct stages of cell development, activation and maturation, and have the potential to induce both immunity and tolerance. Under steady-state conditions, they exist as conventional DCs (cDCs) or precursor DCs (pre-DCs); the latter are able to rapidly adapt their morphology and function in response to stimuli from the extracellular environment. The ability of DCs to respond to endogenous and exogenous danger signals links their crucial roles in innate and adaptive immunity [90].

In mice, three subsets of cDCs (CD11chi) have been identified in the spleen and lymph nodes: CD8α+ DCs, CD4+CD8– DCs and CD4–CD8– DCs. In the steady state, CD8α+ DCs constitute 20–30% of the CD11chi DC population in the spleen, are semi-mature APCs (CD86mid) and reside within the T-cell areas4. The remaining CD8– DCs are immature, located mostly in the marginal zone and throughout the red pulp of the spleen, and migrate to the T-cell areas in response to stimulation by microbial products. Lymph nodes contain two other DC subsets skin-derived DCs (known as Langerhans cells) and tissue interstitial DCs that arrive from the periphery through the lymphatic circulation5. The thymus is mostly populated by CD8α+ DCs. The initial misconception that mouse CD8α+ DCs were derived exclusively from lymphoid-committed

haematopoietic precursors, whereas CD8α– DCs arose only from myeloid-restricted precursors, led to the designation of CD8α+ and CD8α– DCs as lymphoid- and myeloid-related DCs, respectively.

The term ‘CD8α+ DC’ applies only to mice, as human DCs do not express CD8. The best-studied human DCs are those derived from monocytes; several subtypes of human DCs, including myeloid and plasmacytoid DCs (pDCs) and epidermal Langerhans cells, have been described *in vivo* and in vitro [91]. In recent years pDCs have been identified in mice. They do not express T-, B- or myeloid-lineage markers, but exhibit plasmacell- like morphology in the steady state, and are of bone marrow origin. DCs can be isolated fresh from blood, lymphoid or non-lymphoid organs, or generated *in vitro* from haemato poietic or more immediate DC precursors of different sources. Different subsets of freshly isolated or *in vitro*-generated human, mouse or rat DCs have been used extensively to study the role of DCs as modulators of alloreactive and autoreactive T-cell responses. DCs offer potential as therapeutic tools to ameliorate or prevent graft rejection or graft-versus-host disease (GVHD), or to treat autoimmune disorders. DC tolerogenicity is not specific to a DC subset or restricted to the immature state of the APC. Tolerogenic DCs present antigen to antigen specific T cells, but fail to deliver adequate co-stimulatory signals (or deliver net co-inhibitory signals) for effector T-cell activation and proliferation. This may be

manifested as T-cell death, T-cell anergy or regulatory T-cell expansion or generation. DCs that can induce tolerance may need to be resistant to maturation-inducing factors and resident in (or capable of migration to) lymphoid tissues [92].

Although it is well recognized that thymic DCs induce tolerance to self antigens *in vivo* through the clonal deletion of CD4+CD8+ thymocytes, and that tolerogenic DCs have been shown to suppress experimental autoimmune disease much of the literature on DC tolerogenicity has focused on alloimmunity. Transplantation offers the opportunity to target or manipulate DCs before or after initiation of the immune response. Also, uniquely to transplantation, both the direct and indirect pathways of antigen recognition provide potential targets for tolerogenic DCs to promote improved outcomes. A profile of what might constitute a tolerogenic DC has emerged and is described in **BOX 1**. DC manipulation to promote tolerance During the past decade, numerous studies [93, 94] have unveiled mechanisms by which different subsets of DCs induce or maintain self-tolerance *in vivo*. Some of these mechanisms have been exploited for the generation of tolerogenic DCs *in vitro* that, when transferred, induce donor-specific tolerance for therapeutic purposes. *[Adapted from: Morelli; Nat. Rev. Imm. 7, 610-621 (2007)]*

Box 1 | What constitutes a tolerogenic dendritic cell?

- The ability to acquire and present antigen to antigen-specific T cells (either through direct, indirect or semi-direct presentation)
- Low constitutive expression of surface MHC molecules and low net expression of co-stimulatory molecules — for example, CD80 and CD86 compared with PDL1 (programmed cell death ligand 1)
- Low production of interleukin-12p70 (IL-12p70) and high IL-10 and indoleamine 2,3-dioxygenase (IDO) production
- Resistance to maturation in response to 'danger signals,' such as high-mobility group box 1 protein (HMGB1), Toll-like receptor (TLR) ligands or CD40 ligation
- The ability to generate, select and/or expand alloantigen specific, naturally occurring or adaptive regulatory T cells
- The ability to promote apoptotic death of effector T cells
- The ability to respond to regulatory T cells by upregulating the expression of inhibitory molecules (such as IL-10 or IDO) to provide an inhibitory feedback loop
- The ability to migrate to T-cell areas in secondary lymphoid tissue through the expression of relevant surface chemokine receptors and a responsiveness to cognate ligands

- In vivo longevity; resistance to innate natural killer (NK)-cell mediated or T-cell mediated killing
- Sustained expression of immunosuppressive transgene products (genetically modified dendritic cells)

GENERATION OF TOLEROGENIC DENDRITIC CELLS

Tolerogenic DCs are immature, maturation-resistant or alternatively activated DCs that express surface MHC molecules, have a low ratio of co-stimulatory to inhibitory signals, and an impaired ability to synthesize T helper 1 (TH1)-cell-driving cytokines (such as interleukin-12p70 (IL-12p70)) (BOX 1). In an effort to generate tolerogenic DCs, *in vitro*-propagated DCs have been manipulated through exposure to various anti-inflammatory and immunosuppressive agents. These agents target DC differentiation and function by various mechanisms and include IL-10 and transforming growth factor-β1 (TGFβ1), inducers of cyclic AMP (cAMP) such as prostaglandin E2, histamine, β2 agonists, neuropeptides, the vitamin D3 metabolite 1α,25-dihydroxyvitamin D3 (1α,25(OH)2D3) and its analogues, glucosamine, the antioxidant *N*-acetyl-l-cysteine, ligands for inhibitory immunoglobulin-like transcript receptors (such as the MHC class Ib molecule HLA-G), and cobalt protoporphyrin (to induce haem oxygenase-1 expression) [96, 97, 98].

In addition numerous, clinically approved or experimental immunosuppressive drugs such as corticosteroids, cyclosporine, tacrolimus, rapamycin, aspirin, deoxyspergualin (DSG), mycophenolate mofetil (MMF) and sanglifehrin A have been used to target DC differentiation and function. All these

molecules prevent DC maturation and/or activation or impair the capacity of DCs to produce IL-12p70. In addition, some of these agents prevent the nuclear translocation of nuclear factor-κB (NF-κB) family members, which are required for the differentiation of DCs [99].

Rapamycin is a potent immunosuppressive drug that, when complexed with its intracellular receptor FK506- binding protein 12, inhibits the downstream signaling from mTOR (mammalian target of rapamycin) proteins and impairs the maturation and T-cell allostimulatory function of DCs. Rapamycin-conditioned myeloid DCs are poor producers of the cytokines IL-12p70 and tumour-necrosis factor (TNF) and are resistant to maturation induction by Toll-like receptor (TLR) ligands or by signalling through CD40 [100]. When infused systemically into mice, they render allogeneic T cells hyporesponsive to further stimuli with donor antigen. Rapamycin-conditioned myeloid DCs can enrich for naturally occurring forkhead box P3 (FOXP3)+CD4+CD25+ regulatory T (TReg) cells, whereas their ability to expand CD4+ effector T cells is markedly impaired. Moreover, rapamycin-conditioned host DCs that are pulsed with donor antigen and administered to the host before transplantation, followed by a short post-operative course of minimally effective rapamycin, prolongs heart-graft survival indefinitely. This is associated with the infiltration of the graft by TReg cells and with the absence of graft vessel disease. LF15-0195, an analogue of the immunosuppressive drug DSG, inhibits the maturation of bone-

marrow derived DCs *in vitro*. Min *et al.* have reported that the numbers of (tolerogenic) immature DCs and TReg cells are increased in a mouse heart transplant tolerance model by the administration of LF15-0195 and CD45RB-specific antibodies. *In vitro* studies indicate that DCs from tolerant recipients prompt the generation of TReg cells from naive T cells, and that Treg cells from the same recipients can induce tolerogenic DCs, thereby initiating a tolerogenic 'feedback inhibition loop' [101, 102].

Several groups have found that 1α,25(OH)2D3, the active form of vitamin D3, inhibits the differentiation and maturation of DCs *in vitro*. In addition, a combination of 1α,25(OH)2D3 and the corticosteroid dexamethasone induces human and mouse naive T cells to differentiate into IL-10-producing regulatory T cells. The tolerogenic properties of DCs induced by 1α,25(OH)2D3 treatment in combination with the immunosuppressive drug MMF have also been shown *in vivo* by the induction of tolerance to mouse pancreatic islet allografts following the administration of donor-derived DCs treated with 1α,25(OH)2D3 and MMF [103]. This tolerogenic effect was associated with the induction of CD4+CD25+ TReg cells.

Signalling in DCs through the inhibitory immunoglobulin- like transcript 4 (ILT4) receptor in humans and its analogue paired immunoglobulin-like receptor B (PIRB) in mice downregulates the function of DCs. Incubating human DCs with soluble HLA-G

(the natural ligand for ILT4) impairs APC maturation *in vitro*, and administering donor-derived DCs exposed to agonistic PIRB monoclonal antibodies prolongs skin-allograft survival in mice [104].

Advances in gene-transfer technology have resulted in the enhancement of the tolerogenic potential of DCs. These DCs are genetically manipulated to express 'immuno suppressive' molecules, which can inhibit or block the expression of cell-surface co-stimulatory molecules by genetically inducing the expression of IL-10, TGFβ1 or the cytotoxic T-lymphocyte antigen 4 (CTLA4)–immunoglobulin fusion protein; to prevent NF-κB activation by expressing a dominant negative form of IκB-kinase 2 (IKK2); to prevent the proliferation of allogeneic T cells through the expression of indoleamine 2,3-dioxygenase (IDO); to induce and maintain T-cell anergy (by expressing programmed cell antigen-specific T cells through the expression of CD95 ligand (CD95L) or TNF-related apoptosis-inducing ligand (TRAIL) (**Figure 9**).

To date, there have been no reports of achieving tolerance across MHC barriers using genetically modified DCs alone. Of note, a single, pre-transplant infusion of donor myeloid DCs that are treated with NF-κB specific 'decoy' oligodeoxynucleotides (ODNs) and transduced with a CTLA4–immunoglobulin-fusion protein markedly prolongs heart allograft survival. Recently, Hill *et al.* reported that mouse DCs in which the production of IL-12

was silenced by RNA interference showed reduced allostimulatory capacity and promoted TH2-cell differentiation in an antigen-specific manner. These genetically silenced DCs have yet to be tested in transplant models and the future of genetically engineered DCs as ‘negative cellular vaccines’ remains uncertain [105, 106]. *[Adapted from: Morelli; Nat. Rev. Imm. 7, 610-621 (2007)]*

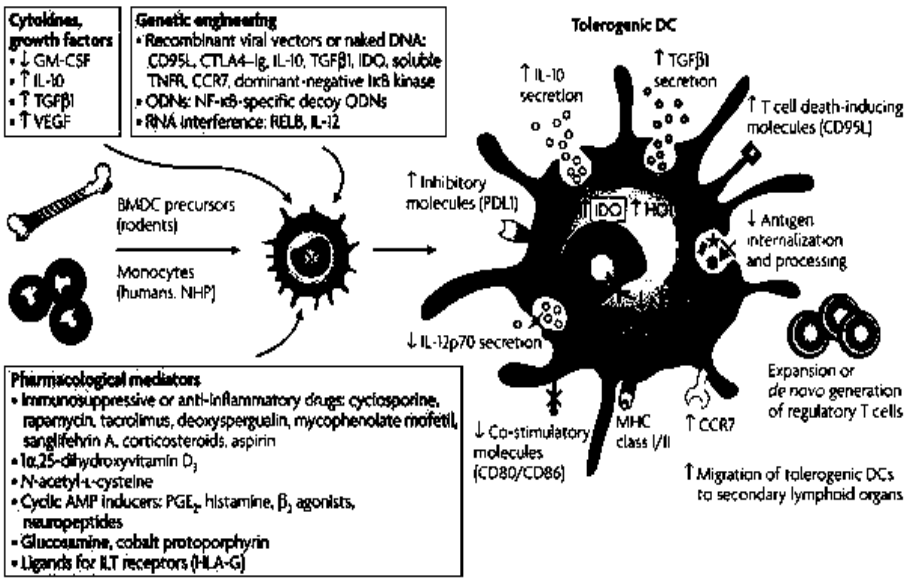

Figure 9: Generation of tolerogenic DCs *in vitro*. Dendritic cells (DCs) that are generated in vitro from bone marrow precursors (BMDC precursors) in rodents or blood monocytes in humans and non-human primates (NHP) have been rendered tolerogenic by controlling their culture conditions through exposure to cytokines, growth factors or pharmacological mediators, or by genetic engineering. DCs generated under such conditions can downregulate the outcome of the T-cell response by a single predominant function or, more frequently, by a combination of complementary mechanisms. *[Adapted from: Morelli; Nat. Rev. Imm. 7, 610-621 (2007)]*

TOLEROGENIC DCs SUBSETS

Until recently, the immature developmental stages of DC differentiation were believed to induce T cell anergy or Treg cells, whereas DC, which are transformed into mature DC by virtue of activation stimuli, were thought to represent immunogenic DC capable of inducing strong, primary T cell responses. This bimodal concept of immature versus mature DC has been challenged recently by studies demonstrating that bone marrow-derived, antigen-bearing mature DC can expand Treg cells with suppressive properties in vitro and in vivo.

Steady-state, immature myeloid DC

The first evidence that immature DC are able to induce tolerance derives from studies showing that peripheral CD4 T cells acquire regulatory properties after repeated in vitro stimulation with immature DC. These T cells were then able to block the proliferation of conventional effector T cells. This inhibition was mediated by cell– cell contact and was independent of soluble mediators [107]. These observations were confirmed in in vivo models with OVA or hen egg lysozyme (HEL) covalently linked to anti-DEC-205 antibodies and injected into mice. These antigen-antibody conjugates target the DC specific antigen receptor DEC-205, which mediates uptake and presentation to OVA- and HEL-specific T cells without further activating the DC in situ. The analysis of

the immune responses induced after targeting DC in the steady-state revealed that tolerance was obtained. Possible mechanisms included the disappearance of antigen-specific T cells and the induction of Treg cells. These mechanisms are not mutually exclusive, as induction of Treg cells was observed approximately 8 days after loading the DC in vivo, and T cell deletion was recorded 3 weeks after the original challenge of the DC [108].

Although the exact mechanism of action is not yet clear, the activation of these two pathways is corroborated further by additional results. For example, the in vivo occurrence of CD8 T cells with regulatory properties has been demonstrated after injection of immature DC. In contrast, direct “killing” of T cells by induction of apoptosis after contact with DC has been reported by Suss and Shortman. They showed that a certain subtype of DC expresses the Fas ligand (FasL), enabling these DC to kill Fas-bearing, activated T cells. Moreover, a subset of DEC-205/CD8 DC identified in mouse spleens was shown to be able to curb proliferation of CD8 and CD4 T cells in vitro [109].

Immature DC may function as “the police” of the immune system, which actively maintains tolerance to self-antigens derived from the processing and presentation of the apoptotic cell. In general, when DC encounter apoptotic cells, a noninflammatory and tolerogenic phenotype typical of immature DC is observed. For example, Stuart et al. showed that DC pretreated with apoptotic cells exhibited an impaired capacity to stimulate T lymphocytes. In addition, Kim et al. demonstrated

that phosphatidylserine from apoptotic cells induces regulation of DC activation. Downstream events following apoptotic cell contact or phosphatidylserine contact with DC resulted in the inhibition of IL-12p35 transcription and thus, IL-12p70 synthesis.

In addition to maintaining an immature phenotype, exposure to apoptotic cells induces the expression of IL-6 and IL-12p40, further enhancing the tolerogenic function of the DC. Consequently, differentiation of naıve T lymphocytes into type 1 effectors is suppressed.

Modulated, immature myeloid DC

Immature myeloid DC treated with agents, which inhibit their maturation, acquire tolerogenic activity. Such agents include vitamin D3, immunosuppressive drugs (such as cyclosporine), and IL-10, which inhibit differentiation, maturation, activation, and survival of DC in vivo and in vitro, leading to impaired activation of alloreactive T cells. The secretion of IL-12 and proinflammatory cytokines and the expression of costimulatory molecules are reduced significantly in these modulated DC, resulting in an impaired capacity of DC to induce allogeneic or autologous T cell activation [110].

The inhibitory effect of IL-10 on the APC function of DC is attributed to the down-regulation of MHC-class II and costimulatory molecules and to the inhibition of inflammatory cytokine synthesis inducing an antigen-specific anergy in

alloreactive and melanoma antigen-specific CD4 and CD8 T cells, and the anergic CD8 cells lose their capacity to lyse target tumor cells. Treatment of immature DC with TGF-b and IL-10 has been shown to have a similar effect, resulting in the generation of CD4 and CD8 Treg cells. It is important that DC with properties similar to the IL-10-modulated DC generated in vitro have also been detected in vivo in IL-10- producing tumors.

Treg cell-treated, immature myeloid DC

Human CD8 CD28 T cells are a distinct Treg cell population, which suppresses antigen-specific CD4 T cell responses by inhibiting their capacity to produce IL-2 and to up-regulate CD40L expression. Chang et al. found that human CD8 CD28 T cells can modulate the function of immature, monocyte-derived DC. These modulated DC do not express CD80 and CD86 and are able to anergize alloreactive memory CD4 T cells. This suppressive effect by CD8 CD28 T cell-treated DC is MHC-restricted and antigen-specific [111]. The induction of CD4 T cell anergy is not caused by a suppressor effect mediated by soluble factors but requires direct interactions between effector CD4 T cells and pretreated DC. The mechanism for generating tolerogenic DC has been analyzed extensively in this system. Exposure of immature DC to CD8 CD28 T cells in an antigen-specific manner results in interference with CD40–CD40L-mediated signaling, which normally induces the functional

maturation of DC and a high level of expression of CD80 and CD86. Moreover, the tolerogenic influence of CD8 CD28 T cells is associated with the induction of the inhibitory molecules Ig-like transcript 3 (ILT3) and ILT4 at the DC surface [28]. It is interesting that CD8 CD28 T cells, which can up-regulate these molecules in donor APC, are present in the circulation of human heart transplant recipients, especially in rejection-free patients [112].

As with CD8 CD28 Treg, CD4 CD25 Treg cells have been shown to act directly on DC in a cytokine-independent manner, inducing the up-regulation of the inhibitory receptors ILT3 and ILT4. These inhibitory receptors are crucial to the tolerogenic phenotype acquired by DC, as the suppressive effect of Treg cells on T cell proliferation can be abrogated by antibodies to ILT3 and ILT4.

Semimature myeloid DC

Lutz and Schuler have described semimature DC, which originate from the exposure of immature DC to tissue-derived TNF-a in the absence of a pathogenic motif. These cells acquire part of the characteristics of fully mature DC, including the expression of costimulatory molecules and the ability to migrate to the draining lymph nodes. However, they produce low levels of proinflammatory cytokines such as IL-1b, IL-6, TNF-a, and IL-12p70. Semimature DC lack the capacity to

produce polarizing signals and as a consequence, predominantly drive the development of adaptive Treg cells. It is important that on subsequent pathogenic challenge, such semimature DC can still develop into fully immunogenic, IL-12 producing mature DC, and they drive effector Th1 cell responses [113]. For example, in vitro-generated, peptide-loaded DC, which have been matured by TNF-a and subsequently injected into mice, have been shown to act in a tolerogenic manner by preventing experimental autoimmune encephalomyelitis through the induction of IL-10-producing CD4 Treg cells. However, the mechanisms used to induce tolerance remained unclear.

Immature pDC

Immature pDC, freshly enriched from human peripheral blood, can induce an anergic state in antigen-specific CD4 T cell lines. This effect is associated with a loss of IL-2 production and is completely or partially reversible in the presence of a high concentration of exogenous IL-2 in the secondary cultures. T cell anergy induction by immature pDC requires cognate contact via TCR-MHC engagement. This process is cytokine-independent, but the precise interactions between pDC and CD4 T cells, which induce T cell anergy, remain unclear. The mechanism for T cell anergy induction could be explained by TCR-MHC engagement without costimulatory signals through CD80 and CD86, as freshly isolated pDC do not express or

express only minimal levels of CD80 and CD86. However, as CD40-activated mature pDC are also tolerogenic, it is unlikely that their capacity to elicit the generation of Treg cells is related only to inefficient T cell stimulation. CD40L-activated pDC The induction of tolerance versus immunity may not simply be determined by the maturity of the DC. For example, naïve CD8_ T cells primed with allogeneic, CD40L-activated pDC differentiate into CD8 T cells, which display poor secondary proliferative and cytolytic responses. In addition, they produce, respectively, high and low levels of IL-10 and IFN-g upon restimulation [115]. These IL-10-producing CD8 Treg cells can be generated directly from naıve CD8 T cells by a single round of stimulation with mature pDC, and they share many similarities with CD4 Treg cells. These CD8 Treg cells, allostimulated with mature pDC, suppress primary T cell activation through IL-10 secretion.

Mature DC

Current literature suggests that T cells recognizing antigen on mature DC differentiate into effector T cells, whereas tolerance is induced when antigen is presented by immature DC. Liu reported the only study of DC requirement for the differentiation of Treg cells from the thymus. Within the medulla of the human thymus, a subset of mature DC positively selects Treg cells, protecting the medium to high-affinity, selfreactive T cells from negative deletion and inducing their differentiation into Treg

cells in the thymus. How DC can have a negative role in the deletion of high-affinity, self-reactive thymocytes and simultaneously, a positive role in the selection of high-affinity, self-reactive Treg cells seems to be a paradox. A recent study by Watanabe et al. showed that the epithelial cells within Hassall's corpuscles are developmentally programmed to express TSLP, which activates a subpopulation of DC in the thymic medulla to express CD80 and CD86. TSLP does not induce activated myeloid DC to produce proinflammatory cytokines such as IL-12, IL-6, TNF-a, and IL-1. Although the immature DC within the cortico-medullary junction may be critical for negative selection, TSLP-DC in the central part of the medulla may be critical for the positive selection of high-affinity, autoreactive T cells and for their differentiation into CD4 CD25 Treg cells [116].

In the periphery, mature DC also expand functional Treg cells. These latter cells may be important during infection, when DC are presenting microbial antigens, as there is likely a concomitant presentation of self and environmental antigens.

When mature DC are inducing immune responses in the lymph node, with concomitant IL-2 production, bystander CD25 CD4 T cells would be expected to expand. In addition, when maturing DC are themselves presenting cognate selfantigens, thymic-derived CD25 CD4 T cells would be expected to expand vigorously and to serve to suppress autoreactive responses by other DC. Moreover, Verhasselt et al. observed that a significant fraction of CD4 T cells cultured with mature,

autologous DC acquired regulatory properties. Indeed, when added to an allogeneic MLR, these CD4 T cells suppressed the response of alloreactive T lymphocytes to the priming DC [117, 118]. *[Adapted from: Hubert; J. Leuk. Biol. 82, 781-794 (2007)]*

INDUCTION OF Treg CELLS BY TOLEROGENIC DCs

Although a role for Foxp3+ Treg cells in the maintenance of immune tolerance has been demonstrated in both humans and mice, the origin of these cells is still not completely understood. Early neonatal thymectomy experiments in mice strongly suggested that Treg cells are generated in the thymus. Studies with Foxp3 reporter mice and transgenic mice that express foreign antigens in thymic tissue have also traced the development of Foxp3+ cells to the thymus. Aside from evidence that natural Foxp3+ Treg cells arise and mature in the thymus, there is mounting evidence that Foxp3+ Treg cells can develop extrathymically under certain conditions. Both murine and human naïve T cells have been shown to express Foxp3 and acquire suppressive activity in vitro after T cell receptor (TCR) stimulation in the presence of TGF-b. In vivo, delivery of subimmunogenic doses of antigen as well as endogenous expression of foreign antigen in a lymphopenic environment can also induce peripheral Foxp3+ Treg cell development [119].

Despite a growing body of literature documenting a potential role for these converted cells in the control of autoimmune or inflammatory diseases, the nature of the antigen-presenting cells (APCs) involved in this conversion process remains poorly understood. Several reports support the idea that immature DCs may be more efficient at inducing Foxp3+ Treg cell development in the presence of TGF-b than activated DCs. For

example, targeting of antigens to immature DCs via the regulatory receptor DEC205 can favor the induction of Foxp3+ T cell development de novo. Although virtually all APCs at steady-state conditions may have the capacity to induce antigen-specific Treg cells, DCs appear to be more efficient at this process than other APCs. Thus, spleen DCs are more potent than DC-depleted APCs for the induction of Treg cells and require lower doses of peptide antigen. In the absence of exogenous IL-2, endogenous IL-2 production by T cells favoring Treg cell conversion can be efficiently triggered by DCs expressing CD80 and CD86 but not by other APCs [120]. However, another study has proposed that B cells are more efficient at inducing Foxp3+ Treg cells than splenic DCs in the presence of TGF-b. This discrepancy is likely to be associated with the level of activation of the APCs in these different settings.

Several reports suggest that the status of activation of DCs as well as inflammatory mediators modulates the capacity of these cells to induce Treg cells de novo. For example, IL-6 and TGF-b in tandem can direct the production of IL-17-secreting T cells (Th17 cells) over Treg cells, and Th1 and Th2 cell effector cytokines have an antagonistic effect on Treg cell conversion. Through the use of an APC-free system in vitro, it has been suggested that strong costimulation provided by extensive CD28 signaling can inhibit Foxp3 induction. In vivo, efficient induction of Foxp3+ Treg cells is also abolished in the presence

of strong costimulation. DCs deficient in CD80 and CD86 induce higher expression of Foxp3 on naive T cells than control DCs. Furthermore, when ex vivo spleen DCs were activated with an agonist anti-CD40 or lipopolysaccharide, induction of Foxp3+ Treg cells was impaired. Thus, activated DCs are poor inducers of Foxp3+ Treg cells in favor of the induction of effector responses [121].

This is in contrast with the observation that activated DCs are more efficient at inducing the proliferation of natural Treg cells than immature DCs. This could suggest that in some defined situations, Treg cells could play a sequential and complementary role. For instance, the endogenous population may preferentially control highly inflammatory settings, whereas converted Treg cells may play a more important role during the downstream of the inflammatory process or in situation of chronic infections.

The demonstration that some DCs subsets from lymphoid tissues could be more efficient at inducing Treg cells than others came from a study showing that CD8+ DCs induce higher conversion than other spleen DC subsets in the presence of TGF-b. Several molecules contribute to the induction of Foxp3+ Treg cell development. For instance, the B7-CTLA-4 axis is important to favor the induction of these cells. A role for PD-L1 expressed by DCs in the induction of

Treg cells has been recently reported, adding another potential role of this molecule in the control of peripheral tolerance [122].

Previous work demonstrates that CD3 antibody treatment transiently depletes large numbers of T cells and subsequently induces long-term immune tolerance. A recent study provides evidence that the mechanism underlying this regulatory effect is an enhanced production of TGF-b by macrophages and immature DCs after engulfment of apoptotic T cells. This increase in TGF-b induces the development of Foxp3+ Treg cells and contributes to immune tolerance. Clearance of apoptotic bodies has been shown to lead to the development of Treg cells. The relative contribution of this pathway in the constitutive generation of Treg cells at sites with a high amount of remodeling (e.g., gut or uterus) remains to be addressed. *[Adapted from: Belkaid; Immunity 29, 362-371 (2008)]*

ROLE OF TISSUE-SPECIFIC DCs IN THE INDUCTION OF Treg CELLS

No other tissue is subjected to more antigenic pressure than the gut. For instance, the adult human intestine contains up to 1014 microorganisms. It is therefore a highly regulated immunologic site that must generate both tolerogenic and immunogenic responses. On one hand, immune reactivity against nonpathogenic gut elements is not only wasteful but also dangerous to the host and is known to lead to severe tissue damages (e.g., inflammatory bowel disease). On the other hand, the development of active immunity is required to protect the host against invasive pathogens. Different subsets of Treg cells have been shown to be instrumental in the maintenance of this complex homeostasis. Additionally, several subsets of DCs with regulatory properties have been described with the capacity to induce IL-10 secretion from T cells or induce oral tolerance at steady-state conditions. Some of these features may have been influenced by conditioning signals received from noninflammatory cytokines constitutively produced by the intestinal epithelia. These cytokines include TGF-b and the Th2 cell response driving thymic stromal lymphopoietin [123, 124].

It has recently been demonstrated that the gut-associated lymphoid tissue is a preferential site for the peripheral induction of Foxp3+ Treg cells. A role for local DCs in this conversion process is supported by the observation that DCs from the

lamina propria of the small intestine and from the mesenteric lymph node (MLN) are noticeably better than splenic DCs at inducing the expression of Foxp3 in naive T cells in the presence of exogenous TGF-b. Similarly, lamina propria macrophages can efficiently induce Foxp3+ T cells. In particular, DCs expressing CD103+ in these two compartments can induce Foxp3+ T cells in the absence of any exogenous factors. This conversion process was associated with their capacity to release bioactive TGF-b, which could be linked with their capacity to activate latent TGF-b. This hypothesis is supported by the observation that DCs lacking the TGF-b-activating integrin avb8 fail to induce Foxp3+ Treg cells in vitro. Loss of v-integrin expression by myeloid cells led to the development of intestinal inflammation, probably through the combined effects of a failure to remove apoptotic cells and a loss of TGF activation [125].

Although DCs derived from the lamina propria and MLN can efficiently induce Treg cells, the exact sites in which these events take place remains unclear. We cannot exclude that Treg cell conversion may occur in the lamina propria, because a small but sizeable population of naive cells can be found in this tissue. However, early after oral feeding with antigen, most of the converted cells can be found in the MLN preceding their accumulation in the lamina propria. Gut DCs constitutively transport apoptotic epithelial cells to the MLN. Recent evidence also suggest that the majority of CD103+ DCs represent a

tissue-derived migratory population that plays an important role in presenting orally derived soluble antigen to T cells. Thus, as predicted by previous studies showing a central role for the MLN in the acquisition of oral tolerance, most of the conversion may occur in this compartment in response to tissue migrating CD103+ DCs. A likely hypothesis would be that these gut-converted Treg cells could become part of the peripheral Treg cell pool. So, over time, the gut flora, oral pathogens, or food may have an important role in shaping the repertoire of peripheral Foxp3+ Treg cells. The relative contribution of these converted Treg cells to peripheral tolerance and the outcome of infections, as well as how pathogens can utilize or interfere with this pathway to favor their own survival, remains to be addressed [126]. Currently, in absence of definitive markers to distinguish endogenous versus converted Foxp3+ Treg cells, these questions will remain difficult to answer. *[Adapted from: Belkaid; Immunity 29, 362-371 (2008)]*

ROLE OF DC-DERIVED PRODUCTS IN IMPRINTING REGULATORY PROPERTIES

Gut DCs also have an important role in dictating the homing potential of lymphocytes. DCs isolated from the Peyer's patches, small-intestinal lamina propria, and MLNs promote the expression of the gut-homing receptors a4b7-integrin and CCR9 by CD4+ and CD8+ T cells. The molecule CCR9 binds to CCL25 produced by epithelial cells of the small intestine, and a4b7-integrin binds to mucosal vascular addressin cell-adhesion molecule 1 (MADCAM1), which is expressed by the vascular endothelium of the gastrointestinal tract. It is also becoming clear that nutrient status can impact an individual's susceptibility to intestinal pathologies. In the case of vitamin A and, in particular, its transcriptionally active metabolite, retinoic acid (RA), prolonged insufficiency not only disrupts the integrity of the intestinal epithelial barrier but also prevents the proper deployment of effector lymphocytes into the gut-associated lymphoid tissue (GALT) after priming. Indeed, the capacity of GALT DCs to imprint gut-homing receptors to lymphocytes is associated with their capacity to release retinoic acid [127]. More recently, it was demonstrated that RA and cytokines produced by DCs in the Peyer's Patches synergized to promote IgA secretion by gut-activated B cells. Importantly, the addition of RA to naturally occurring Treg cells in vitro can promote their expression of gut tropism receptors and subsequently favor their migration to the GALT. Another effect of RA on the

immune regulation of the gastrointestinal (GI) tract is associated with its capacity to enhance the TGF-b-mediated generation of Foxp3+ Treg cells from naive T cells by gut DCs. The induction of Foxp3 observed in the presence of small-intestinal lamina propria DCs and CD103+ MLN DCs can be inhibited by a retinoic-acid receptor (RAR) antagonist. Conversely, incubation of splenic or CD103_ MLN DCs with both TGF-b and RA enhanced their capacity to induce Foxp3 Treg cells [128, 129]. RA produced by intestinal macrophages can also synergize with TGF-b to induce Foxp3+ Treg cells. Importantly, RA can induce the conversion of naïve CD4+ T cells purified from human cord blood into Foxp3+ Treg cells. Reciprocally, RA can inhibit the generation of Th17 cells, suggesting that RA may play an important role in maintaining the balance between effector and regulatory populations in the GI tract. The mechanism by which RA produced by DCs can enhance the capacity of TGF-b to induce Foxp3 on naïve T cells remains unclear but is likely due to a conjunction of effects on both T cells and DCs. One possible role of RA would be via its capacity to enhance TGF-b signaling, e.g., RA can increase the expression of TGF-b receptor subunit. Another possibility could be associated with the capacity of RA to suppress effector cytokines known to suppress the induction of Foxp3 by T cells. In addition to the gut, other compartments could be involved in the generation of Foxp3+ T cells. For instance, a recent report demonstrated that liver plasmacytoid DCs can contribute to the acquisition of tolerance against oral antigens. Of importance,

the liver and in particular Ito cells can store up to 80% of total body retinol. How liver DCs can acquire retinol form these cells and convert it to RA remains to be addressed. Although the capacity of GALT DCs or macrophages to imprint gut-homing receptors and induce Foxp3+ Treg cells is associated with their capacity to release RA, it remains unclear whether these cells are the main producer of this metabolite in the gut [130].

Synthesis of RA from stored or dietary retinol depends on the expression of the appropriate enzymes, which can be expressed directly by GALT DCs. DCs from Peyer's patches and MLNs express Aldh1a1 and Aldh1a2, respectively. DCs from the lamina propria express a large array of this family of enzymes, such as ADH1, ADH4, ADH5, Adh1a1, Adh1a2, and Adh1a3. Supporting the idea of a role for these cells as producers of RA, Peyer's patch and MLN DCs can directly convert retinol to RA in culture. However other cells, including epithelial cells, can express enzymes associated with vitamin A metabolism, suggesting that DCs may also acquire RA from other sources and store it. A recent study demonstrates that monocyte-derived DCs pretreated with RA can acquire several attributes characteristic of mucosal DCs, such as secretion of TGF-b and IL-6 and the capacity to augment mucosal- homing receptor expression and IgA production. In this particular study, these gut-derived features acquired by DCs were associated with the capacity of DCs to become carriers and not producers of RA. The precise factors that govern the activation of some of

these enzymes and how inflammation or infections modify the metabolism of vitamin A remain to be explored. Another important question would be to understand the timing necessary for DCs migrating in the GALT to acquired RA from epithelial cells and how these processes can be modified during inflammatory responses. How RA contributes to oral tolerance and at the same time protective immunity in the GI tract also remains to be addressed. One possibility would be that RA could favor the induction of Treg cells in the absence of secondary signals but enhances effector response after exposure to inflammatory mediators. Indeed, lamina propria DCs stimulated with flagellin can induce the differentiation of Th17 cells in an RA-dependent manner, suggesting that at physiological doses RA does not inhibit but rather promotes this pathway [131, 132].

This observation is consistent with the fact that the gut enriched in RA is home to a large number of IL-17-producing T cells at steady-state condition. After exposure to CpG, lamina propria dendritic cells can prevent the induction of Treg cells while inducing effector T cells expressing a high amount of RA-dependent gut-homing receptors (Y.B., unpublished data). The shared feature by local mediators to imprint homing and regulatory properties is also observed in another defined microenvironment. Vitamin D3 is generated as an inactive form in the skin in response to sunlight and is converted to the active form, 1,25-dihydroxyvitamin D3 [1,25(OH)2D3], by an

enzymatic cascade involving 25-hydroxylases and 1-hydroxylase. The D vitamins have many effects on immune cells. It has been shown that 1,25(OH)2D3 can inhibit the differentiation and maturation of DCs, leading to decreased IL-12 and enhanced IL-10 production and decreased T cell activation [133]. Several experimental evidences support the regulatory function of vitamin D. Mouse models revealed that that 1,25(OH)2D3 can inhibit several autoimmune- disease models in mice. Vitamin D3 in combination with dexamethasone can also favor the induction of cells able to produce IL-10 with strong regulatory properties. Vitamin D3 was also shown to confer T cell tropism in the skin. CCL27 is a skin-specific chemokine ligand expressed by keratinocytes in the epidermis, and the expression of its receptor CCR10 allows T cells to migrate toward this chemokine. Vitamin D3 increases the expression of this receptor. Furthermore, active vitamin D3 suppressed the expression of the gut-homing receptors a4b7-integrin and CCR9 by T cells. Gene-expression analysis of skin DCs showed that they express both 25-hydroxylase and 1-hydroxylase, produce active vitamin D3, and induce CCR10 expression on T cells. Vitamin D can also enhance the suppressive capacity of Foxp3+ T cells from regional lymph node when delivered topically [134].

Defined microenvironments may have evolved self-containing strategies in which local mediators can imprint homing properties while at the same time favoring the induction or

function of Treg cells. Site-specific cells or factors such as neurons or hormones can also favor the induction of Foxp3+ Treg cells. It is therefore tempting to speculate that a link between homing and regulatory function induction may represent a more general mechanism. Such strategy could allow the constant generation and migration of Treg cells to defined compartments. These Treg cells are expected to have the prerequisite antigen specificities (e.g., flora antigens), status of activation, and survival requirement, allowing them to regulate a defined microenvironment [135]. *[Adapted from: Belkaid; Immunity 29, 362-371 (2008)]*

Treg CELLS INDUCED BY MICROBE-MANIPULATED DCs

In order to sustain their transmission and/or reproduction, a large number of microbes have to establish long-term interactions with their host. During this coexistence, the microbe must avoid elimination by the host immune response and sustain its life cycle, while at the same time delaying or preventing host destruction.

Microbe-mediated modulation of innate and acquired immune responses of the persistently infected host has to meet these requirements and restore a homeostatic environment. Failure to establish or maintain homeostatic conditions usually causes disease. This is clearly the case of microflora that invade our gut or our skin, as well as for pathogenic microbes that establish chronic infections. All persistent microbes obey the same principle: the immune system constitutes their ecological niche, and they have coevolved with their host to learn how to manipulate APC function in order to dictate an immune response appropriate to insure their survival. For instance, microbes have been shown to induce a large array of regulatory cells to insure their own survival. Surviving an infection requires the generation of a controlled immune response in the host that recognizes and controls the invading pathogen while limiting collateral damage to self-tissues that may result from an exuberant immune response [136]. This implies that induction of Treg cells also arises as a result of the host response to the infectious process in a bid to maintain or restore a homeostatic

environment and/or that it can be actively induced by the pathogen to promote pathogen survival. The role of IL-10 as an immunoregulatory cytokine in infection has been mainly documented in the context of chronic infections IL-10 can inhibit the immune responses (by both Th1 cells and Th2 cells) to many pathogens in experimental models and in human infectious diseases, such as tuberculosis, malaria, hepatitis C, filariasis, leishmaniasis, and schistosomiasis. The most remarkable example of this control is illustrated by its crucial role during acute infection of mice with Toxoplasma gondii. In this model, IL-10 production by T cells is the key regulator of effector cell responses, because IL-10-deficient mice can control parasite number, but they succumb to lethal immunopathology driven by unrestrained effector cell responses. During Th2 cell-dominated helminths infection, the majority of IL-10 is produced by the Th2 cells. Besides T cells, IL-10 can also be produced by numerous cell types, including B cells, natural killer (NK) cells, macrophages, and DCs In acute Plasmodium yoelii infection, a subset of regulatory DCs expressing CD11cloCD45RBhi and inducing IL-10-secreting T cells becomes the predominant DC population in the spleen. IL-10 can also be produced by natural Treg cells and, in some cases, is associated with their function; however, in most cases, the inducible Tr1 cell population is the relevant source of this cytokine during infection [137]. During various infections, Tr1 cells develop from conventional T cells after encountering with certain signals, such as exposure to deactivated or immature

APCs, repeated exposure to antigen, or IL-10 itself. Of note, these conditions prevail during chronic infection in which APC functions are often targeted by the pathogen and cells of the immune system are chronically exposed to microbial antigens.

Consistent with a role for these cells in human disease, Tr1 cell clones can be isolated from patients who are chronically infected with hepatitis C virus (HCV). Interestingly, these regulatory clones had similar viral antigen specificity to protective Th1 cell clones isolated from the same patient. Defined microbial products can manipulate DCs in a way that induces Treg cell populations. For example, filamentous haemagglutinin (FHA) from Bordetella pertussis was shown to induce IL-10 production by DCs; these DCs favor the differentiation of naïve T cells into Tr1 cells. Similarly, Tr1 cells can be generated from naive T cells in the presence of DCs stimulated with phosphatidylserine from Schistosoma mansoni. One promising therapeutic approach has emerged from the observation that microbial products can favor the induction of Tr1 cell populations in vivo. Exposure of mice to S. mansoni antigen prevents development of type 1 diabetes in NOD mice, as well as experimental colitis [138]. The use of single microbial molecules as therapeutic agents has been recently shown as FHA of Bordetella pertussi can efficiently treat experimental colitis. Although Tr1 cells define a population of T cells that can produce IL-10 and/or TGF-b, some IL-10-producing T cells can also produce IFN-g. The autocrine regulation by IL-10 of Th1

and Th2 cells was initially described in human clones. In the context of an infectious disease, IFN-g and IL-10 double producers were first described in the bronchoalveolar lavage of patients with tuberculosis and in individuals chronically infected with Borrelia burgdorferi. Indeed, in many chronic infections, in humans and experimental animals, the presence of CD4+ T cells that produce high amounts of both IL-10 and IFN-g have been documented. Recently it was shown that IFN-g- and IL-10-producing CD4+ T cells emerge during experimental infection with T. gondii and in a model of nonhealing Leishmaniasis and that these cells share many features with Th1 cells and were the main source of protective IL-10. These T cells were identified as activated T-bet+ Th1 cells and were distinct from Th2 cells, natural Treg cells, or other subsets of inducible regulatory T cells [139]. Unlike IFN-g production, IL-10 production was transient, observed in only a fraction of the IFN-g-producing cells, and was produced more rapidly by recently activated T cells than by resting T cells. The instability of IL-10 synthesis, which was observed only when the Th1 cells were fully activated, is probably necessary to prevent sustained suppression of effector functions. Thus, it appears that, in some cases, cells with regulatory properties could arise from fully differentiated Th1 cells as a negative-feedback loop. It is likely that numerous previous studies of Tr1 cells were in fact incriminating similar populations. These IFN-g- and IL-10-producing T cells may represent a dominant regulatory response to infections that induce highly polarized Th1 cell

responses [140]. The nature of the APC or status of activation required for the imprinting of IL-10 on these Th1 cells remains poorly understood, but some evidence suggests that the cytokines produced by DCs could contribute to this phenotype. For instance, repetitive exposure to IL-12 could induce IL-10 on IFN-g-producing cells. Several recent reports suggested that IL-27 might be an important determinant for the induction of IL-10 on Th1 cells. A role for this cytokine as a regulatory mediator has recently emerged. IL-27 can limit Th1, Th2, and Th17 cell responses in various models of infection and autoimmunity. DCs from the spleen modified by exposure to TGF-b-producing Treg cells acquire a plasmacytoid phenotype and release TGF-b and IL-27, which in turn allow the induction of IL-10-producing T cells [141, 142]. How IL-27 could contribute to the induction of IL-10 in the GI tract and how this pathway could contribute to the maintenance of gut homeostasis remains to be addressed. *[Adapted from: Belkaid; Immunity 29, 362-371 (2008)]*

INDUCTION OF Treg CELLS BY DCs DURING INFECTIONS

Acute infection with Listeria monocytogenes in mice failed to induce Foxp3 by conventional CD4+ T cells. Thus, highly inflammatory environments that will prevail in acute infection may not favor the emergence of Foxp3+ T cells. This hypothesis is supported by the observation that Th1 or Th2 cell-polarizing cytokines can interfere with the induction of these cells. However, chronic infections may require an additional layer of regulation, which would be provided by converted Foxp3+ Treg cells. This hypothesis is supported by the observation that during infection, the downstream effects of inflammatory responses are also often associated with anti-inflammatory processes, including TGF-b production [143]. Furthermore, some pathogens target sites in which TGF-b is highly produced, such as the GI tract, the skin, and the eye, which may assist in the conversion in vivo. TGF-b can be also produced by infected cells or by cells the microorganisms are in contact with, or arise as a result of an inflammatory process. For example, the trypomastigote stage of Trypanosoma cruzi induced TGF-b and IL-10 secretion by DCs. Compelling data in a mouse model of malaria suggest that TGF-b and Treg cells are central regulators of immunopathology and parasite expansion. During late infection with Plasmodium yoelii infection, DCs migrate to the spleen of infected mice and secrete TGF-b together with IL-10 and PGE2 [144]. After experimental malaria infection of human volunteers, enhanced

TGF-b and Foxp3+ Treg cell responses in peripheral blood mononucleated cells correlate with a faster parasitic growth rate. Cells with natural Treg cell characteristics are rapidly induced after blood stage infection and are associated with a decrease of proinflammatory cytokines and antigen-specific responses. Monocytes are a likely source of the early TGF-b production in this infection. Some nematodes can themselves express homologs of TGF-b. Compelling experimental data support the idea that Foxp3+ Treg cells can be induced during Heligmosomoides polygyrus infection. This pathway may not be limited to the GI tract, because we found that the Bacillus Calmette-Guerin (BCG) can induce new populations of Foxp3+ T cells in vivo that accumulate at the dermal site of infection [145]. The relative contribution of these converted Treg cells to peripheral tolerance and the outcome of infections, as well as how pathogens can utilize or interfere with this pathway to favor their own survival, remains to be addressed. Currently, in absence of definitive markers to distinguish endogenous versus converted Foxp3+ regulatory T cells, these questions will remain difficult to answer [146, 147].

However, the interaction with persistent microbes does not always lead to the induction of Treg cells. As discussed above, activation of DCs that can be mediated by TLR ligands impairs Treg conversion. We found that gut flora-derived DNA (gfDNA), but not other TLR agonists, strongly constrained the capacity of lamina propria dendritic cells to induce Treg cell conversion in

vitro and can act as a natural adjuvant for priming intestinal responses via modulation of Treg-Teff (T effector) cell equilibrium. This would suggest that in some highly regulated environment, persistent microbes might limit peripheral conversion by modulating APC function. *[Adapted from: Belkaid; Immunity 29, 362-371 (2008)]*

Treg CELLS POPULATIONS

Treg cells consist of different subsets of T lymphocytes characterized by their ability to suppress proliferation of conventional effector T cells by various mechanisms.

The list of candidate markers for Treg cells increases continuously and includes CD45RB, CD103, or CD122 and the transcriptional repressor forkhead box P3 (Foxp3), which is currently considered as the most promising marker for Treg cells. In addition, Treg cells are characterized by the constitutive expression of GITR family-related protein, L-selectin (CD62L), and CTLA-4 (or CD152). Thus, no specific marker (except the transcription factor Foxp3) has been identified to date. In particular, no defined cell surface molecules can be targeted with antibodies specifically to deplete or to purify these cells [148, 149].
This problem is an ongoing challenge for the study and manipulation of these suppressive cells. Concerning the Foxp3 itself, one question remains: whether its expression is an absolute marker of Treg cells in humans or mice.

In the mouse, there is an excellent correlation between the expression of Foxp3 and CD25, but a minor population of Foxp3+ cells is CD25-. In humans, almost all CD4+CD25hi cells are Foxp3+, whereas a variable percentage of CD25int cells expresses lower amounts of Foxp3.

Moreover, whereas the TGF-b-induced murine Foxp3+ T cells manifest suppressor activity in vitro and in vivo, Foxp3 expression in human T cells does not correlate uniformly with suppressor activity. In addition, populations of CD4+CD25+ Treg cells producing IL-10 do not seem to express Foxp3.
Over the past few years, several phenotypically distinct Treg cell populations have been described. The classic Treg cells are thymus-derived CD4+CD25+Foxp3+ T cells, and many investigators have termed these cells “natural” Treg in contrast to Treg cells, which develop in peripheral lymphoid tissues and are frequently Foxp3– and have been termed “adaptive” or “induced” Treg cells. Several studies have raised the possibility that CD4+ Foxp3+ cells might also be generated in peripheral lymphoid tissues from naıve CD4+ Foxp3– progenitors [150].

Among all the subsets described to date, the most studied are the naturally occurring CD4+CD25+ Treg cells, the antigen-induced CD4+CD25+ Treg cells, and the CD8+CD28– T cells (or T suppressive cells). The naturally occurring CD4+CD25+ Treg cells represent a small subset (5–6%) of the overall CD4+ T cell population and are generated in the thymus. They mediate immune suppression by inhibiting the activation and proliferation of Th and cytotoxic T cells through a cell– cell contact and antigen nonspecific mechanism.

However, in some models, Foxp3+ natural Treg cells have been shown to be antigen specific, suggesting that the distinction

between the different subsets of Treg cells is not at all clear and that the notion of antigen induction is not adequate to distinguish the different Treg cells.

The antigen-induced CD4+CD25+ Treg cells are detected in peripheral tissues after MHC/peptide stimulation of conventional CD4+CD25– precursors. These Treg cells include Tr1 cells, secreting IFN-g, IL-10, and to a lesser extent, TGF-b, and Th3 T cells, secreting high levels of TGF-b and IL-10. The suppressive activity of the antigen-induced Treg cells is cell contact-independent and is mediated by the release of TGF-b and IL-10. Th3 and Tr1 cells display suppressive properties on Th1 and Th2 cells, but only Th3 cells provide help for IgA synthesis. Tr1 cells regulate the function of naıve and memory T cells in vitro and in vivo and can suppress responses to tumors, alloantigens, and pathogens. These two subsets of Treg cells also differ in the expression of distinct integrins, mainly a4b1 and a4b7, which have been shown to be homing receptors for the migration of T lymphocytes to inflamed tissues and to mucosal sites, respectively. These data suggest that a4b1+CD25+ Treg cells migrate in vivo to inflamed tissues, where they inhibit effector T cell responses, whereas a4b7+CD25+ Treg cells could prevent chronic mucosal inflammations by counteracting autoreactive T cells.

It is generally understood that being CD4+ T cells, naturally occurring Treg cells as well as antigen-induced Treg cells need

to be activated by MHC class II-bound epitopes on DC. It is widely believed that although antigen-induced Treg cells need TCR ligands and costimulation, naturally occurring Treg cells need only a TCR-driven signal for functional activation. Nonetheless, as only a limited class of cells expresses MHC class II molecules, naturally occurring Treg cells need DC for their activation. Thus, just as DC are indispensable in activating, naıve effector T cells, they are also needed for the activation of Treg cells [151].

A subpopulation of Treg cells expresses CD8 and can mediate immune suppression in an antigen-dependent manner. CD8+ Treg cells suppress antigen-activated CD4+ T cells by a TCR/MHC class Ib molecule restriction. CD8+CD28+ Treg cells also suppress APC, which present the same peptide/MHC complexes to which the CD8+ Treg cells were primed previously. In contrast to naturally occurring CD4+ CD25+ Treg cells, an important feature of CD8+ Treg cells is that they are generated or induced only after antigen priming. *[Adapted from: Hubert; J. Leuk. Biol. 82, 781-794 (2007)]*

1. Kronenberg M, Gapin L. 2002. *Nat. Rev. Immunol.* 2:557–68

2. Smyth MJ, Crowe NY, Hayakawa Y,Takeda K, Yagita H, Godfrey DI. 2002. *Curr. Opin. Immunol.* 14:165–71

3. Hammond KJ, KronenbergM. 2003. *Curr. Opin. Immunol.* 15:683–89

4. Skold M, Behar SM. 2003. *Infect. Immun.* 71:5447–55

5. Taniguchi M, Harada M, Kojo S,Nakayama T,Wakao H. 2003. *Annu. Rev. Immunol.* 21:483–513

6. Brigl M, BrennerMB. 2004. *Annu. Rev. Immunol.* 22:817–90

7. Matsuda JL, Naidenko OV, Gapin L, Nakayama T, Taniguchi M, et al. 2000. *J. Exp. Med.* 192:741-547

8. Bendelac A, Rivera MN, Park SH, Roark JH. 1997. *Annu. Rev. Immunol.* 15:535–62

9. Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ, Van Kaer L. 2004. *Nat. Rev. Immunol.* 4:231–37

10. Slifka MK, Pagarigan RR, Whitton JL. 2000. *J. Immunol.* 164:2009–15

11. Porcelli S, Yockey CE, Brenner MB, Balk SP. 1993. *J. Exp. Med.* 178:1–16

12. Dellabona P, Padovan E, Casorati G, Brockhaus M, Lanzavecchia A. 1994. *J. Exp. Med.* 180:1171–76

13. Brossay L, Chioda M, Burdin N, Koezuka Y, Casorati G, et al. 1998.*J. Exp. Med.* 188:1521–28

14. Spada FM, Koezuka Y, Porcelli SA. 1998. *J. Exp. Med.* 188:1529–34

15. Benlagha K, Weiss A, Beavis A, Teyton L, Bendelac A. 2000. *J. Exp.Med.* 191:1895–903

16. Karadimitris A, Gadola S, Altamirano M, Brown D,Woolfson A, et al. 2001. *Proc. Natl. Acad. Sci. USA* 98:3294–98

17. Kita H, Naidenko OV, Kronenberg M,Ansari AA, Rogers P, et al. 2002. *Gastroenterology* 123:1031–43

18. Lee PT, Putnam A, Benlagha K, Teyton L, Gottlieb PA, Bendelac A. 2002. *J. Clin. Invest.* 110:793– 800

19. Rogers PR, Matsumoto A, Naidenko O, Kronenberg M, Mikayama T, Kato S. 2004. *J. Immunol. Methods* 285: 197–214

20. Gapin L, Matsuda JL, Surh CD, Kronenberg M. 2001. *Nat. Immunol.*2:971–78

21. Benlagha K, Kyin T, Beavis A, Teyton L, Bendelac A. 2002. *Science* 296:553– 55

22. Pellicci DG, Hammond KJ, Uldrich AP, Baxter AG, Smyth MJ, Godfrey DI. 2002. *J. Exp. Med.* 195:835–44

23. Coles MC, Raulet DH. 2000. *J. Immunol.* 164:2412–18

24. Forestier C, Park SH,Wei D, Benlagha K, Teyton L, Bendelac A. 2003. *J. Immunol.* 171:4096–104

25. Elewaut D, KronenbergM. 2000. *Semin. Immunol.* 12:561 68

26. Sivakumar V, Hammond KJ, Howells N, Pfeffer K, Weih F. 2003. *J. Exp. Med.* 197:1613–21

27. Stanic AK, Bezbradica JS, Park JJ, Matsuki N, Mora AL, et al. 2004. *J. Immunol.* 172:2265–73

28. Schmidt-Supprian M, Tian J, Grant EP, Pasparakis M, Maehr R, et al. 2004 *Proc. Natl. Acad. Sci. USA* 101:4566–71

29. Elewaut D, Shaikh RB, Hammond KJ, De Winter H, Leishman AJ, et al. 2003. *J. Exp. Med.* 197:1623–33

30. Townsend MJ, Weinmann AS, Matsuda JL, Salomon R, Farnham PJ, et al. 2004. *Immunity* 20:477–94
31. Elewaut D, Lawton AP, Nagarajan NA, Maverakis E, Khurana A, et al. 2003. *J. Exp. Med.* 198:1133– 46
32. Cernadas M, Sugita M, van der Wel N, Cao X, Gumperz JE, et al. 2003. *J. Immunol.* 171:4149–55
33. Zhou D, Cantu C 3rd, Sagiv Y, Schrantz N, Kulkarni AB, et al. 2004. *Science* 303:523–27
34. Kang SJ, Cresswell P. 2004. *Nat. Immunol.* 5:175–81
35. Stetson DB, Mohrs M, Reinhardt RL, Baron JL,Wang ZE, et al. 2003. *J. Exp. Med.* 198:1069–76
36. Gadue P, Stein PL. 2002. *J. Immunol.* 169:2397–406
37. Eberl G, Lowin-Kropf B, MacDonald HR. 1999. *J. Immunol.* 163:4091–94
38. Gadue P, Morton N, Stein PL. 1999. *J. Exp. Med.* 190:1189–96
39. Gadue P, Yin L, Jain S, Stein PL. 2004. *J. Immunol.* 172:6093–100
40. Shimamura M, Ohteki T, Beutner U, Mac-Donald HR. 1997. *Eur. J. Immunol.* 27:1576–79
41. Cheroutre H. 2004.*Annu. Rev. Immunol.* 22:217–46
42. Leishman AJ, Gapin L, CaponeM,Palmer E, MacDonald HR, et al. 2002. *Immunity* 16:355–64
43. Yamagata T, Mathis D, Benoist C. 2004. *Nat. Immunol.* 5:597–605
44. Jordan MS, Boesteanu A, Reed AJ, Petrone AL, Holenbeck AE, et al. 2001. *Nat. Immunol.* 2:301–6

45. Bendelac A, Killeen N, Littman DR, Schwartz RH. 1994. *Science* 263:1774–78

46. Hernandez-Hoyos G, Sohn SJ, Rothenberg EV, Alberola-Ila J. 2000. *Immunity* 12:313–22

47. Singer A. 2002. *Curr. Opin. Immunol.* 14:207– 15

48. Prussin C, Foster B. 1997. *J. Immunol.* 159:5862–70

49. Gumperz JE, Miyake S, Yamamura T, Brenner MB. 2002. *J. Exp. Med.* 195:625–36

50. Chun T, Page MJ, Gapin L, Matsuda JL, Xu H, et al. 2003. *J. Exp. Med.* 197:907–18

51. Pellicci DG, Uldrich AP, Kyparissoudis K, Crowe NY, Brooks AG, et al. 2003 *Eur. J. Immunol.* 33: 1816–23

52. Schumann J, Voyle RB, Wei BY, Mac- Donald HR. 2003. *J. Immunol.* 170:5815–19

53. Hayakawa Y, Berzins SP, Crowe NY, Godfrey DI, Smyth MJ. 2004. *Nat. Immunol.* 5:590–96

54. Voyle RB, Beermann F, Lees RK, Schumann J, Zimmer J, et al. 2003 *J. Exp. Med.* 197:919–25

55. Kim CH, Butcher EC, Johnston B. 2002. *Trends Immunol.* 23:516–19

56. Johnston B, Kim CH, Soler D, Emoto M, Butcher EC. 2003. *J. Immunol.* 171:2960–69

57. Thomas SY, Hou R, Boyson JE, Means TK, Hess C, et al. 2003. *J. Immunol.* 171:2571–80

58. Lee PT, Benlagha K, Teyton L, Bendelac A. 2002. *J. Exp. Med.* 195:637–41

59. Nieuwenhuis EE, Matsumoto T, ExleyM, Schleipman RA, Glickman J, et al. 2002. *Nat. Med.* 8:588–93
60. Akbari O, Stock P, Meyer E, Kronenberg M, Sidobre S, et al. 2003. *Nat. Med.* 9:582–88
61. Lisbonne M, Diem S, de Castro Keller A, Lefort J, Araujo LM, et al. 2003. *J. Immunol.* 171:1637–41
62. Mempel M, Ronet C, Suarez F, Gilleron M, Puzo G, et al. 2002. *J. Immunol.* 168:365–71
63. Matsuda JL, Gapin L, Sidobre S, Kieper WC, Tan JT, et al. 2002. *Nat. Immunol.* 3:966–74
64. Ranson T, Vosshenrich CA, Corcuff E, Richard O, Laloux V, et al. 2003 *Proc. Natl. Acad. Sci. USA* 100:2663–68
65. Park SH, Benlagha K, Lee D, Balish E, Bendelac A. 2000. *Eur. J. Immunol.* 30:620–25
66. D'Andrea A, Goux D, De Lalla C, Koezuka Y, Montagna D, et al. 2000. *Eur. J. Immunol.* 30:1544–50
67. Apostolou I, Cumano A, Gachelin G, Kourilsky P. 2000. *J. Immunol.* 165:2481–90
68. Ronet C, Mempel M, Thieblemont N, Lehuen A,Kourilsky P, Gachelin G. 2001. *J. Immunol.* 166:1755–62
69. Matsuda JL, Gapin L, Fazilleau N,Warren K, Naidenko OV, Kronenberg M. 2001. *Proc. Natl. Acad. Sci. USA* 98:12636–41
70. Eberl G, MacDonald HR. 1998 *Immunity* 9:345–53
71. Osman Y, Kawamura T, Naito T, Takeda K, Van Kaer L, et al. 2000. *Eur. J. Immunol.* 30: 1919–28
72. Leite-de-Moraes MC, Herbelin A, Gouarin C, Koezuka Y, Schneider E, Dy M. 2000. *J. Immunol.* 165:4367–71 Annu. Rev

73. Hayakawa Y, Takeda K,Yagita H, Kakuta S, Iwakura Y, et al. 2001. *Eur. J. Immunol.* 31:1720–27

74. Nakagawa R, Nagafune I, Tazunoki Y, Ehara H, Tomura H, et al. 2001. *J. Immunol.* 166:6578–84

75. Wilson MT, Johansson C, Olivares- Villagomez D, SinghAK,StanicAK,et al. 2003. *Proc. Natl. Acad. Sci. USA* 100:10913–18

76. Crowe NY, Uldrich AP,Kyparissoudis K, Hammond KJ, Hayakawa Y, et al. 2003. *J. Immunol.* 171:4020–27

77. Harada M, Seino K, Wakao H, Sakata S, Ishizuka Y, et al. 2004. *Int. Immunol.* 16:241–47

78. Burdin N, Brossay L, Kronenberg M. 1999. *Eur. J. Immunol.* 29:2014–25

79. Singh N, Hong S, Scherer DC, Serizawa I, Burdin N, et al. 1999. *J. Immunol.* 163:2373–77

80. Cui J,Watanabe N, Kawano T, Yamashita M, Kamata T, et al. 1999. *J. Exp. Med.* 190:783–92

81. Gumperz JE, Roy C, Makowska A, Lum D, Sugita M, et al. 2000. *Immunity* 12:211–21

82. Rauch J, Gumperz J, Robinson C, Skold M, Roy C, et al. 2003. *J. Biol. Chem.* 278:47508–15

83. Gadola SD, Dulphy N, Salio M, Cerundolo V. 2002. *J. Immunol.* 168: 5514–20

84. Gui M, Li J, Wen LJ, Hardy RR, Hayakawa K. 2001. *J. Immunol.* 167:6239–46

85. Wu DY, Segal NH, Sidobre S, Kronenberg M, Chapman PB. 2003. *J. Exp. Med.* 198: 173–81

86. Fischer K, Scotet E, Niemeyer M, Koebernick H, Zerrahn J, et al. 2004.*Proc. Natl. Acad. Sci. USA* 101:10685–90

87. Gadola SD, Zaccai NR, Harlos K, Shepherd D, Castro-Palomino JC, et al. 2002. *Nat. Immunol.* 3:721–26

88. Zajonc DM, Elsliger MA, Teyton L, Wilson IA. 2003. *Nat. Immunol.* 4:808–15

89. Batuwangala T, Shepherd D, Gadola SD, Gibson KJ, Zaccai NR, et al. 2004. *J. Immunol.* 172:2382–88

90. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, et al. 1997.*Science* 278:1626–29

91. Brossay L, Naidenko O, Burdin N, Matsuda J, Sakai T, Kronenberg M. 1998. *J. Immunol.* 161:5124– 28

92. Prigozy TI, Naidenko O, Qasba P, Elewaut D, Brossay L, et al. 2001. *Science* 291:664–67

93. Sidobre S, Naidenko OV, Sim BC, Gascoigne NR, Garcia KC, Kronenberg M. 2002. *J. Immunol.* 169: 1340–48

94. Sim BC, Holmberg K, Sidobre S, Naidenko O, Niederberger N, et al. 2003. *Immunogenetics* 54:874–83

95. Cantu C III, Benlagha K, Savage PB, Bendelac A, Teyton L. 2003. *J. Immunol.* 170:4673–82

96. Sidobre S, Hammond KJ, Benazet- Sidobre L, Maltsev SD, Richardson SK, et al. 2004. *Proc. Natl. Acad. Sci. USA* 101:12254–59

97. Leite-De-Moraes MC,HamegA, Arnould A, Machavoine F, Koezuka Y, et al.1999. *J. Immunol.* 163:5871–76

98. Leite-De-Moraes MC, Moreau G, Arnould A, Machavoine F, Garcia C, et al. 1998. *Eur. J. Immunol.* 28:1507–15

99. Brigl M, Bry L, Kent SC, Gumperz JE, Brenner MB. 2003. *Nat. Immunol.* 4:1230–37

100. Chiu YH, Jayawardena J, Weiss A, Lee D, Park SH, et al. 1999*J. Exp. Med.* 189:103– 10

101. Ikarashi Y, Mikami R, Bendelac A, Terme M, Chaput N, et al. 2001. *J. Exp. Med.* 194:1179–86

102. Moody DB, Young DC, Cheng TY, Rosat JP, Roura-Mir C, et al. 2004. *Science* 303:527–31

103. Metelitsa LS, Naidenko OV, Kant A, Wu HW, Loza MJ, et al. 2001. *J. Immunol.* 167:3114–22

104. Miyamoto K, Miyake S, Yamamura T. 2001. *Nature* 413:531–34

105. Stanic AK, Shashidharamurthy R, Bezbradica JS, Matsuki N, Yoshimura Y, et al. 2003. *J. Immunol.* 171:4539–51.

106. Oki S, Chiba A, Yamamura T, Miyake S. 2004. *J. Clin. Invest.* 113:1631–40

107. Schmieg J, Yang G, Franck RW, Tsuji M. 2003. *J. Exp. Med.* 198:1631–41

108. Matsuda JL, Gapin L, Baron JL, Sidobre S, Stetson DB, et al. 2003. *Proc. Natl. Acad. Sci. USA* 100:8395–400

109. Carnaud C, Lee D, Donnars O, Park SH, Beavis A, et al. 1999. *J. Immunol.* 163:4647–50

110. Eberl G, MacDonald HR. 2000. *Eur. J. Immunol.* 30:985 92

111. Arase H, Arase N, Saito T. 1996. *J. Exp. Med.* 183:2391 96

112. Fujii S, Shimizu K, Kronenberg M, Steinman RM. 2002. *Nat. Immunol.* 3:867–74

113. Vincent MS, Leslie DS, Gumperz JE, Xiong X, Grant EP, Brenner MB. 2002. *Nat. Immunol.* 3:1163–68

114. Hermans IF, Silk JD, Gileadi U, Salio M, MathewB, et al. 2003. *J. Immunol.* 171:5140–47

115. Fujii S, Shimizu K, Smith C, Bonifaz L, Steinman RM. 2003. *J. Exp. Med.* 198:267–79

116. Chung Y, Chang WS, Kim S, Kang CY. 2004. *Eur. J. Immunol.* 34:2471–79

117. Kitamura H, Ohta A, Sekimoto M, Sato M, Iwakabe K, et al. 2000. *Cell. Immunol.* 199:37–42

118. Galli G, Nuti S, Tavarini S, Galli- Stampino L, De Lalla C, et al. 2003. *J. Exp. Med.* 197:1051–57

119. Stober D, Jomantaite I, Schirmbeck R, Reimann J. 2003. *J. Immunol.* 170:2540–48

120. Nishimura T, Kitamura H, Iwakabe K, Yahata T, Ohta A, et al. 2000. *Int. Immunol.* 12:987–94

121. Gonzalez-Aseguinolaza G, Van Kaer L, Bergmann CC, Wilson JM, Schmieg J, et al. 2002. *J. Exp. Med.* 195:617–24

122. Kitamura H, Iwakabe K, Yahata T, Nishimura S, Ohta A, et al. 1999. *J. Exp. Med.* 189:1121– 28

123. Tomura M, Yu WG, Ahn HJ, Yamashita M, Yang YF, et al. 1999. *J. Immunol.* 163:93–101

124. Nishikawa H, Kato T, Tanida K, Hiasa A, Tawara I, et al. 2003. *Proc. Natl. Acad. Sci. USA* 100: 10902–6

125. Azuma T, Takahashi T, Kunisato A, Kitamura T, Hirai H. 2003. *Cancer Res.* 63:4516–20

126. Hansen DS, Siomos MA, Buckingham L, Scalzo AA, Schofield L. 2003. *Immunity* 18:391– 402

127. Crowe NY, Smyth MJ, Godfrey DI. 2002. *J. Exp. Med.* 196:119–27

128. Gillessen S, Naumov YN, Nieuwenhuis EE, Exley MA, Lee FS, et al. 2003. *Proc. Natl. Acad. Sci. USA* 100:8874– 79

129. Fuss IJ, Heller F, Boirivant M, Leon F, Yoshida M, et al. 2004. *J. Clin. Invest.* 113:1490–97

130. Exley MA, Bigley NJ, Cheng O, Tahir SM, Smiley ST, et al. 2001. *J. Leukoc. Biol.* 69:713–18

131. Baron JL, Gardiner L, Nishimura S, Shinkai K, Locksley R, Ganem D. 2002. *Immunity* 16:583– 94

132. Durante-Mangoni E,Wang R, Shaulov A, He Q, Nasser I, et al. 2004. *J. Immunol.* 173:2159–66

133. Hammond KJ, Poulton LD, Palmisano LJ, Silveira PA, Godfrey DI, Baxter AG. 1998 *J. Exp. Med.* 187:1047–56

134. Singh AK,Wilson MT, Hong S, Olivares- Villagomez D, Du C, et al. 2001 *J. Exp. Med.* 194:1801– 11

135. Sonoda KH, Faunce DE, Taniguchi M, Exley M, Balk S, Stein-Streilein J. 2001. *J. Immunol.* 166:42–50

136. Beaudoin L, Laloux V, Novak J, Lucas B, Lehuen A. 2002. *Immunity* 17:725–36

137. Smyth MJ, Crowe NY, Pellicci DG, Kyparissoudis K, Kelly JM, et al. 2002. *Blood* 99:1259– 66

138. Heller F, Fuss IJ, Nieuwenhuis EE, Blumberg RS, Strober W. 2002. *Immunity* 17:629– 38

139. Ito K, Karasawa M, Kawano T, Akasaka T, Koseki H, et al. 2000. *Proc. Natl. Acad. Sci. USA* 97:740–44

140. Tupin E, Nicoletti A, Elhage R, Rudling M, Ljunggren HG, et al. 2004. *J. Exp. Med.* 199: 417–22

141. Nakai Y, Iwabuchi K, Fujii S, Ishimori N, Dashtsoodol N, et al. 2004. *Blood* 104:2051–59

142. van der Vliet HJ, von Blomberg BM, Nishi N, Reijm M, Voskuyl AE, et al. 2001. *Clin. Immunol.* 100:144–48

143. Wilson SB, Kent SC, Patton KT, Orban T, Jackson RA, et al. 1998. *Nature* 391:177–81

144. Kukreja A, Cost G, Marker J, Zhang C, Sun Z, et al. 2002. *J. Clin. Invest.* 109:131–40

145. Berzins SP, Kyparissoudis K, Pellicci DG, Hammond KJ, Sidobre S, et al. 2004. *Immunol. Cell. Biol.* 82:247–52

146. Tahir SM, Cheng O, Shaulov A, Koezuka Y, Bubley GJ, et al. 2001. *J. Immunol.* 167:4046–50

147. Araki M, Kondo T, Gumperz JE, Brenner MB, Miyake S, Yamamura T. 2003. *Int. Immunol.* 15:279–88

148. Levy O, Orange JS, Hibberd P, Steinberg S, LaRussa P, et al. 2003. *J. Infect. Dis.* 188:948– 53

149. Nieda M, Okai M, Tazbirkova A, Lin H, Yamaura A, et al. 2004. *Blood* 103:383–89

150. Giaccone G, Punt CJ, Ando Y, Ruijter R, Nishi N, et al. 2002. *Clin. Cancer Res.* 8:3702–9

151. Zhou D, Mattner J, Cantu Iii C, Schrantz N, Yin N, et al. 2004. *Science* 306:1786–89 Annu.

CHAPTER 2 :

Papers

120 In agreement with Italian law 22 april 1941 n. 633, Art. 70

On/off TLR signaling decides pro-inflammatory or tolerogenic dendritic cell maturation upon iNKT cell contact.

Simone Caielli,[1] Cristina Conforti Andreoni,[1] Ester Badami,[1] Denis V. Baev,[1] Maria Luisa Malosio,[2] Marika Falcone.[1,*]

[1]Experimental Diabetes Unit; Division of Immunology, Transplantation and Infectious Diseases San Raffaele Scientific Institute. Via Olgettina 60 - 20132 Milan, Italy

[2]Cell Imaging Unit; Division of Immunology, Transplantation and Infectious Diseases San Raffaele Scientific Institute. Via Olgettina 60 - 20132 Milan, Italy

*Correspondence : falcone.marika@hsr.it

Running title: NKT cell contact and tolerogenic DC maturation

SUBMITTED FOR THE PUBBLICATION TO IMMUNITY

SUMMARY

Mature tolerogenic dendritic cells (tolDCs) maintain immune tolerance towards self-tissues by inducing differentiation and/or expansion of regulatory T cells. The physiological conditions that regulate tolerogenic DC maturation are unknown. We found that cell contact with activated invariant Natural Killer (iNK) T cells directly induced tolerogenic DC maturation via CD1d engagement and activation of the ERK1/2 signaling pathway. iNKT cell-matured DCs showed a tolerogenic cytokine profile with predominant IL-10 secretion, induced ex novo differentiation of Treg cells and promoted immune tolerance in vivo thus preventing autoimmune diabetes in NOD mice. While the iNKT cell-contact under steady-state conditions triggered tolerogenic DC maturation, in the presence of a pathogen-derived signal, i.e. LPS stimulation of TLR4, iNKT cells played a completely opposite effect and enhanced pro-inflammatory DC maturation. Our data emphasize that cell-cell contacts between DCs and other immune cells like iNKT cells do not result in a single adjuvant or suppressive effect but are integrated within a complex network of simultaneous stimuli that orchestrate the final DC decision between tolerance and immunity.

INTRODUCTION

Dendritic cells (DCs) play a central role in the immune system decision to start immunity or maintain peripheral tolerance. DCs perceive environmental stimuli and appropriately modulate adaptive immune responses by acting as antigen-presenting cells and producing different effects on T cell precursors (Banchereau and Steinman, 1998). In the steady state, immature DCs maintain immune tolerance by uploading self-antigens in peripheral tissues and carrying them to the lymphoid organs where they induce anergy of self-reactive T cells that escaped negative selection in the thymus. When DCs receive a maturation signal in the peripheral tissue, e.g. during an infection, they undergo terminal maturation and move to the draining lymph nodes to trigger the activation of naïve T cells and built a protective immune response. A growing body of evidence indicates the existence of a third functional DC subset that in steady-state conditions, i.e. in the absence of danger signals, undertake a tolerogenic maturation pathway leading to expression of intermediate levels of co-stimulatory molecules, secretion of modulatory cytokines like interleukin-10 and capacity to prime regulatory T cells (Treg cells) (Morelli and Thomson, 2007). The signals that drive terminal maturation and pro-inflammatory function of DCs are well known and include toll-like receptor (TLR) engagement by pathogen-derived molecules, modulation by inflammatory cytokines like TNF-a and cognate interaction with other lymphocytes such as NK

cells (Steinman and Banchereau, 2007). Conversely, the mechanisms that promote the maturation of mature tolerogenic DCs (tolDCs) are still undefined. There is indication that conditioning DCs with cytokines like GM-CSF, IL-10, TGF-b and thymic stromal lymphopoietin (TSLP) or with 1,25-dihydroxyvitamin D3 induces their tolerogenic maturation (Besin et al., 2008; Letterio and Roberts, 1998; Penna and Adorini, 2000; Pestka et al., 2004). Disruption of E-cadherin-mediated intercellular adhesion induces tolDC differentiation by breaking DC contact with other cells (Jiang et al., 2007). Although it is clear that tolDCs play a crucial immune regulatory function, the physiological conditions under which immature DCs undergo a tolerogenic maturation pathway are unknown. It has been hypothesized that regulatory T cells could promote tolDC differentiation (Bayry et al., 2007). However, although there is evidence that natural $FoxP3^+$ Treg cells interact with DCs (Tang et al., 2006) and down-modulation their pro-inflammatory maturation (Wing et al., 2008), so far there is no report indicating that the cell-cell interaction with regulatory T cells triggers the tolerogenic maturation of DCs.

Invariant Natural Killer (iNK) T cells represent an important regulatory T cell subset that perceives signals of danger or cellular distress and modulate the adaptive immune response accordingly (Kronenberg, 2005). In the presence of pathogens, iNKT cells acquire an adjuvant function that is fundamental to boost anti-microbial and anti-tumor immunity. In steady-state conditions, iNKT cells perform a regulatory function to maintain

peripheral T cell tolerance towards self-antigens and prevent autoimmune diseases (Miyamoto et al., 2001; Sharif et al., 2001; Yang et al., 2003). Both effects of iNKT cells involve the cell-cell interaction and downstream activation of other immune cells including DCs (Vincent et al., 2002). Because of the pivotal role of DCs in shaping the immune responses, the crosstalk between iNKT cells and DCs has been a field of extensive study but the effect and mechanism of this interaction remains controversial. Some reports showed that iNKT cell activation induced rapid maturation of DCs followed by enhancement of Th1 immune responses (Fujii et al., 2002; Kitamura et al., 1999). In contrast, in vivo studies on experimental model of transplantation and autoimmune diseases clearly demonstrated that iNKT cell activation promoted differentiation of tolerogenic DCs and immune tolerance (Chen et al., 2005; Kojo et al., 2005). Those findings opened questions on how the iNKT cell subset could play opposite effects on DC differentiation and which are the events that regulate the fate of DCs upon iNKT cell modulation.

Here we demonstrated that in steady-state conditions, i.e. in the absence of pathogen-driven signals, intercellular contact with regulatory iNKT cells induces the tolerogenic maturation of DCs. iNKT cell-modulated DCs showed the classical features of tolDCs such as intermediate expression levels of MHC class II and co-stimulatory molecules, a tolerogenic cytokine profile with high secretion of IL-10 and minimal release of pro-inflammatory cytokines IL-12, IL-6 and TNF-a, and capacity to prime

regulatory T cells and prevent autoimmune diabetes *in vivo*. iNKT cells exerted their modulatory effect by establishing a direct cell-cell contact with DCs and activating the ERK1/2 signaling pathway via CD1d engagement. The tolerogenic effect of iNKT cells on DCs was abolished by simultaneous toll-like receptor stimulation. In fact, the concomitant activation of the TLR-4 and CD1d by iNKT cells intensified the pro-inflammatory maturation of DCs dramatically increasing their IL-12 secretion.

The iNKT cell-DC interaction is central for modulation of adaptive immune responses and represents a critical crossroad between T cell immunity and immune tolerance. We demonstrated that the iNKT cell-DC communication does not produce a single adjuvant or downregulatory immune effect but it is integrated in a complex network of concomitant stimuli and intercellular interactions that orchestrate the DC decision to undergo pro-inflammatory or tolerogenic maturation.

RESULTS

Invariant NKT cells Induce Tolerogenic DC Maturation

In vivo activation of iNKT cells through administration of their antigen, a-GalCer, induced immune tolerance through DC modulation (Naumov et al., 2001). Although DCs of a-GalCer-treated mice showed some features of tolerogenic DCs, it remains unclear whether iNKT cells were directly responsible for tolerogenic DC maturation (Chen et al., 2005; Kojo et al., 2005). Although different lymphocyte subsets interact with DCs and directly modulate their maturation and function, so far there is no evidence that a specific lymphocyte subset can directly elicit the tolerogenic maturation of DCs. To assess whether iNKT cells promote differentiation of tolDCs, we performed co-culture experiments with purified iNKT cells and bone marrow-derived myeloid DCs. iNKT cell lines were established in vitro as previously described (Baev et al., 2008) and iNKT cells were purified prior to the addition to DCs (Figure S1A). The expansion and purification procedures did not alter the phenotypical and functional features of iNKT cells that resembled that of ex-vivo iNKT cells (Figure S1B and S1C). A genome-wide microarray analysis performed on $CD11c^+$ DCs after four hours of co-culture with iNKT cells revealed significant DC activation with more than 350 genes differentially regulated (Figure S2A). After 20 hours of co-culture with iNKT cells DCs acquired all the morphological and functional features of mature

DCs. These included the redistribution of MHC class II molecules from the lysosomial compartment to the cell surface (Figure 1A and 1B), the up-regulation of co-stimulatory molecules CD80, CD86, CD200 and PVR (Figure 1B and Figure S2B) and the acquisition of antigen-presenting capacity (Figure S2C). Moreover, nktDCs showed a tolerogenic cytokine profile with high secretion of IL-10 and minimal release of pro-inflammatory cytokines such as IL-12, TNF-a and IL-6 (Figure 1C).

Next, we evaluated whether nktDCs owned the capacity to upload soluble antigens and acquired the migratory properties of tolerogenic DCs. First, we compared the endocytic capacity of nktDCs with that of immature DCs and LPS-matured DCs by measuring the uptake of two fluorescent compounds, the FITC-dextran and the Lucifer Yellow, to assess mannose receptor-mediated and fluid phase endocytosis. DCs matured by iNKT cell modulation were able to upload both dyes thus integrally maintaining the phagocytic properties of immature DCs, a feature that was lost upon TLR-induced maturation (Figure 1D). The key function of steady-state tolerogenic DCs is to transport self-antigens from peripheral tissues to draining lymph nodes where they prime T cells in a tolerogenic fashion. The homeostatic migration of tolDCs is regulated by expression of specific chemokine receptors such as CCR5 and CCR7 (Forster et al., 2008). A cytofluorimetric analysis revealed that nktDCs expressed CCR5 like immature DCs (Figure 1E). At the same time, the iNKT cell-induced maturation significantly up-

regulated the expression of CCR7 thus conferring nktDCs with the capacity of LPS-matured DCs to migrate towards peripheral LN (Figure 1E). All together those data demonstrated that, upon interaction with iNKT cells, immature DCs adopt a unique maturation status with several phenotypical and functional features of tolerogenic DCs.

TolDCs matured by iNKT cells elicited ex-novo differentiation of regulatory T cells and prevented autoimmune diabetes.

The hallmark of tolDCs is their capacity to expand and induce ex novo differentiation of regulatory T cells from naïve T cell precursors and to induce immune tolerance in vivo (Morelli and Thomson, 2007). To demonstrate that nktDCs play such pro-tolerogenic function, we first tested their capacity to promote ex novo differentiation of regulatory T cells. We stimulated naïve $CD4^+$ T cells from TCR transgenic BDC2.5 mice with nktDCs or LPS-matured DCs previously loaded with the BDC2.5 peptide 1040-1051 and assessed whether they acquired two major features of regulatory T cells: anergy and suppressive capacity. Antigenic stimulations of BDC2.5 T cells with LPS-DCs induced significant proliferation (Figure 2A) and cell expansion (Figure S2A). Conversely, BDC2.5 T cells became completely anergic after antigenic stimulation with nktDCs as demonstrated by their lack of expansion (Figure S3A) and failure to proliferate even if challenged with peptide-pulsed LPS-matured DCs (Figure 2A).

Next, we assessed the suppressive capacity of BDC2.5 T cells stimulated by nktDCs. To this aim, we added nktDC-induced BDC2.5 Treg cells to naïve BDC2.5 $CD4^+$ T cells together with peptide-pulsed LPS-DCs as APCs. Our data showed that nktDC-induced Treg cells inhibited proliferation of BDC2.5 $CD4^+$ T cells in a dose dependent manner (Figure 2B). nktDC-induced Treg cells did not belong to previously known Treg cell subsets such as knokn $FoxP3^+CD25^+CD4^+$ Treg or Tr1 cells. Indeed, although they expressed GITR, a typical marker of $CD4^+CD25^+FoxP3^+$ Treg cells, they were FoxP3 negative and did not express other Treg cell markers such as LAG-3 or OX-40 (Figure 2C). Moreover, although they predominantly secreted IL-10 like human Tr1 cells (Figure 2D) (Groux et al., 1997), they also release IL-4, a Th2 cytokine that is not normally secreted by Tr1 cells (Figure S3B) and did not suppress bystander T cell proliferation through IL-10 (Figure 3SC).

Next, we determined whether nktDCs induce peripheral tolerance in vivo by injecting SJL/J mice with PLP peptide-loaded nktDCs or LPS-matured DCs and then challenged them with the same antigen in CFA two days after the DC injection. When we recalled the antigen-specific T cell response in vitro, we observed that antigen-specific T cells from mice immunized with antigen-pulsed nktDCs proliferated poorly as compared to T cells from untreated control mice or mice immunized with LPS-DCs (Figure 3A). To be sure that $CD4^+$ T cell tolerance was related to nktDCs, we tested the tolerogenic properties of

nktDCs in a different immune tolerance setting. TCR transgenic BDC2.5 $CD4^+$ T cells were transferred into non-transgenic NOD mice 24 hr prior to injection untreated, LPS-matured or nktDCs previously previously loaded with the BDC2.5 peptide 1040-1051. $CD4^+$ BDC2.5 T cells injected into untreated NOD mice rapidly reached the PLN as previously shown (Hoglund et al., 1999). When we isolated $CD4^+$ BDC2.5 T cells from PLN and recall their antigen-specific response in vitro, we again observed that $CD4^+$ T cells from mice immunized with nktDCs proliferated significantly less well than did $CD4^+$ T cells from mice immunized with LPS-matured DCs (Figure 3B). In addition, BDC2.5 $CD4^+$ T cells from PLN of nktDC-treated mice showed a regulatory cytokine profile with a predominant IL-10 secretion and minimal release of IFN-g in stark contrast with that of $CD4^+$ T cells from LPS-DC-treated mice that mostly secreted IFN-g with no IL-10 (Figure 3C). As an independent measure of immune tolerance, we asked whether injection of nktDCs could prevent the onset of spontaneous autoimmune diabetes in NOD mice. As shown in Figure 3D, the administration of nktDCs into 4 week-old NOD mice completely prevented autoimmune diabetes. Those results imply that tolerogenic nktDCs reached the pancreatic tissues of NOD mice, carried the islet antigens to PLN and induced islet-specific immune tolerance.

iNKT cell induce tolerogenic maturation of DCs independent of TLR signaling and through activation of the ERK MAPK Signaling Pathway.

TLR signaling is associated with the activation of the transcription factors NFkB and the P38 MAP kinase pathway (Barton and Medzhitov, 2003) and leads to pro-inflammatory DC maturation, IL-12p70 production and Th1 immune response generation. The intracellular cell-signaling network generated within DC by pro-tolerogenic maturation stimuli are completely different from that triggered by TLR (Jiang et al., 2007). Next, we compared the signaling events induced by iNKT cells with those generated by LPS stimulation of TLR4. As expected, LPS induced the phosphorylation of both IkBa (an hallmark of NF-kB activation) and p38 MAP kinases (Figure 4A). In contrast, neither p38 MAP kinases nor IkB was detectably phosphorylated after iNKT cell-modulation (Figure 4A). This excluded that iNKT cell-induced DC maturation was due to contaminating LPS in our cell preparation and suggested that alternative signaling pathways were involved in iNKT cell-induced tolerogenic DC maturation. We asked whether iNKT cells could drive the activation of ERK1/2, a signaling pathway that was previously linked to tolerogenic DC maturation induced by splenic stromal cells or zimosan (Dillon et al., 2006; Tan and O'Neill, 2007). A western blot analysis and intracellular protein staining on $CD11c^{+}$ DCs revealed that nktDCs expressed significantly increased and sustained levels of phosphorylated

ERK1/2 compared to immature DCs. ERK1/2 phosphorylation is also induced by TLR4 stimulation as previously shown but it is dispensable for the LPS-induced pro-inflammatory maturation of DCs (Rescigno et al., 1998), Conversely, ERK1/2 activation was instrumental for the iNKT cell-induced tolerogenic DC maturation and the iNKT cell-modulation in the presence of U0126, a selective inhibitor of ERK1/2 phosphorylation, was completely blocked (Figure 4C and 4D).

iNKT cell-driven tolerogenic maturation of DCs required cell-cell contact and CD1d engagement.

Next, we sought to gain insights into the mechanism by which iNKT cells induced tolerogenic DC maturation. Previous studies postulated that the iNKT cell action on DCs is mediated through secretion of modulatory cytokines (Kojo et al., 2005) or cell-cell contact mechanism (Diana et al., 2009; Vincent et al., 2002). To discriminate between those two possibilities, we added anti-IFN-g, anti-IL-4 and anti-IL-10 receptors mAbs to the iNKT cell-DC co-cultures. None of the cytokine blocking reagents abolished iNKT cell-mediated tolerogenic maturation of DCs, even when all three mAbs were added together (Figure 5SA). Next, we performed the iNKT cell-DC co-culture experiments in transwell to assess the importance of the cell-cell contact. In those experiments, iNKT cells were placed in the upper chamber and physically separated from immature DCs residing in the lower chamber. Strikingly, the tolerogenic maturation of

DCs, i.e. up-regulation of maturation markers MHC II, CD80 and CD86 and secretion of IL-10, was completely inhibited when cell-cell contact between immature DCs and activated iNKT cells was prevented (Figure 5A and 5B). In order to exclude the possibility that, in our transwell cultures, iNKT cells did not modulate DCs because lacked important activating signals from DCs, we added a-GalCer-pulsed LPS-DCs in the upper chamber along with iNKT cells. Once again, the modulatory action of iNKT cells on immature DCs was lost in the absence of intercellular contact (data not shown).

Next, we asked which molecules were involved in the pro-tolerogenic iNKT cell-DC interaction. Previous reports demonstrated that the CD40-CD40L interaction is crucial for the adjuvant effect of iNKT cells on LPS-matured DCs. (Fujii et al., 2004). However, when we performed the iNKT cell-DC co-culture experiments by using CD40 knockout DCs, we observed that the CD40-CD40L was totally dispensable for tolerogenic DC maturation. On the contrary, the absence of CD1d molecule completely inhibited iNKT cell-induced tolerogenic maturation, i.e. up-regulation of MHC II and co-stimulatory molecules (Figure 5A) as well as IL-10 secretion (Figure 5B). CD1d is and MHC class I-like molecule responsible for glycolipid antigen presentation to iNKT cells. Although restriction molecules are primarily known for their role in antigen presentation, additional biological functions have long been reported. For example, cross-linking of MHC class II molecules is able to induce a series of cellular events including proliferation/activation and/or

cell death (Andreae et al., 2003). Along this line, the cross-linking of CD1d molecules produced activating signals within human monocytes and intestinal intraepithelial cells (Colgan et al., 1999; Yue et al., 2005). The short cytosolic domain and the absence of a signaling motif in the CD1d structure appears inconsistent with its capacity to trigger the complex signaling pathway that regulates tolerogenic DC maturation. One possible explanation is that membrane-associated signaling components such as ITAM-containing adapters might provide CD1d molecule with the capacity to transmit intracellular signals within the cell. A Western blot analysis performed on total cell lysate of immature $CD11c^+$ DCs demonstrated that in steady-state DCs express all ITAM-bearing adapters proteins (data not shown) and the FcRg co-precipitated with the CD1d molecule (Figure 5D). These data indicate that, under steady-state conditions, the CD1d molecule is associated with the ITAM-bearing FcRg and forms a stable receptor complex capable to transmit the iNKT cell-induced tolerogenic maturation signal.

Simultaneous TLR signaling and iNKT cell-contact abolished tolerogenic maturation and enhanced pro-inflammatory DC maturation.

We showed that under steady-state conditions the iNKT cell-contact promotes tolerogenic DC maturation. Next, we asked what is the consequence of the iNKT cell-contact on DC maturation in the presence of pro-inflammatory maturation

signals. While $CD4^+CD25^+FoxP3^+$ Treg cells inhibit DC maturation and/or activation elicited by TLR-ligands (Wing et al., 2008), during infections iNKT cells do not inhibit DC maturation, but play a strong adjuvant effect on pro-inflammatory DC maturation (Diana et al., 2009; Vincent et al., 2002). To assess whether iNKT cell-contact synergizes with TLR ligands or countervail pro-inflammatory DC maturation, we performed iNKT cell-DC co-culture experiments in the presence of simultaneous LPS stimulation of TLR4. As shown in figure 6, the presence of iNKT cells during LPS stimulation enhanced the TLR4-ligand effect and significantly intensified the expression of all maturation markers tested (Figure 6A) and IL-12p70 secretion (Figure 6B). Notably, the synergistic effect of iNKT cells was limited to IL-12p70 secretion and the levels of other pro-inflammatory cytokines such as IL-6 and TNF-a were unaltered by the iNKT cell-contact (Figure 6B). As previously shown (Fujii et al., 2004), the CD40-CD40L interaction was crucial for the adjuvant effect of iNKT cells on LPS-induced DC maturation (Figure 6C), thus suggesting that the receptors and molecular pathway involved in the adjuvant effect of iNKT cells on DCs are different from those implicated in their pro-tolerogenic action. Interestingly, both the ERK1/2 and NFkB signaling pathways were activated by iNKT cell-contact in the presence of LPS (Figure 6D).

DISCUSSION

The DC interaction with T cells represents a critical crossroad between immunity and tolerance. During infections and anti-tumoral immunity effector T cells favor DC maturation and pro-inflammatory function (Xu et al., 2007). Conversely, in steady state conditions or when T cell responses are no longer needed, regulatory T cells inhibit DC maturation to maintain immune homeostasis and prevent autoimmunity. The interaction between $CD4^+CD25^+$ Treg cells and DCs takes place under steady-state conditions through the formation of stable clusters in the peripheral lymph nodes (Tang et al., 2006). Although it is clear that classical $CD25^+CD4^+$ Treg cells interact with DCs to down-modulate their pro-inflammatory maturation (Wing et al., 2008), so far there is no direct evidence that regulatory T cells can induce the tolerogenic maturation of DCs. Tolerogenic mature DCs result from the activation of a unique maturation pathway leading to a specific phenotype and functional state with intermediate expression levels of MHC class II and co-stimulatory molecules, secretion of modulatory cytokines IL-10 and TGF-b, and, most importantly, capacity to induce the differentiation of Treg cells and promote immune tolerance (Morelli and Thomson, 2007). Our results provide the first evidence that the cell-cell contact of immature DCs with a regulatory T cell subset, the iNKT cells, can directly trigger their tolerogenic maturation.

iNKT cells are regulatory T cells whose capacity to down-regulate T cell immunity has been clearly demonstrated in several models of immune tolerance induction *in vivo* (Sonoda et al., 1999). For example, the activation of iNKT cells through administration of their antigen, a-Galactosylceramide, protected mice against pre-clinical models of several autoimmune diseases (Miyamoto et al., 2001; Sharif et al., 2001; Yang et al., 2003). There is previous evidence indicating that iNKT cells, like classical $CD4^+CD24^+FoxP3^+$ Treg cells, promote immune tolerance through DC modulation. iNKT cell-mediated protection from autoimmune diabetes was associated with the recruitment of tolerogenic myeloid CD8a DCs within the pancreatic lymph nodes (PLN) of NOD mice. Those DCs transferred immune tolerance towards islet-antigens and prevent diabetes when injected into young NOD mice (Naumov et al., 2001). A subsequent study demonstrated that the stimulation of iNKT cells *in vivo* lead to maturation of DCs with up-regulation of restriction and co-stimulatory molecules (MHC class II, CD80 and CD86) and significant IL-10 secretion (Kojo et al., 2005). Although those findings suggested that activated iNKT cells could promote differentiation of tolDCs, they did not clarified whether iNKT cells play the leading role in the tolerogenic DC maturation. In fact, in those in vivo models, the DC acquisition of tolerogenic features could have been an epiphenomenon related to iNKT cell-induced protection from autoimmunity and reduction of inflammation. Our data clearly indicate that iNKT cells can directly interact with myeloid DCs

through cell-cell contact mechanism and instruct them towards tolerogenic maturation. After four hours from the initial cell-cell contact with iNKT cells, DCs were already activated at the transcriptional level with more than 350 genes differentially regulated compared to immature DCs. At twenty hours from the iNKT cell-contact, DCs had acquired a mature phenotype with typical features of tolDCs including up-regulation of MHC class II and co-stimulatory molecules, secretion of IL-10 and no pro-inflammatory cytokines and, most importantly, capacity to induce immune tolerance and prevent autoimmune disease, i.e. diabetes in NOD mice. Interestingly, tolerogenic nktDCs showed all the phenotypical and functional traits of other mature tolDCs such as the one triggered by alterations in E-cadherin-mediated cell-cell adhesion or by in vitro culturing with TSLP or IL-10 as well as intestinal DCs residing in the lamina propria (Besin et al., 2008; Coombes et al., 2007; Jiang et al., 2007; Wakkach et al., 2003). nktDCs share also a unique expression pattern of chemokine receptors with other mature tolDCs. In fact, nktDCs as other tolDCs expressed the CCR5 chemokine receptor that directs their homing towards peripheral tissues where they upload self-proteins. At the same time, tolerogenic nktDCs up-regulated the CCR7 receptor that drives them towards peripheral lymph nodes where they prime self-reactive T cells and induce the ex novo differentiation of inducible Treg cells or expansion of naturally occurring Treg cells.

The Treg cells induced in vitro and in vivo by antigen-presentation with nktDCs shared many phenotypical and

functional features with human IL-10-secreting regulatory Tr1 cells like a predominant IL-10-secreting cytokine profile and strong suppressive capacity upon effector autoreactive T cells (Nature 1997). However, in contrast with human Tr1 cells whose IL-4 release is consistently undetectable, a large fraction of the Treg cells induced by nktDCs secreted IL-4.

Originally, the observation that iNKT cells release large amounts of cytokines upon TCR-stimulation led to the hypothesis that cytokines are instrumental for iNKT cells to mediate their immune regulatory function. Specifically, it was proposed that iNKT cells could down-regulated Th1 cell immunity through secretion of Th2 cytokines IL-4, IL-5 and IL-13 and down-modulatory cytokines IL-10 and TGF-□. Although that hypothesis was weakened by the observation that regulatory iNKT cells secrete significant amounts of the pro-inflammatory IFN-g, it was legitimate to suppose that iNKT cell-secretion of IL-10, a cytokine able to directly trigger tolerogenic DC maturation (Levings et al., 2005), was implicated in the differentiation of tolerogenic DCs (Taniguchi JI 2005). Here we clearly showed that iNKT cell-modulation of DCs did not required neither IL-10 nor other cytokines such as IL-4 or IFN-□ but was mediated by the direct contact between the two cell subsets. However, IL-10 could be involved downstream in the immune regulatory process triggered by iNKT cells thus explaining its requirement for iNKT cell-induced immune tolerance in vivo (Kojo et al., 2005; Sonoda et al., 2001).

The relevance of the iNKT cell-DC contact in the adjuvant function of iNKT cells was demonstrated by others (Diana et al., 2009; Vincent et al., 2002). Here we established that cell-cell contact of iNKT cells with DCs is the mechanism that underlies the pro-tolerogenic iNKT cell function and identified the molecules involved in such intercellular interaction. We found that the CD1d molecule is crucial for iNKT cell to bind immature DCs and trigger their tolerogenic maturation. There is previous reports indicating that the CD1d molecule does not only acts as restriction molecule to present glycolipid antigens to iNKT cells, but can also transmit intracellular activating signals within the cell. Specifically, CD1d ligation on intestinal epithelial cells induced the release of IL-10 and dampened pro-inflammatory signals in the intestinal mucosa. Interestingly, that report indicated that the short cytoplasmic tail of CD1d was required for signaling through surface CD1d and was blocked by a tyrosine kinase inhibitor (Colgan et al., 1999). Our data extended that finding and demonstrated that the cytoplasmic portion of the CD1d molecule physically associate with an ITAM-containing adapter, FcRg, containing tyrosine that is phosphorilated by Src family kinases and trasmits activating signal within the cell. This observation supports the idea that the CD1d molecule can function as a transmembrane receptor and be directly responsible for the transmission of a pro-tolerogenic maturation signal from a yet unidentified ligand on the iNKT cell surface towards the immature DC.

Our results contribute to answer another important open question in the field of iNKT cell biology: how can a single immune cell subset play a dual function? It has been postulated that iNKT cells can acquire either regulatory or adjuvant phenotype and function according to the strength of the antigenic stimulation, the cytokine mircoenvironment and accessory co-stimulatory signals (Kronenberg, 2005). According to that theory the two iNKT cell functional subsets exist and play different roles by secreting specific sets of cytokines: IL-10, TGF-b and Th2 cytokines for the regulatory iNKT cells and pro-inflammatory cytokines IFN-g, IL-12 and IL-17 for the adjuvant iNKT cells. Here we showed that the same iNKT cells subset that release a diverse array of cytokines can play a dual function on DCs and promote either their pro-inflammatory or tolerogenic maturation. The nature of the DC maturation produced by iNKT cells was the result of the integration between the CD1d ligation, directly triggered by iNKT cell-contact, with other physiological stimuli simultaneously acting on DCs. In steady-state conditions, the iNKT cell-induced triggering of the CD1d receptor complex switched on the ERK1/2 signaling pathway leading to the tolerogenic DC maturation. In the same experimental setting, the concomitant CD1d ligation by iNKT cell-contact and TLR-4 triggering enhanced the pro-inflammatory DC maturation. Interestingly, while the CD40-CD40L interaction was dispensable for the pro-tolerogenic action of iNKT cells on DCs,

it was necessary for the iNKT cell adjuvant effect as previously demonstrated (Fujii et al., 2004).

The iNKT cell function is to enter in contact and activate DCs. However, the final decision to induce protective immunity or immune tolerance is on the DC side. DCs integrate different signals, including iNKT cell-modulation, and then appropriately orchestrate immune responses to either promote resistance to infections or tolerate self-antigens. Cellular therapy with DCs could have important medical implications (Steinman and Banchereau, 2007) but requires full knowledge of the physiological conditions that regulate the differentiation of tolerogenic or pro-inflammatory DCs. Here we present evidence that activated iNKT cells triggers the tolerogenic DC maturation through CD1d ligation and activation of the ERK1/2 signaling pathway. A better understanding of the different molecules and pathways involved in the differentiation of mature tolerogenic DCs could pave the way to novel therapeutic approaches to silence immune responses in allergy, autoimmunity and transplant rejection.

EXPERIMENTAL PROCEDURES

Reagents and antibodies

For flow cytometry: anti-CD86, CD80, CD40, MHCII I-A^b, CD11c, TCRb, CD4, biotin and PE anti-mouse IgG1, IFN-g, IL-4 IL-10 were from BD PharMingen. Anti-CCR7, CCR5, CD152, GITR, LAG3, FR4, OX-40, PVR, CD200 were from Biolegend. Anti-p-ERK1/2 Alexa-647 were from Cell Signaling Technology. Anti-mouse FoxP3 was from eBioscience. For blocking experiments: anti-TCRb, anti-LAG3, anti-IL10, anti-IL4 and anti-IFNg were from Biolegend. For western blot and immunoprecipitation: anti-CD1d was form BD PharMingen; anti-FcRg, anti-p-ERK1/2, anti-p-P38, anti-p-IkBa were from Santa Cruz Biotechnology; anti-GAPDH was from Cell Signaling Technology; anti-phosphotyrosine was from Biolegend. For immunofluorescence microscopy: anti-CD11c and anti-MHCII I-A^b were form BD PharMingen; anti-LAMP2 was from Abcam; fluorochrome-conjugated secondary antibodies were from Jackson Immunoresearch. Other reagents: recombinant dimeric mouse CD1d:Ig fusion protein (DimerX®) was from BD PharMingen. rmGM-CSF, rmFlt3-L, CFSE®, TRIzol® reagent and SuperScript-III® cDNA synthesis kit were from Invitrogen. Lipopolysaccharides (LPS), FITC-dextran, Lucifer Yellow and all chemicals suppliers were from Sigma-Aldrich. U0126 was a gift from Dr. Angela Palmigiano (San Raffaele Scientific Institute, Milan). BDC2.5 peptide (YVRPLWVRME) was purchased from PRIMM (Milan, Italy). PLP peptide

(EKLIETYFSKNYQD) was a gift from Roberto Furlan (San Raffaele Scientific Institute, Milan). a-GalCer was form Alexis Biochemical. rmIL-7, rmIL-15, rmIL-2 and Leucocyte Activation Cocktail were from BD PharMingen. Anti-CD11c, anti-CD4 and anti-Biotin microbeads were from Miltenyi Biotechnology.

Mice

C57BL/6, SJL/J and NOD/Ltj mice were purchased from Charles River Laboratories. BDC2.5/NOD mice were bred in the animal facility at San Raffaele Scientific Institute. $CD1d^{-/-}$ and $CD40^{-/-}$ C57BL/6 mice were provided by Paolo Dellabona (San Raffaele Scientific Institute, Milan). All mice were maintained under specific pathogen-free conditions in the animal facility at San Raffaele Scientific Institute and all experiments were conducted in accordance with the Institutional Animal Care and Use Committee.

DCs generation and modulation

Bone marrow cells were cultured in RMPI-5 supplemented with 10 ng/ml of rmGM-CSF and rmFlt3-L. After 6 days non-adherent cells were removed and the adherent fraction containing > 90% $CD11c^{+}$ was stimulated with LPS (1 mg/ml), co-cultured with iNKT cells (1:2 DC:NKT cell ratio) or left untreated (immature DCs). In some experiments U0126 (10 mM), neutralizing anti-TCRb, anti-LAG3, anti-IL10, anti-IL4 or anti-IFNg (10 mg/ml) mAbs were added to the co-cultures. For the Transwell experiments, iNKT cells were placed in the upper chamber of a Transwell insert (Corning) while DCs adhered to the well in the lower chamber.

Flow cytometric analysis

Cells were stained for 30 min on ice with fluorochrome-conjugated primary antibodies, washed and then analyzed on a FACS-Calibur (Becton Dickinson). For intracellular cytokines staining of T cells, BDC2.5 T cells were rested for 7 days after antigenic stimulation, stimulated with Leucocyte Activation Cocktail for 4-6 hrs and then subjected to intracellular staining with BD Cytofix/Cytoperm kit (BD PharMingen) following manufacturer's protocol. For intracellular p-ERK1/2 determination, nktDCs were collected at different time-points and stained for surface CD11c. Then, cells were fixed with 4% PFA, permeabilized with 95% ice-cold methanol and subsequently incubated with anti-p-ERK1/2 Alexa-647 antibody and FACS analyzed.

Endocytosis

DCs were incubated in complete RPMI-5 (1 ml) containing FITC-dextran or with Lucifer Yellow for 60 min. Afterward incubation, cells were collected, washed extensively in FACS buffer, stained with CD11c-APCand FACS analyzed.

Cytokines determination

The levels of cytokines in the culture supernatans were measured with BD Flex Set technology and FACSCanto-II (BD Pharmingen) following manufacturer's protocol. Data were analyzed with FCAP Array software v1.0.1 (Soft Flow).

Western blotting and immunoprecipitation

DCs were homogenized for 30 min in ice-cold RIPA buffer. Total cell lysates (30-60 mg of total proteins) were boiled in

Laemmli buffer and subjected to 12% SDS-PAGE before being immunoblotted with phospho-specific mAbs. For immunoprecipitation, total cell lysate was precleared with normal-rat IgG/protein G-agarose before being subjected to immunoprecipitation with anti-CD1d, or isotype matched antibody, overnight at 4℃. Immunoprecipitates were collected, washed extensively in PBS, boiled in Laemmli buffer and subjected to 15% SDS-PAGE before being immunoblotted with anti-FcRg antibody.

Real Time RT-PCR

Total RNA was isolated from CD11c positive cells (purity >98%) by using TRIzol reagent and converted into cDNA by reverse transcription by using SuperScript III kit (Invitrogen) according to the manufacturer's instructions. Quantitative real-time RT-PCR was carried out with the DNA Engine Opticon 2 real-time detection system and SYBR Green system, and data were normalized by the expression levels of b-actin.

Immunofluorescence analysis

Cells were fixed in 4% PFA and permeabilized with 0.2% Triton X-100 followed by 30 min each with primary antibody and secondary antibody, labeled with appropriate fluorochrome, before mounting in Fluoromount-G solution (Southern Biotech). Confocal microscopy was performed with Perkin Elmer Ultraview ERS microscope with a 63x oil immersion objective. Images were processed with Adobe® Photoshop CS (Adobe).

BDC2.5 CD4 T cell lines generation and characterization

Naïve BDC2.5 CD4 T cells were cultured with the different DCs (LPS-matured DCs or nktDCs) previously irradiated and loaded with the BDC2.5 peptide 1040-1051. 50 U/ml rmIL-2 was added to the cell lines and replaced every 3-4 days. After two rounds of antigenic stimulation, the BDC2.5 T cells were analyzed for their suppressive capacity and anergic state. Briefly, the BDC2.5 T cells were antigen-stimulated with peptide-loaded LPS-matured BMDC and their proliferation was measured by mean of $[H]^3$ thymidine incorporation in the last 18 hours of a three-day period. To test for the capacity of nktDC-induced Treg cells to suppress proliferation of naïve effector T cells, BDC2.5 $CD4^+$ T cells were freshly isolated from BDC2.5 mice and stimulated with peptide-loaded LPS-matured BMDC in the presence of increasing numbers of nktDC-Treg cells. In some experiments neutralizing anti-IL-10 or anti-IL-4 mAbs (10 mg/ml) were added. In parallel experiments, naïve BDC2.5 T cells were labeled with 5 mM CFSE following the manufacturer protocol, stimulated with peptide-loaded LPS-matured BMDC in the presence of increasing numbers of nktDC-Treg cells and their proliferation was evaluated by mean of CSFE dilution.

In vivo tolerance induction

For in vivo tolerance induction 6 week-old SJL/J mice were injected intravenously with 10^6 LPS-matured or nktDCs, pulsed in vitro with PLP peptide, or with PBS alone. Two days later mice were immunized subcutaneously with 100 mg PLP peptide in CFA. Two weeks later total cells from pooled draining lymph nodes (10^5 cells) were incubated with increasing concentrations

of peptide in a 96 well plate. Proliferation of T cells was measured by incorporation of ^{3}[H]-thymidine during the last 18 hrs of 96 hour-incubation. For in vivo induction of regulatory $CD4^{+}$ T cells, 2.5 x 10^{6} naïve BDC2.5 $CD4^{+}$ T cells were injected intravenously into the lateral tail vein of 4 week-old NOD mice. 24 hrs later, 10^{6} DCs previously loaded with the BDC2.5 peptide antigen 1040-1051 were injected into the same mice. 7 days after the DC injection, PLN were collected and $CD4^{+}$ T cells were purified with magnetic microbead-conjugated anti-CD4 mAb and stimulated in vitro with BDC2.5 peptide-loaded or unloaded LPS-matured DCs (1 X 10^{5}). Supernatants were collected after 72 hrs and different cytokine concentration was determined as described above. Cells proliferation was measured by mean of ^{3}[H]-thymidine incorporation during the last 18 hours of a 72 hour-period.

Diabetes protection

Three groups of 4 week-old female NOD mice received one intravenous injection of LPS-matured DCs, nktDCs (10^{6} cells/mouse) or PBS vehicle alone (200ml/mouse). Diabetes was monitored by testing blood glucose levels with Accu-Check glucometer (Roche Diagnostic). The animals were considered diabetic after two consecutive blood glucose measurements > 250 mg/dl.

Statistics

Statistical analysis of the results between groups was performed by Student's *t* test. The log-rank test was used for

the comparison of diabetes incidence rates. p values < 0,05 were considered statistically significant.

ACKNOWLEDGMENTS

We thank Massimo Degano for critical reading of the manuscript and Carlo Ruberto for technical assistance. We also thank Marianna Esposito and Roberto Furlan for experiments with SJL/J mice and Giulia Casorati and Paolo Dellabona for *CD1d*$^{-/-}$ and *CD40*$^{-/-}$ mice. This work was supported by the NIH (R01DK065128-01A2), the Juvenile Diabetes Research Foundation and the Italian Telethon Foundation.

REFERENCES

Andreae, S., Buisson, S., and Triebel, F. (2003). MHC class II signal transduction in human dendritic cells induced by a natural ligand, the LAG-3 protein (CD223). Blood *102*, 2130-2137.

Baev, D.V., Caielli, S., Ronchi, F., Coccia, M., Facciotti, F., Nichols, K.E., and Falcone, M. (2008). Impaired SLAM-SLAM homotypic interaction between invariant NKT cells and dendritic cells affects differentiation of IL-4/IL-10-secreting NKT2 cells in nonobese diabetic mice. J Immunol *181*, 869-877.

Banchereau, J., and Steinman, R.M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245-252.

Barton, G.M., and Medzhitov, R. (2003). Toll-like receptor signaling pathways. Science *300*, 1524-1525.

Bayry, J., Triebel, F., Kaveri, S.V., and Tough, D.F. (2007). Human dendritic cells acquire a semimature phenotype and lymph node homing potential through interaction with CD4+CD25+ regulatory T cells. J Immunol *178*, 4184-4193.

Besin, G., Gaudreau, S., Menard, M., Guindi, C., Dupuis, G., and Amrani, A. (2008). Thymic stromal lymphopoietin and thymic stromal lymphopoietin-conditioned dendritic cells induce regulatory T-cell differentiation and protection of NOD mice against diabetes. Diabetes *57*, 2107-2117.

Chen, Y.G., Choisy-Rossi, C.M., Holl, T.M., Chapman, H.D., Besra, G.S., Porcelli, S.A., Shaffer, D.J., Roopenian, D.,

Wilson, S.B., and Serreze, D.V. (2005). Activated NKT cells inhibit autoimmune diabetes through tolerogenic recruitment of dendritic cells to pancreatic lymph nodes. J Immunol *174*, 1196-1204.

Colgan, S.P., Hershberg, R.M., Furuta, G.T., and Blumberg, R.S. (1999). Ligation of intestinal epithelial CD1d induces bioactive IL-10: critical role of the cytoplasmic tail in autocrine signaling. Proc Natl Acad Sci U S A *96*, 13938-13943.

Coombes, J.L., Siddiqui, K.R., Arancibia-Carcamo, C.V., Hall, J., Sun, C.M., Belkaid, Y., and Powrie, F. (2007). A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med *204*, 1757-1764.

Diana, J., Griseri, T., Lagaye, S., Beaudoin, L., Autrusseau, E., Gautron, A.S., Tomkiewicz, C., Herbelin, A., Barouki, R., von Herrath, M.*, et al.* (2009). NKT cell-plasmacytoid dendritic cell cooperation via OX40 controls viral infection in a tissue-specific manner. Immunity *30*, 289-299.

Dillon, S., Agrawal, S., Banerjee, K., Letterio, J., Denning, T.L., Oswald-Richter, K., Kasprowicz, D.J., Kellar, K., Pare, J., van Dyke, T.*, et al.* (2006). Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance. J Clin Invest *116*, 916-928.

Forster, R., Davalos-Misslitz, A.C., and Rot, A. (2008). CCR7 and its ligands: balancing immunity and tolerance. Nat Rev Immunol *8*, 362-371.

Fujii, S., Liu, K., Smith, C., Bonito, A.J., and Steinman, R.M. (2004). The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. J Exp Med *199*, 1607-1618.

Fujii, S., Shimizu, K., Kronenberg, M., and Steinman, R.M. (2002). Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. Nat Immunol *3*, 867-874.

Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J.E., and Roncarolo, M.G. (1997). A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature *389*, 737-742.

Hoglund, P., Mintern, J., Waltzinger, C., Heath, W., Benoist, C., and Mathis, D. (1999). Initiation of autoimmune diabetes by developmentally regulated presentation of islet cell antigens in the pancreatic lymph nodes. J Exp Med *189*, 331-339.

Jiang, A., Bloom, O., Ono, S., Cui, W., Unternaehrer, J., Jiang, S., Whitney, J.A., Connolly, J., Banchereau, J., and Mellman, I. (2007). Disruption of E-cadherin-mediated adhesion induces a functionally distinct pathway of dendritic cell maturation. Immunity *27*, 610-624.

Kitamura, H., Iwakabe, K., Yahata, T., Nishimura, S., Ohta, A., Ohmi, Y., Sato, M., Takeda, K., Okumura, K., Van Kaer, L.*, et al.* (1999). The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and

IL-12 receptor expression on NKT cells. J Exp Med *189*, 1121-1128.

Kojo, S., Seino, K., Harada, M., Watarai, H., Wakao, H., Uchida, T., Nakayama, T., and Taniguchi, M. (2005). Induction of regulatory properties in dendritic cells by Valpha14 NKT cells. J Immunol *175*, 3648-3655.

Kronenberg, M. (2005). Toward an understanding of NKT cell biology: progress and paradoxes. Annu Rev Immunol *23*, 877-900.

Letterio, J.J., and Roberts, A.B. (1998). Regulation of immune responses by TGF-beta. Annu Rev Immunol *16*, 137-161.

Levings, M.K., Gregori, S., Tresoldi, E., Cazzaniga, S., Bonini, C., and Roncarolo, M.G. (2005). Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25+CD4+ Tr cells. Blood *105*, 1162-1169.

Miyamoto, K., Miyake, S., and Yamamura, T. (2001). A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. Nature *413*, 531-534.

Morelli, A.E., and Thomson, A.W. (2007). Tolerogenic dendritic cells and the quest for transplant tolerance. Nat Rev Immunol *7*, 610-621.

Naumov, Y.N., Bahjat, K.S., Gausling, R., Abraham, R., Exley, M.A., Koezuka, Y., Balk, S.B., Strominger, J.L., Clare-Salzer, M., and Wilson, S.B. (2001). Activation of CD1d-restricted T cells protects NOD mice from developing diabetes by regulating dendritic cell subsets. Proc Natl Acad Sci U S A *98*, 13838-13843.

Penna, G., and Adorini, L. (2000). 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. J Immunol *164*, 2405-2411.

Pestka, S., Krause, C.D., Sarkar, D., Walter, M.R., Shi, Y., and Fisher, P.B. (2004). Interleukin-10 and related cytokines and receptors. Annu Rev Immunol *22*, 929-979.

Rescigno, M., Martino, M., Sutherland, C.L., Gold, M.R., and Ricciardi-Castagnoli, P. (1998). Dendritic cell survival and maturation are regulated by different signaling pathways. J Exp Med *188*, 2175-2180.

Sharif, S., Arreaza, G.A., Zucker, P., Mi, Q.S., Sondhi, J., Naidenko, O.V., Kronenberg, M., Koezuka, Y., Delovitch, T.L., Gombert, J.M.*, et al.* (2001). Activation of natural killer T cells by alpha-galactosylceramide treatment prevents the onset and recurrence of autoimmune Type 1 diabetes. Nat Med *7*, 1057-1062.

Sonoda, K.H., Exley, M., Snapper, S., Balk, S.P., and Stein-Streilein, J. (1999). CD1-reactive natural killer T cells are required for development of systemic tolerance through an immune-privileged site. J Exp Med *190*, 1215-1226.

Sonoda, K.H., Faunce, D.E., Taniguchi, M., Exley, M., Balk, S., and Stein-Streilein, J. (2001). NK T cell-derived IL-10 is essential for the differentiation of antigen-specific T regulatory cells in systemic tolerance. J Immunol *166*, 42-50.

Steinman, R.M., and Banchereau, J. (2007). Taking dendritic cells into medicine. Nature *449*, 419-426.

Tan, J.K., and O'Neill, H.C. (2007). Concise review: Dendritic cell development in the context of the spleen microenvironment. Stem cells (Dayton, Ohio) *25*, 2139-2145.

Tang, Q., Adams, J.Y., Tooley, A.J., Bi, M., Fife, B.T., Serra, P., Santamaria, P., Locksley, R.M., Krummel, M.F., and Bluestone, J.A. (2006). Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. Nat Immunol *7*, 83-92.

Vincent, M.S., Leslie, D.S., Gumperz, J.E., Xiong, X., Grant, E.P., and Brenner, M.B. (2002). CD1-dependent dendritic cell instruction. Nat Immunol *3*, 1163-1168.

Wakkach, A., Fournier, N., Brun, V., Breittmayer, J.P., Cottrez, F., and Groux, H. (2003). Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. Immunity *18*, 605-617.

Wing, K., Onishi, Y., Prieto-Martin, P., Yamaguchi, T., Miyara, M., Fehervari, Z., Nomura, T., and Sakaguchi, S. (2008). CTLA-4 control over Foxp3+ regulatory T cell function. Science *322*, 271-275.

Xu, J., Chakrabarti, A.K., Tan, J.L., Ge, L., Gambotto, A., and Vujanovic, N.L. (2007). Essential role of the TNF-TNFR2 cognate interaction in mouse dendritic cell-natural killer cell crosstalk. Blood *109*, 3333-3341.

Yang, J.Q., Saxena, V., Xu, H., Van Kaer, L., Wang, C.R., and Singh, R.R. (2003). Repeated alpha-galactosylceramide administration results in expansion of NK T cells and alleviates inflammatory dermatitis in MRL-lpr/lpr mice. J Immunol *171*, 4439-4446.

Yue, S.C., Shaulov, A., Wang, R., Balk, S.P., and Exley, M.A. (2005). CD1d ligation on human monocytes directly signals rapid NF-kappaB activation and production of bioactive IL-12. Proc Natl Acad Sci U S A *102*, 11811-11816.

FIGURE LEGENDS

Figure 1. iNKT cells induce mature tolerogenic DCs

A) nktDCs exhibit MHC class II molecules redistribution. DCs from different treatments were fixed with 4% PFA, permeabilized with 0,2% Triton X-100 and labeled with CD11c (blue), MHCII (green) and LAMP2 (red).

B) nktDCs express intermediate levels of maturation markers. $CD11c^+$ were assayed for the expression of the indicated markers by flow cytometry. Dotted lines represent staining with isotype controls.

C) nktDCs produce IL-10. The indicated cytokines in nkt-DC supernatants were assessed by BD Flex Set. Results are the mean +/- SD from one representative experiment out of four.

D) nktDCs retained a phagocytic capacity. $CD11c^+$ were assayed for endocytosis of FITC-Dextran or Lucifer Yellow by flow cytometry. Dotted lines represent cells with medium alone.

E) nktDCs acquire a tolerogenic chemokine receptor pattern. DCs were stained with CD11c and CCR5 or CCR7 mAbs. Dotted lines represent staining with isotype controls. [*, $p<0.0001$].

Figure 2. Tolerogenic nktDCs elicit de novo differentiation of regulatory T cells

A) T cells antigen-stimulated with nktDCs are anergic. After two rounds of antigenic stimulation with LPS-matured or nktDCs,

BDC2.5 $CD4^+$ T cells were stimulated with peptide-loaded (filled square) or unloaded (open square) LPS-DCs. Proliferation was measured by mean of ^{3}[H]-thymidine incorporation. Results are the mean +/- SD from one triplicate experiment out of four.

B) nktDC-induced Treg cells show suppressive capacity. nktDC-induced BDC2.5 $CD4^+$ Treg cells were added at different ratios to naïve BDC2.5 $CD4^+$ T cells in the presence of peptide-loaded LPS-DCs. T cells proliferation was determined by ^{3}[H]-thymidine incorporation (Top) or by CFSE dilution assay (Bottom). Results are the mean +/- SD from one triplicate experiment out of four.

C) nktDC-induced Treg cells express nTreg markers. nktDC-Treg cells were assessed for the expression of nTreg markers. $CD4^+$ $CD25^-$ T cells were used as negative controls (Teff cells) and $CD4^+$ $CD25^+$ as positive controls (nTreg cells). Results are the mean +/- SD from two independent experiments.

D) nktDC-Treg cells secrete IL-10. Top: 72 hrs after antigenic stimulation with the different DC types, supernatants were collected and assessed for IL-10 and IFN-g secretion. Bottom: nktDC-Treg cells were activated with Leucocyte Activation Cocktail and assessed for intracellular cytokines. Values indicate the percentage of positive cells in the indicated quadrant. Results are the mean +/- SD from one triplicate experiment out of four independent experiments.

Figure 3. nktDCs are tolerogenic in vivo

A) nktDCs induce peripheral tolerance in vivo. SJL/J mice were injected i.v. with PLP peptide-loaded LPS-matured or nktDCs (10^6 cells/mouse) or PBS vehicle alone. Two days later the mice were challenged with 100 mg of PLP peptide in CFA. After 2 weeks, peptide-specific T cell response was tested on total draining LN cells by mean of ^{3}[H]-thymidine incorporation. Results are the mean +/- SD from one representative experiment out of two.

B)-C) DCs induce anergic IL-10 T cells in vivo. LPS-matured or nktDCs were loaded with BDC2.5 peptide in vitro and injected (10^6 cells/mouse) intravenously into NOD mice previously treated with BDC2.5 CD4 T cells (2.5 x 10^6 cells/mouse). One week later the proliferative response (A) or the cytokine profile (B) of $CD4^+$ T cells from pancreatic LN were tested. Results are the mean +/- SD from one representative experiment out of three.

D) nktDCs protect NOD mice from autoimmune diabetes. 4 week-old NOD female mice received a single i.v. injection of PBS (n = 5), LPS-matured DCs (10^6 cells/mouse; n=7) or nktDCs (10^6 cells/mouse; n=7). Diabetes was diagnosed after two consecutive measurements of glycemia > 250 mg/dl [*, $p<0.001$; **, $p<0.0001$].

Figure 4. ERK1/2 pathway is activated within the DC by the iNKT cell-modulation

A) The P38 MAPK and NFkB signaling pathways are not activated by iNKT cell-modulation. Total cell lysates from

different treatments were analyzed for phospho-p38 MAPK (left) or phospho-IkBa (right).

B) iNKT cells induce the activation of ERK1/2 pathway. DCs were co-cultured with iNKT cells for 0, 30 or 60 minutes. The expression of p-ERK1/2 was evaluated by western blot (left) or by flow cytometry (right).

C) – D) ERK1/2 inhibition block the iNKT cell-induced tolerogenic DC maturation. DCs were co-cultured with iNKT cells in the presence of ERK inhibitor U0126 (10 mM). The expression levels of the maturation markers (C) and the IL-10 production (D) were then analyzed. b-actin or GAPDH were used as a positive controls. Data are the mean +/- SD from one representative experiment out of three independent experiments. [*, $p<0.0001$]

Figure 5. iNKT cells modulate DCs with a cell-to-cell contact and CD1d-dependent mechanism

A) – B) iNKT cells modulation requires cell-cell contact and the CD1d molecule. To assess the role of cell-cell contact on iNKT cells modulation iNKT cells were added directly to DCs or physically separated in a Transwell system. To assess the role of CD1d and CD40 molecules on iNKT cells modulation we used DCs form wildtype, CD40 or CD1d-ko mice. The expression levels of the maturation markers (A) and the IL-10 production (B) were analyzed. b-actin was used as positive control. Results are the mean +/- SD from one triplicate experiment out of three.

C) The CD1d molecule associates with the ITAM-bearing adapter FcRg. Total cell lysate was immunoprecipitated with anti-CD1d or isotype-matched mAbs. Immunoprecipitates were resolved on 15% SDS-PAGE before being immunoblotted with anti-FcRg mAb. [*, $p<0.0001$]

Figure 6. iNKT cells synergize with LPS in triggering pro-inflammatory DC maturation

A) iNKT enhanced the LPS-induced expression of maturation markers on DCs. DCs were stimulated with medium, iNKT cells alone (1:2 DC:NKT ratio), LPS alone (1mg/ml) or iNKT cells plus LPS and FCAS analyzed. Dotted lines represent staining with isotype control mAbs.

B) iNKT cells increased IL-12p70 secretion on LPS-stimulated DCs. DCs were treated as in A) and supernatants were analyzed for the presence of IL-12p70, TNF-a and IL-6. Results are the mean +/- SD from one triplicate experiment out of two.

C) The absence of CD40 impairs the synergetic effect of iNKT cells on LPS-matured DCs. DCs from wild type or CD40-KO mice were treated as described and the levels of IL-12p70 were assessed. Results are the mean +/- SD from one triplicate experiment out of two.

D) Simultaneous iNKT cell-contact and LPS-stimulation of DCs activates both ERK1/2 and NFkB signaling pathways. Total cell lysates from different treatments were analyzed by immunoblotting with anti-phospho-p38 MAPK or anti-phospho-ERK1/2. GAPDH was used as a positive control. Data are

representative of two independent experiments. [*, $p<0.0001$; **, $p<0.01$].

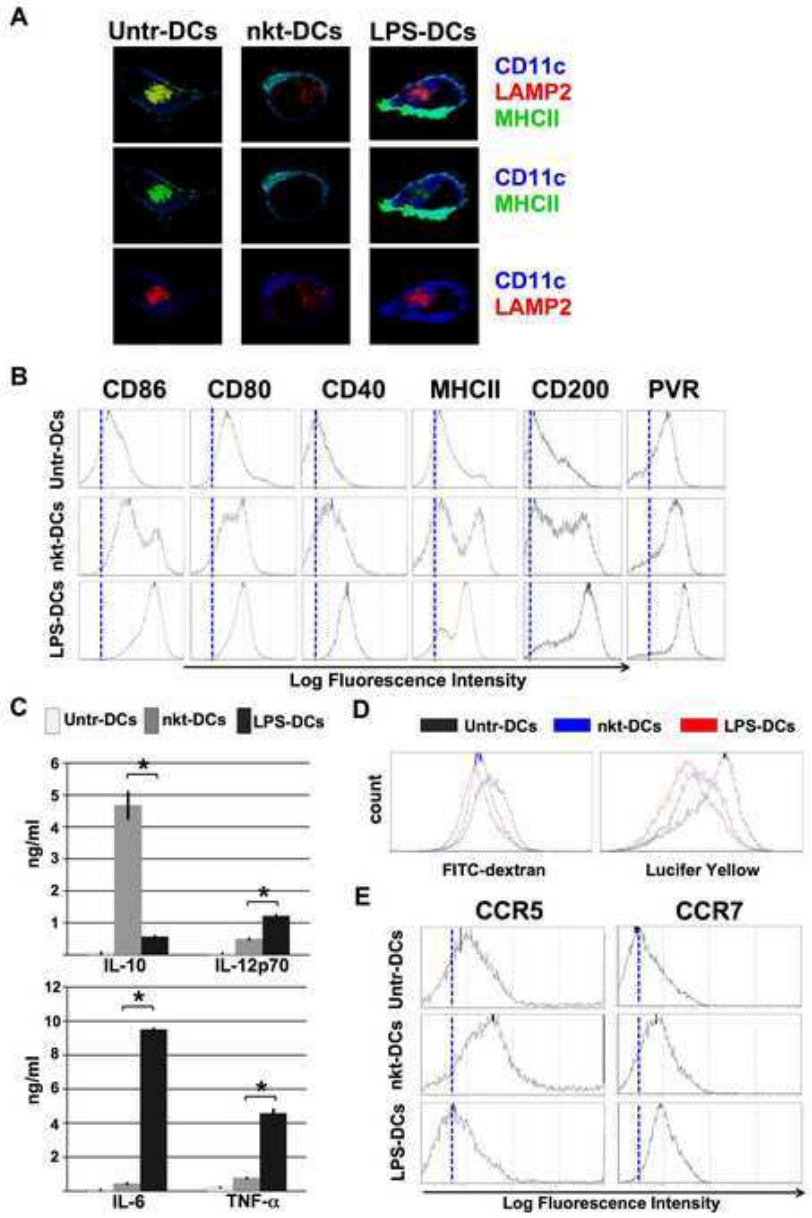

Figure 1

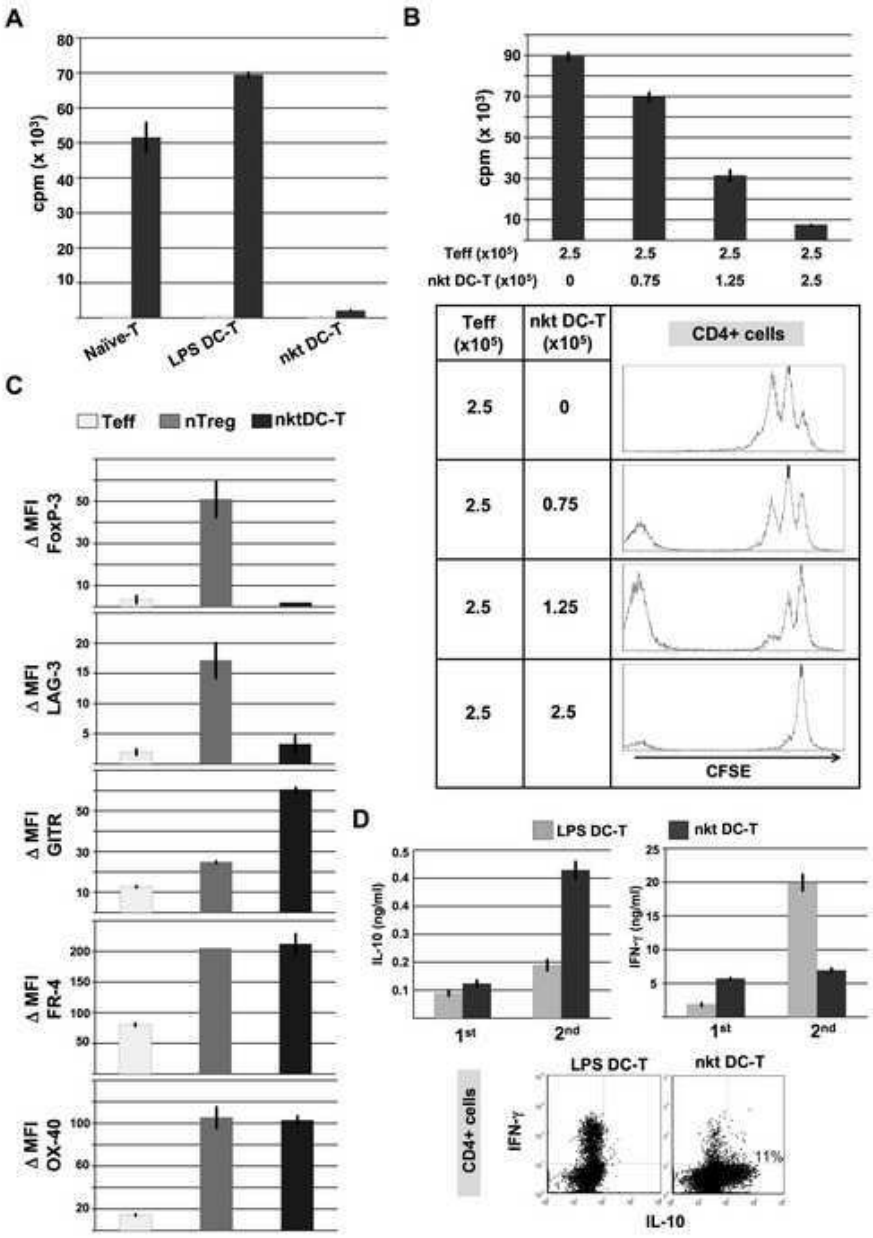

Figure 2

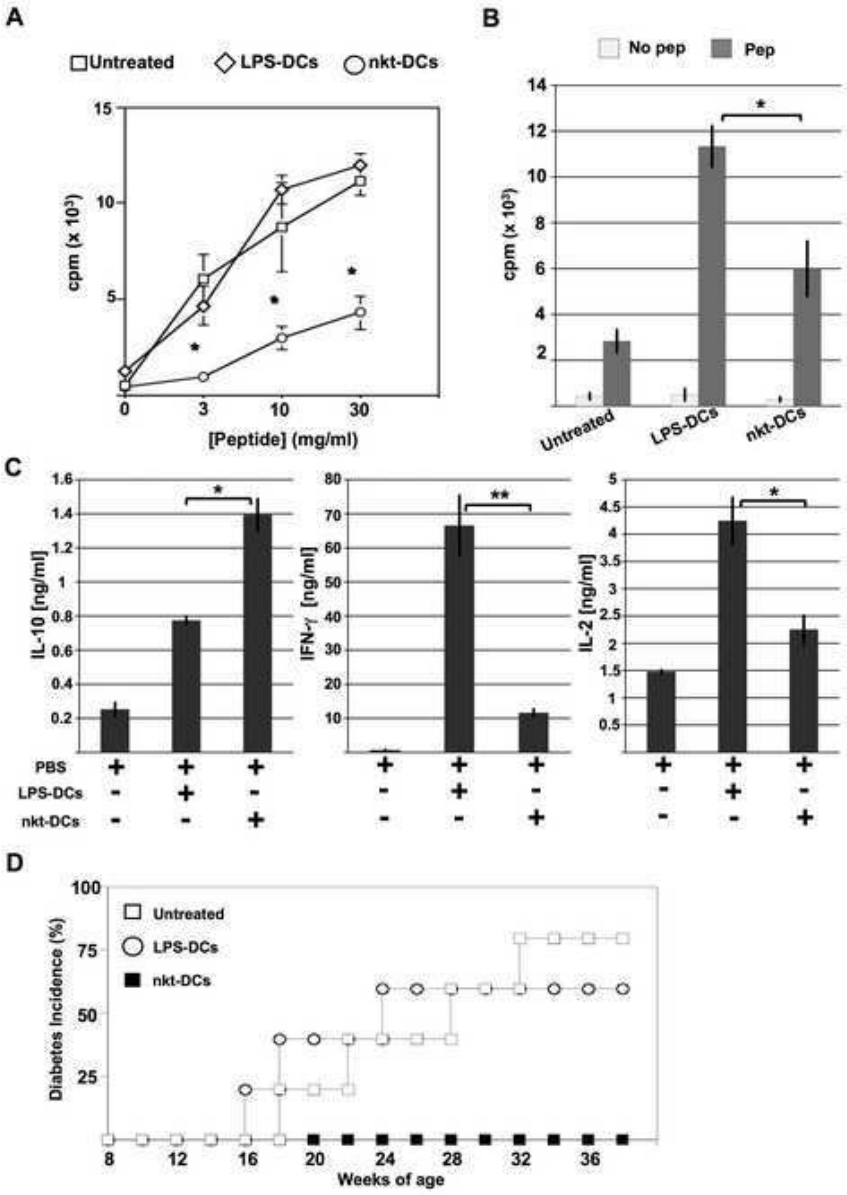

Figure 3

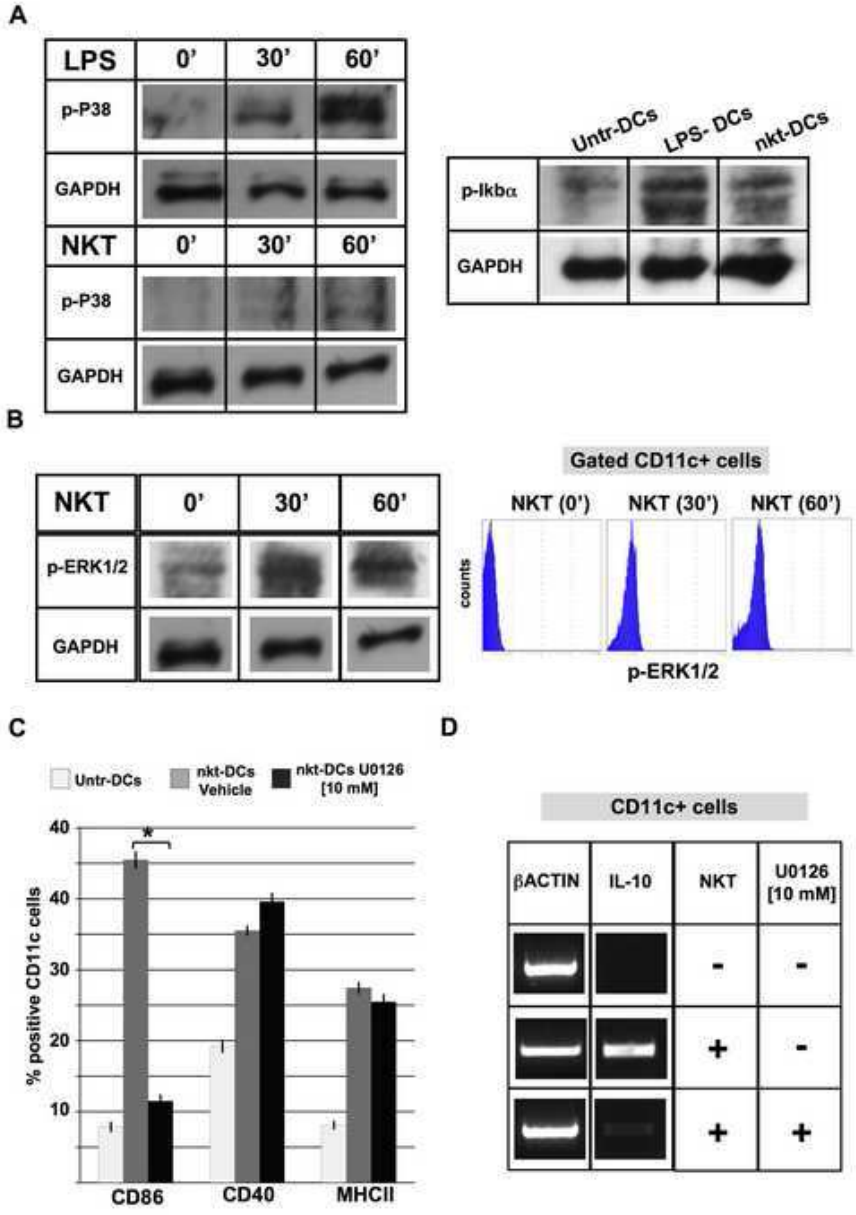

Figure 4

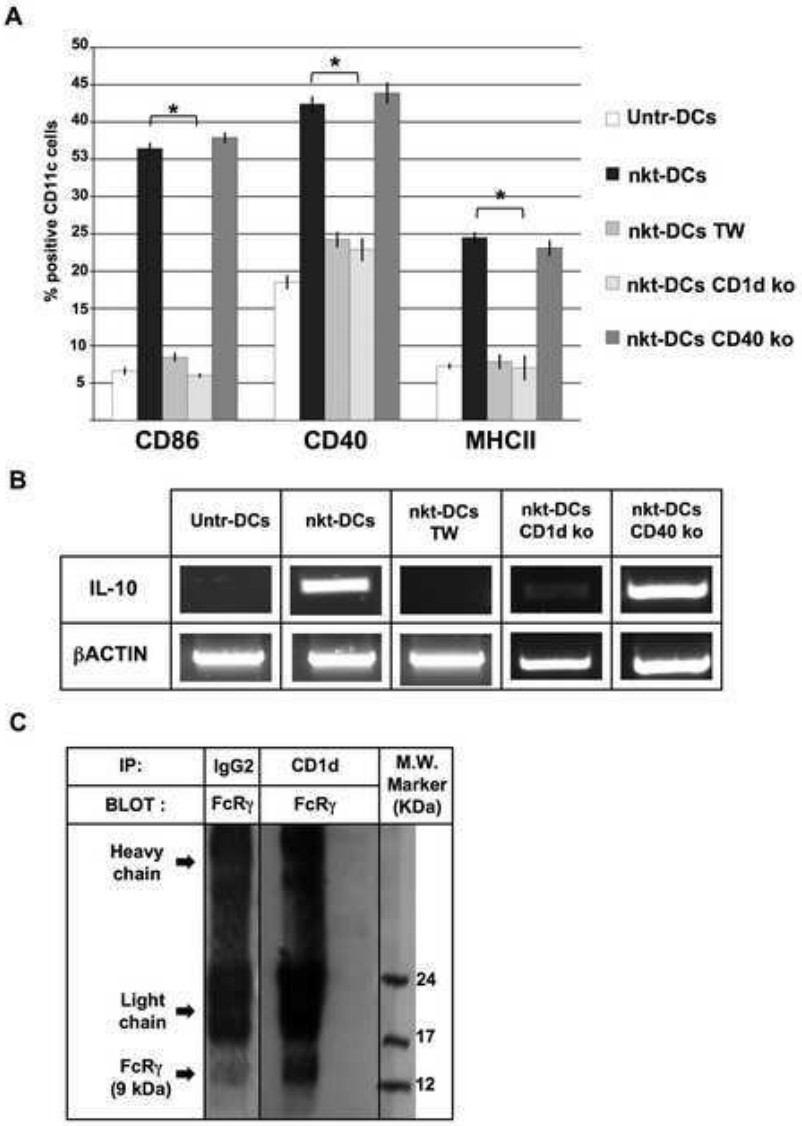

Figure 5

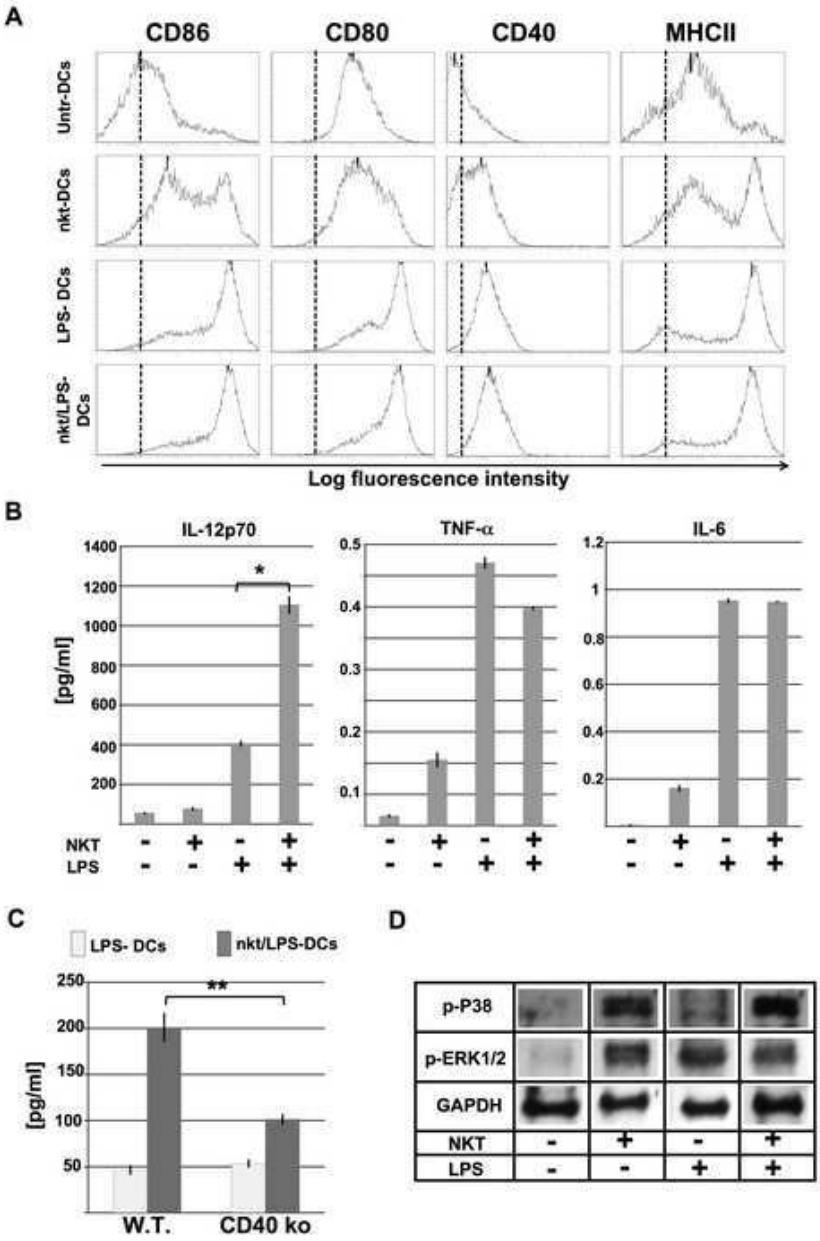

Figure 6

SUPPLEMENTAL DATA

On/off TLR signaling decides immunogenic or tolerogenic dendritic cell maturation upon NKT cell contact

Simone Caielli, Cristina Conforti Andreoni, Ester Badami, Denis V. Baev, Maria Luisa Malosio and Marika Falcone

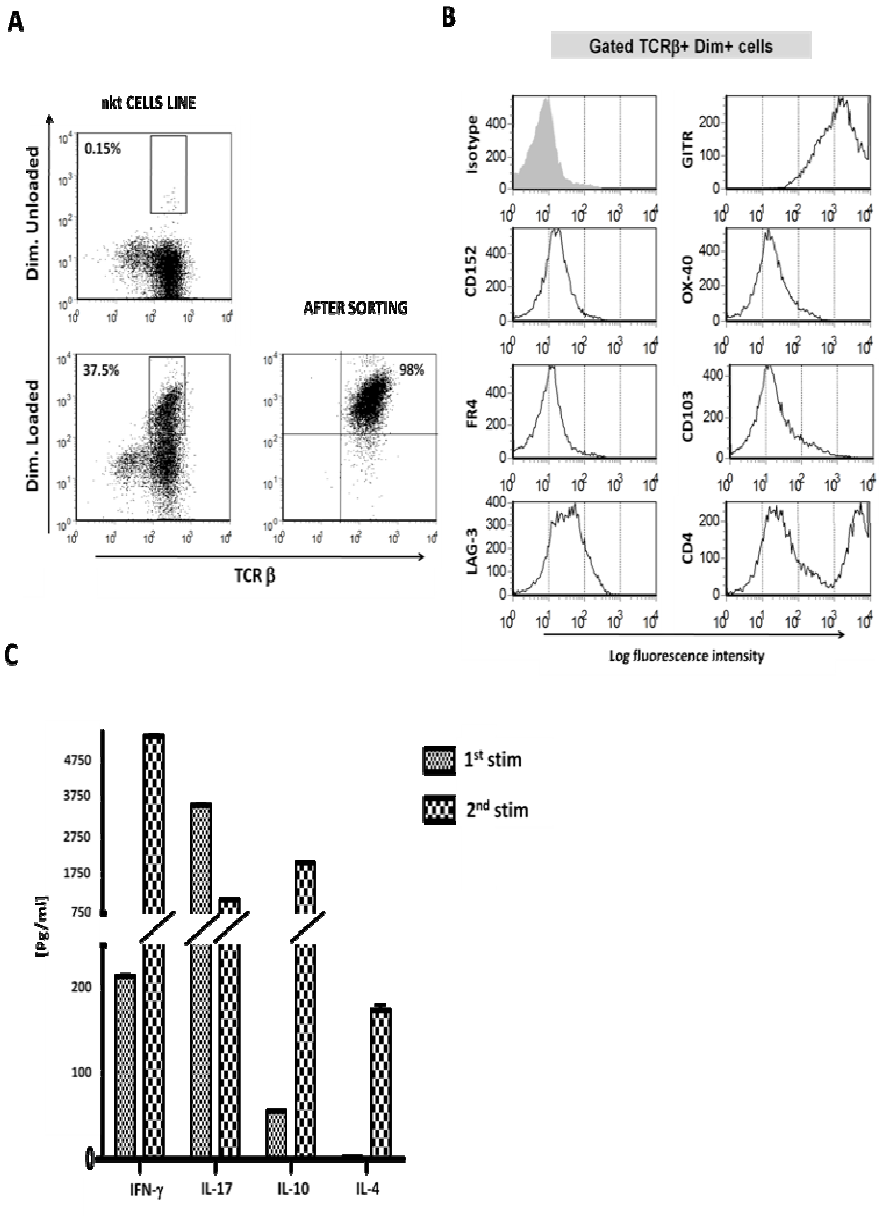

Figure S1

Figure S1. Inkt cells expansion, sorting and characterization

A) Expansion and sorting of Inkt cells. After two round of antigenic stimulation, Inkt cells line was harvested, double stained with TCRb-FITC and a-GalCer loaded or unloaded DimerX following PE – anti-mouse IgG1 before being analyzed by flow cytometry (left). Alternatively Inkt cells line was subsequently incubated with a-GalCer loaded DimerX, PE – anti-mouse IgG1 and anti-PE microbeads. Cells suspension was then passed over a LS columns and Inkt cells was recovered by positive selection. The purity of positive fraction was assessed by flow cytomety analysis by labeling the cells with TCRb-FITC (right).

B) iNKT cells line phenotype. iNKT cells line were analyzed for the expression of the indicated cell surface markers by an array of mAbs. Histogram plots represent cells gated on the basis of their double positivity for TCRb and a-GalCer loaded DimerX. The gray filled histograms show staining whit isotype control.

C) iNKT cells line have a mixed cytokine profile. 72 hrs after the first and the second antigenic stimulation, supernatants form nkt cells line were collected and assessed for IFN-g, IL-17, IL-10 and IL-4 by BD Flex Set. Results are the mean +/- SD from one triplicate experiment. All data are representative of three independent experiments.

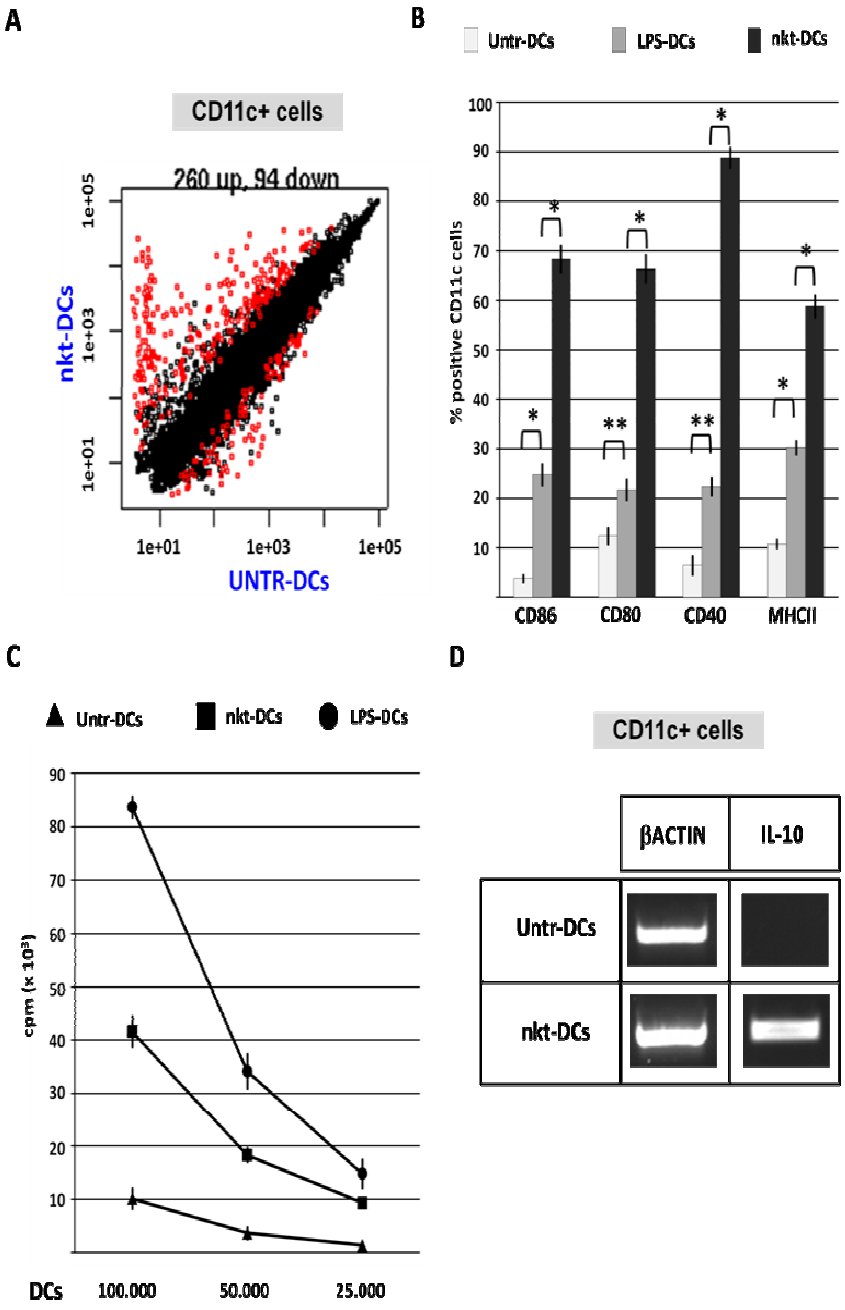

Figure S2

Figure S2. Tolerogenic proprieties of nkt-DCs

A) Nkt-DCs acquire a characteristic gene expression profile. Amplify cRNAs, obtained from untreated and nkt-DCs, were hybridized to Affimetrix GeneChip® mouse genome 430 2.0 arrays and analyzed with Affimetrix GeneChip® scanner 3000. Red dots represent the differentially expressed genes between the two cells populations.

B) Nkt-DCs express intermediate levels of some maturation markers. DCs from different treatments were double stained with CD11c and CD86, CD80, CD40 or MHCII and analyzed by flow cytometry. Results are the mean +/- SD from one triplicate experiment. Data are representative of three independent experiments.

C) NKt-DCs acquire an antigen-presenting capacity. Untr-, LPS- or nkt-DCs, pulsed for 18 hrs with BDC2.5 peptide (1 mg/ml), were added at different ratios to purified syngeneic BDC2.5 naïve CD4 T cells (200.000 cells/well). ^{3}[H]-thymidine (1 mCu/well) was added in the final 18 hrs of a 72 hrs assay culture. Cultures without BDC2.5 peptide did not proliferate. Results are the mean +/- SD from one triplicate experiment. Data are representative of three independent experiments.

D) NKt-DCs actively produce IL-10**.** DCs were magnetically sorted by CD11c microbeads and purified cells were assessed for IL-10 m-RNA expression by semi-quantitative RT-PCR. b-actin was used as a positive loading control. Data are

representative of three independent experiments. [*, $p<0.0001$; **, $p<0.001$]

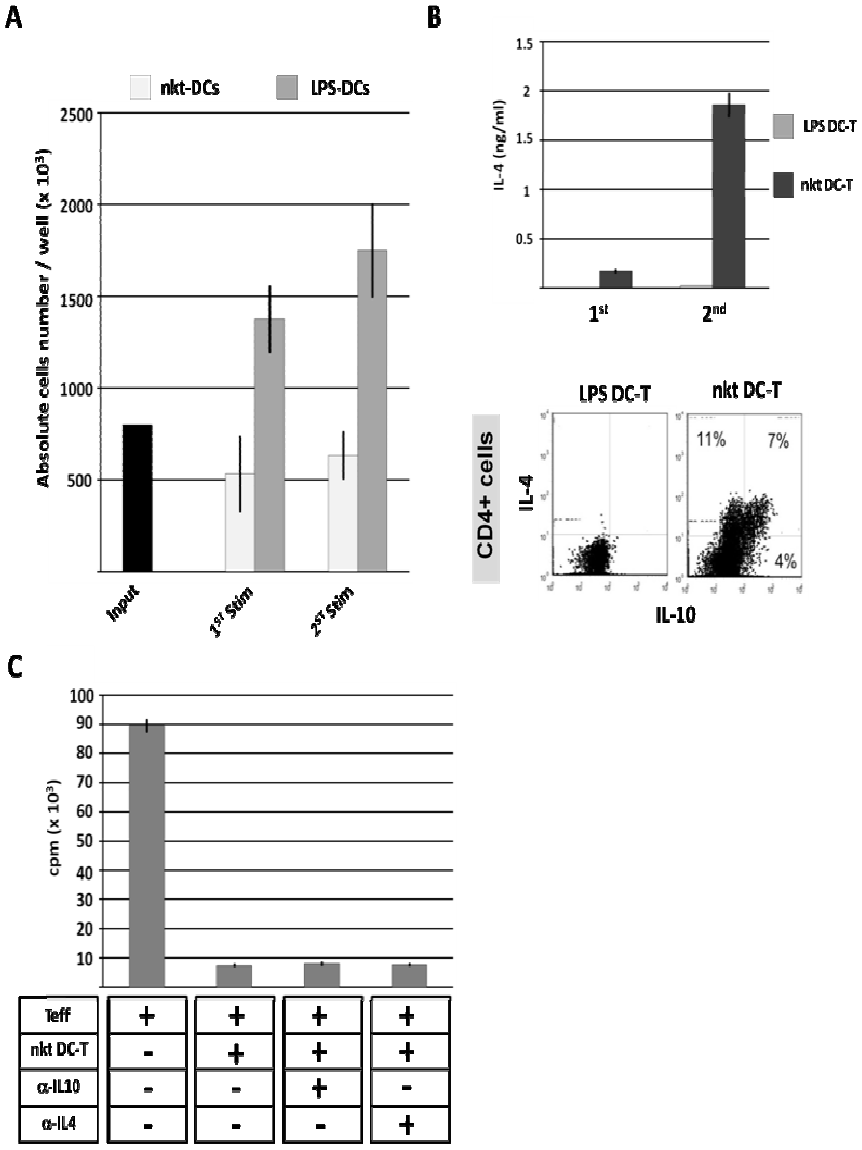

Figure S3

Figure S3. Nkt-DCs favor ex-novo differentiation of regulatory T cells

A) Nkt-DCs poorly sustain the expansion of BDC2.5 CD4 T cells. BDC2.5 CD4 T cell lines were generated as described. 72 hrs after the first or the second antigenic restimulation, cells were harvested and counted with an hemocytometer. The absolute number of live cells / well was plotted. Input represent the number of cells at the beginning of the culture (800.000 cells/well). Each result is the mean +/- SD of three independent experiments.

B) Nkt DC-T have a mixed IL-10/IL-4 secreting cytokine profile. Top: 72 hrs after the first or the second antigenic restimulation supernatants, from DC-T cells cocultures, were collected and assessed for IL-4 by BD Flex Set. Cultures without BDC2.5 peptide did not produce cytokines. Bottom: One week after the second antigenic restimulation CD4 T cells were recovered and reactivated with Leucocyte Activation Cocktail (2 ml/ml). Cells were then fixed and stained for detection of intracellular cytokines using FITC- and APC-labeled mAbs. Values indicate the percentage of positive cells in the indicated quadrant. Results are the mean +/- SD from one triplicate experiment. Data are representative of three independent experiments.

C) IL-4 and IL-10 are not involved in nkt DC-T mediated suppression. Nkt DC-T were added at ratio 1:1 to purified naïve BDC2.5 CD4 T cells in the presence of fully mature peptide-loaded BMDC. Blocking antibodies (10 mg/ml) were added as

indicated. Responder T cells proliferation was determined by adding ^{3}[H]-thymidine (1 mCu/well) in the final 18 hrs of a 72 hrs coculture assay. Results are the mean +/- SD from one triplicate experiment. Data are representative of three independent experiments.

A

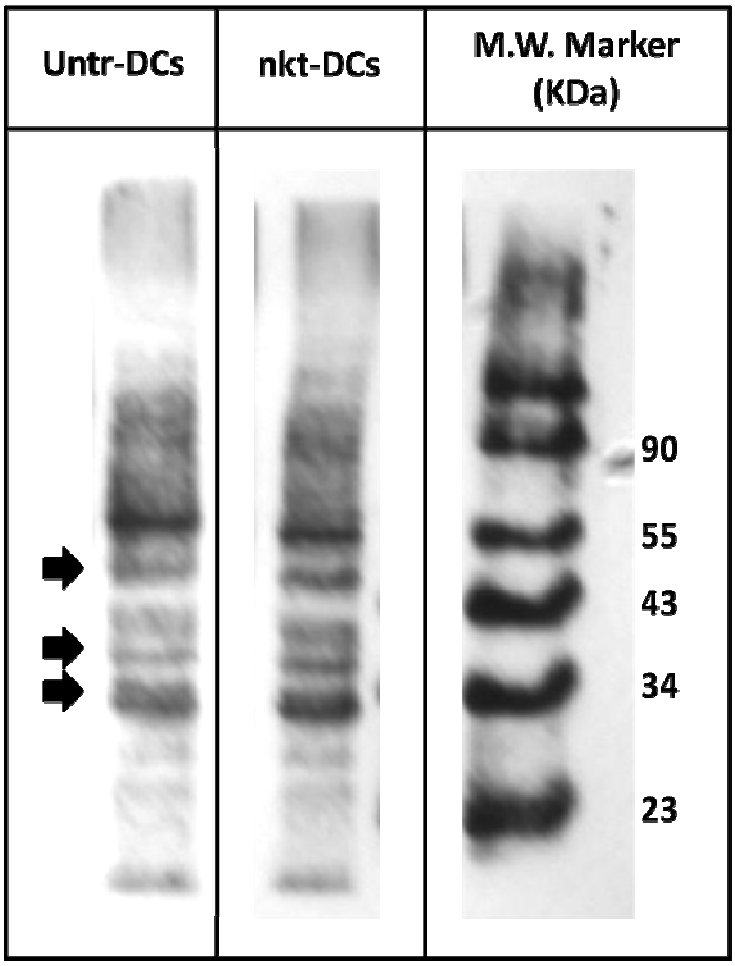

Figure S4

Figure S4. iNKT cells activate PTK

A) iNKT cells induce thyrosine phosphorylation in DCs. DCs were treated with nkt cells or medium alone for 60 min before being lysed with complete RIPA buffer. Whole cell extracts (30 mg protein/lane) were resolved on 12% SDS-PAGE and immunoblotted for P-Tyr with a specific mAb (PY20). Arrows indicate proteins which become phosphorylated. Molecular weight markers are shown on the right.

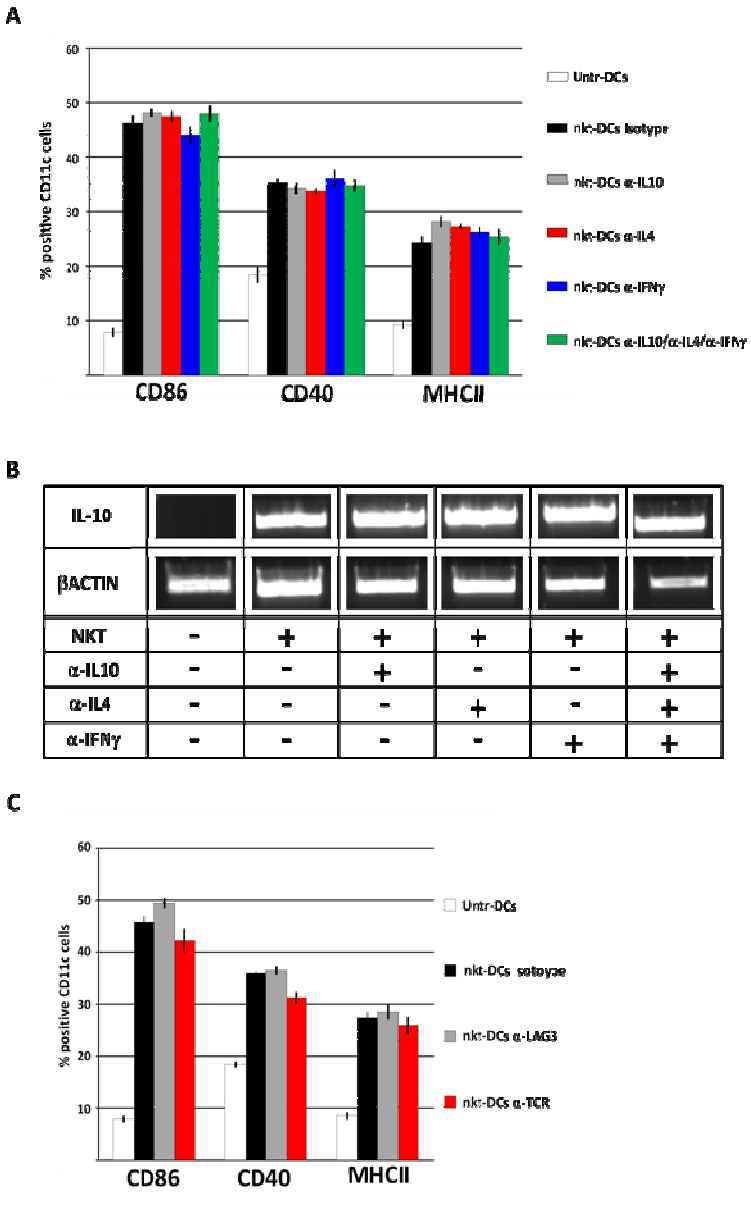

Figure S5

Figure S5. DCs modulation by iNKT cells don't require cytokines and is LAG-3 and TCR independent

A) – B) iNKT cells modulation is cytokine independent. DCs were co-cultured with iNKT cells in the presence of indicated blocking mAbs (10 mg/ml). A) 20 hrs form the beginning of the coculture DCs were harvested, double stained with anti-CD11c and anti-CD86, CD40 or MHCII before being analyzed by flow cytometry. B) 4 hrs from the beginning of the coculture CD11c positive cells have been purified by N418 microbeads and assayed for IL-10 m-RNA expression by semi-quantitative RT-PCR. b-actin was used as a positive loading control. Results are the mean +/- SD from one triplicate experiment. Data are representative of three independent experiments.

C) LAG-3 and TCRb have a dispensable role in iNKT cells modulation. DCs were co-cultured with iNKT cells in the presence of indicated blocking mAbs (10 mg/ml). 20 hrs form the beginning of the coculture DCs were harvested, double stained with anti-CD11c and anti-CD86, CD40 or MHCII before being analyzed by flow cytometry. Results are the mean +/- SD from one triplicate experiment. Data are representative of three independent experiments.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

NKT cell lines generation, sorting and characterization

Splenocytes were passed over a Ficcol gradient and subsequently plated in RPMI-10 for 2 hrs at 37℃ to allow non-T cells to adhere. After 2 hrs non-adherent cells were collected and cultured with autologous irradiated mature BMDC loaded with a a-GalCer (100 ng/ml) at a ratio 1 BMDC : 5 splenocytes. After 72 hrs cells were rested in complete RPMI-10 supplemented with 10 ng/ml of rhIL-7 and rhIL-15 for 96 hrs. Cytokines were added and the culture medium was replaced every 48 hrs. iNKT cells lines were used for the experiments after two rounds of antigenic stimulation. Expansion of the iNKT cells population within the cells line was routinely assessed by FACS analysis with PE-labeled a-GalCer/dimers and TCRb FITC. For iNKT cells sorting the cells suspension were labeled with dimerX/a-GalCer complex following by anti-mouse IgG1 biotin and anti-biotin microbeads and sorted with LS columns (Milteniy Biotechnology). The purity of sorted iNKT cells (< 98%) were routinely assessed by FACS analysis. For cytokine determination the supernatants were collected 72 hrs after the first or the second antigenic stimulation and stored at -20℃ until analysis.

Microarrays and data analysis

Total RNA was extracted from CD11c cells with TRIzol Reagent, purified with RNeasy Kit (Qiagen) and subsequently amplificated using the one cycle target labeling kit according to the AffymetrixGeneChip® Expression Analysis Technical Manual (Affimetrix). Fragmented cRNAs were probed to AffymetrixGeneChip® Mouse Genome 430 2.0 arrays for 16 hours at 45℃ in the AffymetrixHybridization Oven 6 40. The arrays were then washed and stained with streptavidin-phycoerythrin using the AffymetrixFluidic Station 450/250 and scanned using the AffymetrixGeneChip® Scanner 3000. The images were analyzed using GCOC® Software and comparison analyses carried out according to the instructions provided by Affymetrix. Each experiment was performed in triplicates and repeated two times. Data handling was mainly done using Bioconductor Software. The probe set-based summary data were log transformed and normalized for probe set intensity-dependent biases. The identification of differentially expressed genes was addressed using linear modeling approach and empirical Bayer methods together with false discovery rate correction of the p-value. We considered a gene to be differentially increased in nkt-DCs only if its expression intensity was significantly changed ($p<0.001$) compared to Untr-DCs.

CHAPTER 3 : Summary, Conclusions and Future Prospectives

186 In agreement with Italian law 22 april 1941 n. 633, Art. 70

Dendritic cells (DCs) include a heterogeneous family of professional APCs involved in initiation of immunity and in immunologic tolerance. Specifically, peripheral tolerance can be achieved and maintained by promoting regulatory T-cell (Treg) responses and/or T-cell anergy or deletion. Until recently, immature developmental stages of DC differentiation were believed to induce T-cell anergy or Treg cells, whereas DCs transformed into mature DCs by activation stimuli were thought to represent immunogenic DCs capable of inciting primary T-cell responses.

This paradigm has been challenged by the demonstration of Treg-cell expansion by antigen-bearing, fully mature DCs. Similarly, semimature DCs with a distinctive interleukin 10 (IL-10)$^{+}$IL-12^{-} cytokine production profile might be endowed with tolerogenic functions, supporting the concept that DC maturation per se should no longer be considered as a distinguishing feature of immunogenic as opposed to tolerogenic DCs (TDCs).

Cytokine-modulated TDCs reflect an incomplete or altered status of monocyte differentiation and promote in vitro induction of Treg cells and/or in vivo protection from autoimmune diseases. Several growth factors, including IL-10, transforming growth factor β (TGF-β), granulocyte colony-stimulating factor (G-CSF), hepatocyte growth factor (HGF), and vasoactive intestinal peptide (VIP), modulate DC maturation and favor the differentiation of TDCs. From a therapeutic standpoint, cytokine-

modulated TDCs might be beneficial for prevention and/or treatment of posttransplantation graft-versus-host disease (GVHD) and autoimmunity.

Dendritic cells (DCs) are highly specialized antigen (Ag)-presenting cells (APCs) that integrate a variety of incoming signals and orchestrate the immune response [1]. Bidirectional interactions between DCs and Ag-experienced T cells initiate either an immunogenic or a tolerogenic pathway and are of crucial importance in autoimmune diseases and in transplantation medicine [2]. Conventional DC subsets described in humans include myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) [2] Myeloid DCs develop from $CD11c^{+}HLA\text{-}DR^{+}$ blood precursors and undergo activation and maturation in response to triggering of toll-like receptors (TLRs), a class of pattern recognition receptors engaged by microbial products [1]. DC maturation is paralleled by up-regulation of major histocompatibility complex (MHC) class II and costimulatory CD80/CD86 molecules and by production of interleukin (IL)-12. Plasmacytoid DCs might differentiate either from a common blood DC precursor or from a committed lymphoid progenitor; express CD123, CD4, and CD62L; and secrete type I interferon (IFN) in response to viruses and/or TLR9 ligands [3]. In secondary lymphoid organs of mice, different subpopulations of DCs have been identified, namely, $CD8a^{+}$ lymphoid DCs, $CD8a^{-}$ myeloid DCs, and Langerhans cell-derived DCs [4]. In addition, expression of B220 on mouse DCs identifies a functional counter-part of human pDCs [4].

DC ability to induce tolerance has been demonstrated initially by experiments on immature DCs residing in peripheral lymphoid tissues [5]. Under steady-state conditions, immature DCs capture apoptotic bodies arising from cell turnover and, upon migration to draining lymph nodes, silence T cells to self-Ags [6]. Short-lived, migratory DCs might transfer tissue-derived peptides to longerlived tolerogenic DCs (TDCs) upon reaching the lymph node. Interestingly, self-Ag transport, processing, and presentation for tolerance induction by DCs require partial maturation [7]. In the absence of inflammation or TLR triggering, DCs will not produce IL-12, and DCs will be arrested at a semimature stage. Also, DC residence in a tolerizing milieu, for example, in mucosal or immune-privileged sites, affects DC capacity of priming Treg cells. DCs isolated from Peyer patches, lungs, or the anterior chamber of the eye display a mature phenotype, secrete IL-10 but not IL-12, and drive the development of IL-10-producing regulatory T (Treg) cells [8]. Even more intriguing, fully mature, immunologically competent DCs can generate "tolerogenic" peptides upon processing of a self-Ag, thyroid peroxidase. Accordingly, Ag-loaded, mature DCs can expand $CD4^{+}CD25^{+}$ Treg cells with retained ability to suppress the proliferation of non regulatory T cells [9]. Thus, a growing body of experimental evidence indicates that DC maturation per se is neither a distinguishing feature of immunogenic as opposed to TDCs nor a control point for initiating immunity.

Given the remarkable functional plasticity of both mDCs and pDCs, it is presently believed that the net effect of Ag dose, DC lineage and maturational status, DC stimulation by pathogen-derived products, and cytokine milieu at sites of inflammation determines whether an immunogenic or a tolerogenic T-cell response will develop [1, 5].

Because DCs play an undisputed role in inciting autoimmune diseases and in instigating transplant rejection, therapeutic harnessing of peripheral tolerance by TDCs represents an attractive avenue for future clinical applications. Animal models of autoimmunity have provided proof of principle in favor of therapeutic benefits of TDCs.

In mice, Ag-pulsed, TGF-β2-treated TDCs induce regulatory $CD8^+$ T cells and suppress ongoing EAE [6]. TNF-a-differentiated, semimature DCs induce IL-10-secreting, Ag-specific Treg cells and protect from EAE [7]. Host-matched TDCs differentiated with IL-10, TGF-β, and GM-CSF reduce serum levels of proinflammatory cytokines, induce mixed $CD25^+$ and $IL\text{-}10^+$ subpopulations of Tregs with retained graft-versus-leukemia (GVL) responses, and protect from lethal GVHD [8]. Semimature DCs induced by GM-CSF expand TG-specific, IL-10-secreting $CD4^+CD25^+$ Treg cells, which suppress experimental autoimmune thyroiditis (EAT) upon adoptive transfer into TG-primed mice [9]. Similarly, TNF-a-matured DCs pulsed with TG inhibit EAT by inducing $CD4^+CD25^+$ Treg cell activation and secretion of IL-10 [10].

Treatment with G-CSF is beneficial in spontaneous type 1 diabetes in NOD mice through reciprocal effects on TDCs and Treg cells [11]. TDCs in protected mice are enriched in $CD11c^+B220^+Gr\text{-}1^+$ cells producing IFN-a in the absence of measurable IL-12p70 release [12]. G-CSF-recruited pDCs exhibit a semimature phenotype with reduced expression of MHC molecules and CD80, but normal levels of CD86 and CD40, and trigger the accumulation of $CD4^+CD25^+$ Treg cells upon adoptive transfer to secondary NOD recipients.

VIP-differentiated TDCs possess therapeutic effect in murine EAE and rheumatoid arthritis [13]. $CD4^+$ T cells isolated from TDC-treated animals are enriched in $CD4^+CD25^+FoxP3^+$ Treg cells and IL-10^+ Tr1 cells. Additionally, host-matched, VIP-generated TDCs with an in vivo half-life of 17 days induce the differentiation of $CD4^+$ Treg cells, protecting mice from acute GVHD in a haplotype- and TGF-β/IL-10-dependent manner and, remarkably, leave GVL responses unaffected [14].

DCs participate in active regulation of allogeneic graft rejection. In rodents, administration of host-type immature DCs promotes Treg responses and prolongs graft survival [15]. Although most attempts to induce donor Ag-specific tolerance have employed donor-derived DCs to interfere with the direct pathway of allorecognition, several techniques have been developed to load recipient DCs with donor MHC allo-Ags, thus modulating the indirect pathway of allorecognition. In this respect, in vivo targeting of recipient-type DCs with intravenous administration

of donor MHC^+ apoptotic cells prolongs the survival of vascularized heart allografts in the absence of concomitant immunosuppression [16].

Permanent acceptance of organ allografts has not been generally achieved with TDC therapy alone. Blockade of the CD40-CD154 signaling pathway might be synergistic with DC therapy, thus promoting the survival of skin allografts [17]. Pharmacologically treated DCs that are decommissioned from full maturation might also contribute to the maintenance of transplantation tolerance through the promotion of Treg-cell differentiation [18]. Simultaneous targeting of DCs and Treg cells might be a desirable approach to induce transplantation tolerance, and a self-maintaining regulatory loop has been recently described in a murine model of cardiac transplantation, where TDCs induce Treg cells and Treg cells, in turn, program the generation of TDCs [19]. Importantly, inclusion of TDCs in future therapeutic regimens might minimize dependence on nonspecific immunosuppressive drugs currently administered for transplant rejection.

Theoretically, TDCs for clinical application might be obtained after in vivo cytokine administration or might be generated ex vivo from monocyte populations with good manufacturing practice (GMP)-grade cytokine cocktails. Cytokines possess drug like properties, such as potency, but also have disadvantages, such as a short half-life. Considerable effort has been devoted to engineering cytokines with enhanced half-lives

and, for instance, pegylated G-CSF manifests a remarkable potency in inducing the differentiation of Treg cells that protect against mouse acute GVHD [20].

Pharmacologic arrest of DC maturation or use of genetically engineered DCs expressing immunosuppressive molecules might selectively enhance DC tolerogenicity. Among the various drugs implicated in the promotion of DC tolerogenicity, 1a,25-dihydroxivitamin D_3 induces the differentiation of TDCs with up-regulated expression of ILT3, low IL-10, and enhanced IL-12 secretion [21]. The armamentarium of inhibitory pharmacologic agents is expected to increase as more compounds are evaluated for the ability to affect DC functions.

Transfection of DCs with a gene construct encoding a modified CTLA4 molecule translates into deficient expression of CD80/CD86 and induction of T-cell anergy and might represent an attractive means of restoring tolerance in autoimmune diseases [22]. A human HGF expression vector administered in liposomal formulation decreases IL-12, IFN-g, and TNF-a expression in tissues, thus improving mice survival from acute GVHD [23]. It is tempting to speculate that the beneficial effects of HGF treatment on GVHD reported in this study might be, at least in part, attributed to the cytokine-driven promotion of DC tolerogenicity [24].

An important issue to be considered when designing DC-based immunotherapy protocols is whether TDCs might inadvertently

receive in vivo maturation signals in an inflammatory microenvironment and incite unwanted T-cell responses as fully mature DCs. To date, several reports have demonstrated a stable phenotype of cytokine-modulated DC preparations, indicating that TDCs differentiated in the presence of G-CSF, IL-21, VIP, or low-dose GM-CSF might be resistant to further maturation-inducing stimuli [25, 26]. This concern is further mitigated by recent findings that endogenous modulators produced at sites of inflammation, for example, PGE_2 and histamine, might interfere with DC maturation and promote DC tolerogenic functions [27]. *[Adapted from: Rutella; Blood 108, 1435-1440 (2006)]*

ARE TOLEROGENIC DCs READY FOR THE CLINIC?

Support for the clinical translation of tolerogenic DC therapy can be found in DC vaccine trials for cancer. The first report of a clinical study using a DC vaccine was published 12 years ago. Subsequently, many patients have received DC vaccines in an effort to promote immunity to tumours. Most of these studies have used mDCs generated from circulating blood monocytes (or alternatively CD34+ cells) as monocytes can readily be recovered and mDCs are easily generated from monocytes. Testing of DC immunotherapy has generally proved to be safe, with minimal side effects and has been effective in some patients (even though most patients had late-stage, advanced cancer). Some of the early DC vaccine trials used immature rather than mature DCs, without untoward effects. Although it has been suggested that more preclinical work is needed before clinical trials, it was also suggested that well-performed, phase I–II studies with quality control measures and appropriate clinical and immunological outcomes should proceed.

There is still much to learn about optimisation of tolerogenic DC therapy for clinical transplantation or autoimmune disease therapy from a scientific perspective. Variables such as cell dose, single versus multiple doses (and frequency), route of administration (although intravenously seems the most appropriate route for systemic tolerance and has been well

tolerated with unmodified bone marrow cell infusion after organ transplantation as well as DC vaccines) and timing relative to transplantation or disease onset remain in question and warrant further preclinical investigation. However, given the relative permissiveness of small animals models to tolerance induction and perhaps species differences in DC biology, many of these questions may not be fully answered until phase I–II trials are initiated in human transplant recipients. We may not be far from that position. Cell therapy (bone marrow infusion, DC vaccines and others) has a good safety record, and for a patient group with significant need, the potential benefits may justify the risks.

An obvious patient group to which regulatory DC therapy could apply is live donor organ allograft recipients, with kidney the most prevalent organ transplanted in this manner. Tolerogenic DCs could be generated, modified and administered, well in advance of transplantation during steady-state conditions. The prospective infusion of mobilised tolerogenic donor DCs or tolerogenic DCs propagated from leukapheresis products into graft recipients, followed by conventional immunosuppression cover, with the goal of inducing immunoregulation, is applicable in the clinic albeit in the context of live donor transplants. Further exploratory work with tolerogenic DCs given at the time of transplant and/or subsequently is needed to ascertain the efficacy/applicability of these cells in deceased donor transplantation. Well-designed, phase I–II studies for

allotransplantation with appropriate safety, as well as immunological monitoring, may not be far off.

In conclusion, there are strong indications that tolerogenic DCs will one day play a role in the treatment of clinical transplantation and chronic inflammatory disease. Indeed, suppressive DCs are in the clinic for type 1 diabetes and soon will be in the clinic for the treatment of rheumatoid arthritis. As we contemplate the widespread introduction of this treatment in the clinic, the safety of DC vaccines is encouraging and it is reassuring that this treatment can be introduced in the context of current immunosuppressive strategies.

1. Banchereau J, Steinman RM. Nature. 1998;392: 245-252.
2. Vlad G, Cortesini R, Suciu-Foca N. J Immunol. 2005;174: 5907-5914.
3. Colonna M, Trinchieri G, Liu YJ. Nat Immunol. 2004; 5: 1219-1226.
4. Pulendran B, Banchereau J, Maraskovsky E, Maliszewski C. Trends Immunol. 2001;22: 41-47.
5. Steinman RM, Hawiger D, Nussenzweig MC. Annu Rev Immunol. 2003;21: 685-711.
6. Steinman RM, Turley S, Mellman I, Inaba K. J Exp Med. 2000;191: 411-416.
7. Lutz MB, Schuler G. Trends Immunol. 2002; 23: 445-449.
8. Quaratino S, Duddy LP, Londei M. Proc Natl Acad Sci U S A. 2000;97: 10911-10916.
9. Yamazaki S, Iyoda T, Tarbell K, et al. J Exp Med. 2003;198: 235-247.
10. Smits HH, de Jong EC, Wierenga EA, Kapsenberg ML. Trends Immunol. 2005; 26: 123-129.
11. Cella M, Dohring C, Samaridis J, et al. J Exp Med. 1997;185: 1743-1751.
12. Probst HC, McCoy K, Okazaki T, Honjo T, van den Broek M. Nat Immunol. 2005;6: 280-286.
13. Mahnke K, Knop J, Enk AH. Trends Immunol. 2003;24: 646-651.

14. Munn DH, Sharma MD, Lee JR, et al. Science. 2002;297: 1867-1870.
15. Lewkowich IP, Herman NS, Schleifer KW, et al. J Exp Med. 2005;202: 1549-1561.
16. Vigouroux S, Yvon E, Biagi E, Brenner MK. Blood. 2004;104: 26-33.
17. Battaglia M, Gregori S, Bacchetta R, Roncarolo MG. Semin Immunol. 2006;18: 120-127.
18. Yvon ES, Vigouroux S, Rousseau RF, et al. Blood. 2003;102: 3815-3821.
19. Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. J Exp Med. 2001;193: 233-238.
20. Jonuleit H, Schmitt E, Schuler G, Knop J, Enk AH. J Exp Med. 2000; 192: 1213-1222.
21. Brinster C, Shevach EM.. J Immunol. 2005;175: 7332-7340.
22. Tarbell KV, Yamazaki S, Olson K, Toy P, Steinman RM. J Exp Med. 2004;199: 1467-1477.
23. Munn DH, Sharma MD, Baban B, et al. Immunity. 2005;22: 633-642.
24. Munn DH, Sharma MD, Hou D, et al. J Clin Invest. 2004;114: 280-290.
25. Orabona C, Puccetti P, Vacca C, et al. Blood. 2006;107: 2846-2854.
26. Terness P, Chuang JJ, Bauer T, Jiga L, Opelz G. Blood. 2005;105: 2480-2486.

27. Braun D, Longman RS, Albert ML. Blood. 2005;106: 2375-2381.