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Cytotoxic activity of invariant NKT cells in human allergic contact dermatitis

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Introduction

Natural killer T cells (NKT cells) are a unique subset of T-lymphocytes that express a T-cell receptor (TCR) a/ß, with a restricted repertoire (Godfrey *et al*., 2004). This distinguishes them from NK cells, although NKT cells share some markers characteristic of NK cells such as CD161 (CD161c is known as NK1.1 in mice) or NKR-P1 (Godfrey *et al*., 2004). In contrast to conventional T-lymphocytes and other Tregs, the NKT cell TCR does not interact with peptide antigen presented by classical major histocompatibility complex (MHC)-encoded class I or II molecules, but instead it recognizes glycolipids presented by CD1d, a nonclassical antigen–presenting molecule that associates with ß2 microglobulin (Godfrey *et al*., 2000 and Kronenberg *et al*., 2002).

Polymorphic MHC molecules present peptide antigens that are specifically co-recognized by aß TCRs expressed on T-cells. These TCRs are highly specific and restricted to recognizing the MHC molecules of the individual from which they were derived; this concept is known as MHC-restriction.

In contrast, CD1 family members are monomorphic MHC class-I-like glycoproteins that present lipid-based antigens recognized by T-cells, even across species. The antigen-binding cleft of the CD1 family contains large hydrophobic pockets that are ideally suited to binding lipid antigens and, on the basis of sequence identity and chromosomal location, are divided into CD1a, CD1b, CD1c, CD1d and CD1e family members. Although humans express all family members, only the CD1d genes are present and expressed in mice and rats. CD1a, b and c are most homologous to each other and are expressed by thymocytes, dendritic cells and activated monocytes, (Dougan *et al*., 2007) while

B cells express only CD1c, among these 3 family members (Small *et al*., 1987). CD1d is divergent in sequence from CD1a, b and c (Balk *et al*., 1989) and its tissue distribution is more widespread, including cells outside the lymphoid and myeloid lineages (Canchis *et al*., 1993; Exley *et al*., 2000). In addition CD1d is expressed in epidermal keratinocytes (KCs) in the skin, and by several other peripheral epithelia or stromal cells in diverse organs such as the intestine, liver, kidney, pancreas, uterus, and conjunctiva (Sieling, 2000). Some bone marrow-derived cells, such as B cells and monocyte-derived dendritic cells, also express CD1d (Sieling, 2000). Thus, the wide distribution of CD1d in widely divergent tissues by predominantly nonprofessional antigen presenting cells (APCs) suggests that it plays an important, yet poorly defined, role in both health and disease states (Fishelevich *et al*., 2006). CD1a, b and c molecules, which can present self and foreign (microbial) lipid antigens, are generally recognized by polyclonal T-cells expressing aß TCRs or by some ?d T cells that recognize CD1c. The recognition of glycolipid antigens in association with CD1d means that NKT cells recognize a class of antigens ignored by conventional T-cells. Relatively few examples of these antigens are defined, although presentation of several mycobacterial cell wall antigens by the 3 other human CD1 molecules, CD1a, CD1b and CD1c, has been well characterized (Gumperz *et al*., 2001), as has presentation of several autologous antigens, including gangliosides and some phospholipids. The compound most efficient for activating the majority of NKT cells is a synthetic glycolipid (originally derived from a marine sponge) known as a-galactosylceramide (aGalCer) (Hayakawa *et al*., 2004 and Hong *et*

al., 1999). This compound binds effectively to CD1d, and the complex of glycolipid plus CD1d binds the NKT cell TCR (Sidobre *et al*., 2002). aGalCer is widely used as a highly specific antigen for both human and murine NKT cells. In both species, these cells use precisely rearranged homologous TCR variable (V)a and junctional (J)a segments.

In mice, NKT cells most express an invariant TCR a chain rearrangement (Va14-Ja18) with a conserved CDR3 region, and they typically coexpress either Vß8.2, Vß2, or Vß7. Similar T-cells are present in other mammalian species, including humans. The homologous population of human invariant NKT cells express a Va24-Ja18 rearranged TCR a chain with a Vß11-containing ß chain (Dellabona *et al*., 1994 and Porcelli *et al*., 1993). The evolutionary conservation of these cells is striking, as mouse NKT cells recognize human CD1d plus glycolipid antigen and vice versa (Brossay *et al*., 1998).

NKT cells may be double negative for CD4/CD8 or may be single positive for CD4 or CD8. During development in the thymus, rare CD4+ CD8+ (DP) cortical thymocytes that successfully rearrange the semi-invariant TCR are directed to the Va14 NKT cell lineage via interactions with CD1d-associated endogenous glycolipids expressed by other DP thymocytes. As they mature, Va14 NKT lineage cells upregulate activation markers such as CD44 and subsequently express NK-related molecules such as NK1.1 and members of the Ly-49 inhibitory receptor family (MacDonald HR *et al*., 2007).

The distribution of NKT œlls and NK cells is different; NKT cells are found everywhere that conventional T-cells are found. In mice, NKT cells represent

approximately 30% of the total lymphocytes in the liver (50%of aßTCR+ T cells), 20% of the aß T-cells in the bone marrow, and 3% of the aßT cells in the spleen, but are rare in the lymph node (Berzins *et al*., 2004; Emoto & Kaufmann, 2003; Godfrey *et al*., 2000). In humans, only 0.2% of peripheral blood T-cells are NKT cells. They are also present in the human liver but their numbers are lower than in the liver of mice (Prussin & Foster, 1997; Benlagha *et al*., 2000; Norris *et al*., 1999). NKT cells predominate in the liver, while conventional T cells prevail in blood and lymph node. In contrast, the order for NK cell frequency is lung > liver > peripheral blood mononuclear cells (PBMCs) > spleen > bone marrow > lymph node > thymus, where NK cells are almost undetectable (Gregoire *et al*., 2007).

NK cells are lymphocytes that play a vital role in cell-mediated immunity, and serve as the first line of defense against cancerous and virally infected cells. Their cytolytic activity is not regulated by antigenic specificity, but through a balance of activating or inhibitory signals mediated through cell surface receptors. These receptors specifically bind human leukocyte antigen (HLA) ligands (Bashirova *et al*., 2006), which are expressed in virtually all nucleated cells but downregulated in aberrant cells, thus allowing NK cell reconnaissance. This mode of recognition was first referred to as the 'missing self' hypothesis (Ljunggren *et al*., 1990).

Although most NKT cells express NK cell markers such as CD161, they also contain a small population of cells that are negative for NK cell markers (Godfrey *et al*., 2004). Importantly, CD1d-restricted T cells also contain T-cells that neither express the canonical TCR a-chain nor respond to aGalCer (Duarte *et al*., 2004

and Terabe *et al.*, 2005). To avoid confusion, it has recently been recommended that NKT cells should be defined by their reactivity to aGalCer loaded onto CD1d multimers, instead of expression of NK cell markers (Godfrey *et al*., 2004). It is apparent, now, that the simple classification of NKT cells as T-lymphocytes that also express NK receptors is inadequate.

Robson MacDonald reviewed the discovery of NKT cells through time. Early in the history, there was no connection with the NK cell lineage for either Vß8-biased DN thymocytes or Va14-expressing peripheral T-cells. He reported that this could probably be traced to the fact that only T-cell biologists were studying these rare populations and there was no reason at the time to suspect any shared markers between T and NK cells. On the other hand, NK cell biologists often were required to exclude T-cell markers from their analysis, putting them in a better position to identify shared phenotypic properties (Robson MacDonald, 2007). Around 1990, it was recognized that a subset of lymphocytes in spleen and bone marrow shared NK and T-cell markers (Yankelevich *et al*., 1989; Sykes, 1990). Importantly, this was also shown to be true for the DN Vß8-biased thymocyte subset (Ballas & Rasmussen, 1990). At this point in time "NKT" cells finally had a birth certificate but still were not linked to expression of the Va14 chain.

Regulatory role of NKT cells

NKT cells influence and regulate a wide range of immune responses. They share some characteristics with CD25+CD4+ Tregs, which, also regulate different types

of immune responses and which also have recently attracted much attention (Godfrey and Kronenberg, 2004).

CD4+CD25+ Tregs are essential for the control of immune responses in inflammatory, autoimmune, or cancer diseases, and it is well established that in particular the lineage-specific transcription factor Foxp3, as well as cytokines (including IL-2, IL-10, and TGF-ß), characteristic surface markers such as CTLA-4, and members of the TNF superfamily (e.g., RANKL) are critically involved in the thymic development, peripheral maintenance, and suppressive activity of CD4+CD25+ Tregs (Kim, 2007).

There are some clear similarities between NKT cells and CD4+CD25+ Tregs, including expression of CD25 by human CD4+ NKT cells (Lee *et al*., 2002). This raises the possibility of confusion between these distinct types, such that some activity ascribed to CD25+CD4+ Tregs might really be due to NKT cells. It is therefore important to carefully distinguish these cells (Godfrey and Kronenberg, 2004). In Table1 similarities and differences among NKT cells, NK cells, T cells and CD25+CD4+ Tregs are summarized.

Ligands

Since aGalCer, was discovered as a potent ligand for NKT cells (Kawano *et al*., 1997), a synthetic aGalCer has widely been used for study of NKT cells as a surrogate ligand. It is now established that two lipid chains of aGalCer are inserted to hydrophobic grooves of the CD1d glycoprotein expressed by APCs (McCarthy *et al*., 2007), whereas the a-linked sugar moiety is accessible and

recognized by the TCR of NKT cells. Recently, the crystal structure of the invariant TCR and CD1d loaded with aGalCer has shown a very unique orientation of TCR towards CD1d (Borg *et al*., 2007), which allows a selective involvement of the invariant a-chain for recognition of the a-linked sugar. Comparison of aGalCer with its structurally altered analogues has provided important insights into how NKT cells may differentially respond to glycosphoingolipids with lipid tail variants (Brutkiewicz, 2006; Miyake & Yamamura, 2007). An aGalCer analogue called OCH (Miyamoto *et al*., 2001; Oki *et al*.,2004, 2005), with a shorter sphingosine, would selectively stimulate IL-4 production from NKT cells, whereas aGalCer stimulation induces both IL-4 and IFN-?. Accordingly, OCH stimulation of NKT cells favors a Th2 bias of immune responses in vivo, as compared to aGalCer stimulation. a-linked sugars such as aGalCer are not recognized as a product of mammalian cells, implying that aGalCer is not a physiological ligand for NKT cells. Currently, it is well recognized that NKT cells can be activated during infectious diseases (Tupin *et al*., 2007). Interestingly, it has been reported that aGalCer-like glycosphingolipids are rather ubiquitously found in the environment, indicating that aGalCer may be actually derived from bacteria residing with the marine sponge.

Other analogs of aGalCer have been shown to have various effects on NKT cell activation *in vitro*, as well as antimicrobial and antitumor activity *in vivo*. For example, modifying the acyl chain of the aGalCer molecule KRN7000 to create a diunsaturated 20 carbon chain results in IL-4 production with a reduction in IFN? secretion (Yu *et al*., 2005). In contrast to the effects of acyl chain modification on

aGalCer activity, Gonzalez-Aseguinolaza and colleagues used a different approach. In their studies, they used a C-glycosidic form of aGalCer in analyses of the adjuvant properties of aGalCer. It was found that the C-glycoside (a-C-GalCer) induced more of a Th1 response, was longer lasting, and was actually a better adjuvant than the parental compound itself in murine models of malaria and metastatic melanoma (Gonzalez-Aseguinolaza *et al*., 2000 and 2002; Schmieg *et al*., 2003 and 2005; Yang *et al*., 2004).

Ortaldo *et al* (Ortaldo *et al*., 2004), as well as Parekh *et al* (Parekh *et al*., 2004), have found that a 12 carbon acyl chain form of galactosylceramide (ßGalCer (C12)) can stimulate NKT cells in a CD1d-dependent manner. The administration of the ßGalCer (C12) in vivo caused the apparent loss of NKT cells as found with aGalCer, but without cytokine expression or activation of NK cells. Tetramers loaded with ßGalCer (C12) could stain NKT cells, although lower numbers were detected as compared with when aGalCer-loaded tetramers are used (Wilson *et al*., 2003; Crowe *et al*., 2003; Harada *et al*., 2004). However, some contamination with minute amounts of aGalCer cannot be completely ruled out in either of the ßGalCer preparations. Thus, ßGalCer may have some similar yet distinctly different actions on NKT cells.

In 2005 three groups independently found that a predominant glycosphingolipid from the LPS-free *Sphingomonas* (*S. capsulata*, *S. paucimobilis*, and *S. wittichii*) and *Ehrlichia muris* bacteria could stimulate human and/or murine NKT cells in a CD1d-dependent manner (Kinjo *et al*., 2005 and 2006; Mattner *et al*., 2005; Sriram *et al*., 2005; Wu *et al*., 2005). All of these groups identified

aglucuronosylceramide (GSL-1) as this glycosphingolipid, with agalacturonosylceramide (GS-1`) also able to activate NKT cells. An additional unique aspect of GSL-1 as opposed to aGalCer is the NKT cell populations identified by CD1d1 tetramers or dimers loaded with this glycolipid. Approximately one-half of those NKT cells that are aGalCer/CD1d1 tetramer positive are GSL-1/CD1d1 dimer (or tetramer) positive. Two other sources of microbial products have been shown to be able to activate NKT œlls. The Schaible group reported that Phosphatidylinositol mannoside (PIM) could stimulate both human and murine NKT cells (Fischer *et al*., 2004). Additionally, PIM-loaded CD1d tetramers could stain both human (PBL) and mouse (liver) NKT cells, although the latter detected a substantially smaller population than CD1d tetramers loaded with aGalCer. Despite this interesting observation clearly identifying PIM as a CD1d ligand, how PIM actually participates in the immune response against mycobacteria is not yet clear.

Search for an endogenous ligand of NKT cells has led to the identification of lysosomal glycosphingolipid isoglobotrihexosylceramide (iGb3), a ß-linked sugar capable of stimulating NKT cells as a potential endogenous ligand for mouse and human NKT cells (Mattner *et al*., 2005; Zhou *et al*.,2004). With regard to the role of iGb3 in adaptive immune responses, Mattner *et al*. reported that Gram-negative, LPS positive Salmonella typhimurium activates NKTcells through the recognition of iGb3, presented by LPS-activated dendritic cells.

However, very recent works have cast doubt on the meaning of the iGb3 discovery (Porubsky *et al*., 2007; Speak *et al*., 2007). The study by Zhou *et al*.

(2004) indicated that iGb3 presented by CD1d-expressing CD4+CD8+ thymocytes should be involved in the thymic positive selection of NKT cells. Porubsky *et al*. has then generated iGb3 synthetase deficient mice and examined if NKT cells are really missing in the mice lacking expression of iGb3. They found that the number and function of NKT cells were as normal as those seen in wild-type mice. Using highly sensitive HPLC assay, Speak *et al*. sought for the presence of iGb3 in various mouse and human tissues. The only tissue containing iGb3 was the dorsal root ganglion of mice. No iGb3 was detected in any human tissue (Porubsky *et al*., 2007; Speak *et al*., 2007). These new findings do not support the idea that iGb3 is central in the selection of NKT cells and re-opened the search for endogenous ligands for NKT cells.

Two more natural lipid components of the cell membrane, the disialoganglioside (GD3) and Phosphatidylinositol (PI), have been shown to be CD1d presented Ags capable of stimulating (at least some) NKT cells (Brutkiewicz, 2006).

Functional subsets of NKT cells

During their development in the thymus, conventional aßT-cells acquire the expression of either CD4 or CD8, MHC coreceptors that define functionally distinct MHC-restricted T-cell subsets. In response to antigen stimulation, CD4+T-cells differentiate into T helper, Th1 or Th2 cells. Th1 cells produce the signature cytokine interferon IFN?, whereas Th2 cells produce interleukin IL-4, IL-5, IL-13, and IL-10. Similarly, effector CD8+T cells can be classified as Tc1 or Tc2 cells, which produce type 1 or type 2 cytokines, respectively (Woodland &

Dutton, 2003).

The Th1/Th2 paradigm has recently been reevaluated to include a third population of T helper cells, producing IL-17 and designated Th17. These pro-inflammatory T cells have been mostly studied in the mouse. Th17 cells exhibit a cytokine profile distinct from Th1 and Th2 cells, producing cytokines, such as IL-17 and IL-22, and express a characteristic transcription factor retinoic acid receptor (RAR)-related orphan receptor gamma t (ROR?t). The differentiation of Th17 cells requires the coordinate and specific action of the proinflammatory cytokine IL-6 and the immunosuppressive cytokine TGF-ß. In addition, the IL-12 family member IL-23 is involved in the maintenance of these cells (Korn *et al*., 2007). A pro-inflammatory role in autoimmune diseases has been reported for IL-17; furthermore IL-17 has been shown to be important for host defense against pathogens such as Klebsiella pneumoniae and Bacteroides fragilis (Stockinger and Veldhoen, 2007). The detection of IL-17-producing T cells in humans with inflammatory diseases, such as multiple sclerosis (MS), contact dermatitis (CD), rheumatoid arthritis (RA), and Lyme arthritis (Steinman, 2007) suggests that in humans these cells have a proinflammatory role similar to that described in mouse models (Rautajoki *et al*., 2008).

From this perspective, the cytokine profiles of T-lymphocytes are thought to reflect their functional activities. In Figure 1 a model of the differentiation of CD4+CD8+ T-cells and activation of NKT cells is depicted.

NKT cells are different from functionally differentiated conventional a/ß TCR bearing T-cells in that they are autoreactive (i.e., they can recognize self-

glycolipids) and produce both Th1 and Th2 cytokines, including IL-4, IL-10, and IFN?, upon stimulation with their ligands (Taniguchi *et al*., 2003).

Two major subsets of NKT cells with distinct effector functions have been identified.

Lee *et al* hypothesized that NKT cells could promote Th2 responses in some conditions and Th1 in others for the presence of different characteristics. They reported that CD4+ and CD4-CD8- NKT cell subsets represent functionally distinct lineages with marked differences in their profile of cytokine secretion and pattern of expression of chemokine receptors, integrins, and NK receptors. CD4+NKT cells were the exclusive producers of IL-4 and IL-13 upon primary stimulation, whereas CD4-CD8- NKT cells had a strict Th1 profile and prominently expressed several NK lineage receptors (Lee *et al*., 2002).

Gumperz *et al* also reported different characteristics for 2 distinguished subsets of NKT cells. CD4- NKT cells selectively produced Th1 cytokines as IFN?, TNFa and expressed NKG2d; this subset up-regulated perforin after exposure to IL-2 or IL-12. In contrast, CD4+ NKT cells potently produced both Th1 and Th2 cytokines, up-regulated perforin in response to stimulation by phorbol myristate acetate and ionomycin but not IL-2 or IL-12, and could be induced to express CD95L (Gumperz *et al*., 2002).

In attempt to better understand the contribution of NKT-DC crosstalk governing the Th1/2 balance, Liu *et al* demonstrated that 2 major distinct subsets of NKT cells, CD4+ and CD4−CD8−, express functional CD40 ligand (CD40L), but they differentially regulate the DC function by reciprocal NKT-DC interactions, thereby

influencing the subsequent Th response. The CD4+ subset stimulated by a-GalCer-loaded DC immediately produced massive amounts of IL-4 and IL-13, which together with IFN? enhanced CD40L-induced IL-12 production by DC.

In contrast, the CD4–CD8– subset eliminated the DC by cytolysis and changed the living DC into a default subtype, in turn markedly down-regulating the levels of IL-12. Therefore, the DC stimulated by the CD4+ subset preferentially induced Th1 responses, whereas the DC reacted with the CD4–CD8– subset induced a shift toward Th2 responses (Liu *et al*., 2008).

Although human NKT cell subsets with distinct effector functions have been identified, it is unclear whether the effector functions of these subsets are imprinted during development or can be selectively reprogrammed in the periphery.

Eger *et al* demonstrated that NKT cells from neonates are phenotypically and functionally distinct from adult NKT cell subsets (Eger *et al*., 2006). In comparison to adult CD4+ NKT cells, neonatal CD4+ NKT cells exhibit higher expression of the differentiation markers CCR7 and CD62L, lower expression of the NK cell markers CD161 and CD94, and are more amenable to effector function reprogramming. They also found wide heterogeneity within the adult CD4+ NKT cell subset for expression of memory markers, suggesting that the pool of CD4+ NKT cells in adult peripheral blood represents a more heterogeneous mixture of cells with respect to their phenotype and stage of maturation. They have hypothesized that adult NKT cells are not recent thymic

emigrants, but have undergone some form of peripheral maturation (Eger *et al*., 2006).

The *invariant* Va14+NKT cells (iVa14+NKT), also called *type I NKT cells*, include two defined populations: a CD4+ and a CD4-CD8- DN population; they express TCR repertoire consisting of Va14-Ja18 (in mice) or Va24-Ja18 (in humans).

In addition to type I NKT cells, a population of CD1d-reactive NKT cells that express diverse non-Va14TCRs, referred to as *type II NKT cells or noninvariant*, has been described. These cells were identified based on the fact that NKT cell function was still detectable in mice lacking type I NKT cells but not in mice lacking CD1d (Godfrey *et al*., 2004). Although type II NKT cells are also restricted by CD1d, they do not recognize aGalCer. It is important to note, however, that some type II NKT cells are also autoreactive, as they recognize the endogenous myelin-derived glycolipid sulfatide and help protect mice against the development of experimental autoimmune encephalitis (Jahng *et al*., 2004).

Type III or CD1d-independent NKT cells express a diverse repertoire restricted to classical MHC class I and II molecules, but not CD1d molecule. They are classified by surface expression of Ly49 or NK1.1 (CD161 in humans) and CD3. (Meyer *et al*., 2007). However some authors consider these cells as not belonging to NKT cells family and classify them as NKT-like cells (Godfrey *et al*., 2004). Thus, different types of NKT cells appear to have distinct functional capabilities, but these functional differences have not been well characterized yet. Type I iVa14+NKT cells (referred to hereafter as NKT cells) have been shown to mediate both protective and regulatory immune functions. These

include tumor rejection, protection against infectious microbes, maintenance of transplant tolerance, and inhibition of autoimmune disease development (Taniguchi *et al*., 2003). However, it remains unclear whether distinct subsets or subtypes of CD1d-reactive NKT cells mediate different functions.

Two new studies, suggest that a distinct subset of NKT cell mediates tumor rejection (Crowe *et al*., 2005), and that type II NKT cells suppress antitumor immunosurveillance (Terabe, 2005). These new studies might help explain the seemingly contradictory functions that have been ascribed to CD1d-reactive NKT cells. They also raise new questions about how subsets of NKT cells acquire distinct functional capabilities (Seino & Taniguchi, 2005).

When activated, NKT cells respond with vigorous cytokine production within 1–2 hours of TCR ligation (Godfrey and Hammond, 2000; Kronenberg and Gapin, 2002). These cells release Th1-type cytokines including IFN? and TNFa, as well as Th2-type cytokines including IL-4 and IL-13 (Smyth and Godfrey, 2000; Wilson and Delovitch, 2003), and at least in mice, they seem to store or pre-form some mRNA for these cytokines even before activation with exogenous antigens (Matsuda *et al*., 2003 ; Stetson *et al*., 2003). Moreover, individual NKT cells are able to make both Th1- and Th2-type cytokines simultaneously following stimulation in vivo (Matsuda *et al*., 2000, 2003; Crowe *et al*., 2003), an unusual characteristic that at face value seems paradoxical, as Th1 cytokines often antagonize the action of Th2 cytokines and vice versa. Despite this Th0-like cytokine pattern, NKT cells can “go both ways,” as their activation in some cases can polarize the immune response in a Th1 direction, while in other cases a Th2

response is generated (Godfrey and Kronenberg, 2004). Adding to the unpredictability surrounding the functional consequences of NKT cell activation, these lymphocytes have been implicated as immunosuppressive cells in some systems, usually via their production of the Th2-type cytokines or IL-10, while in other systems, they appear to promote enhanced cell-mediated immunity via production of Th1-type cytokines (Smyth and Godfrey, 2000; Wilson and Delovitch, 2003). The mechanisms that determine the cytokine polarity of the NKT cell response, and the influence of the NKT cells response on the systemic immune system, are not well understood, and indeed, this problem represents a key challenge in the field of NKT cell research.

In humans, during early phase of activation, NKT cells produce both Th1 and Th2 cytokines; during later phases of activation, they predominantly produce proinflammatory cytokines such as IFN? and TNFa (Gumperz *et al*., 2002; Lee *et al*., 2002). Accordingly, the CD4+ cells are thought to be the major source of Th2 cytokines for controlling Th1 cell-mediated inflammation or promoting Th2-dependent pathologies. Although earlier studies have tended to focus on the ability of NKT cells to down-modulate inflammatory responses, more recent works have shown that they could promote joint inflammation in models of arthritis (Kim *et al*., 2005; Chiba *et al*., 2005; Ohnishi *et al*., 2005) or mediate airway inflammation in bronchial asthma (Akbari *et al*., 2003; Lisbonne *et al*., 2003). The divergent effects of NKT cells in inflammatory pathologies are thought to reflect a broad spectrum of their functions in vivo. In fact, NKT cells explosively produce a number of pro- and anti-inflammatory cytokines after nonphysiological

stimulation with aGalCer or anti-CD3 mAb (Chen and Paul, 1997), although stimulation with alternative ligands such as aGalCer analogues may lead to selective Th1 (Schmieg *et al*., 2003) or Th2 cytokine production (Miyamoto *et al*., 2001; Miyake and Yamamura, 2005).

NKT cells usually exhibit only a marginal response in response to endogenous ligand bound with CD1d. However, when cytokines are added exogenously, the cells that recognize the endogenous ligand would produce a large amount of selected cytokines. For example, IL-12 induces production of IFN? (Brigl *et al*., 2003), whereas IL-2 provokes IL-5 and IL-13 (Sakuishi *et al*., 2007).

IL-12 induced production of IFN? (Brigl *et al*., 2003; Mattner *et al*., 2005) as well as IL-2 induced production of IL-5 (Sakuishi *et al*., 2007) depends upon the recognition of endogenous ligand via TCR. However, NKT cells could also produce a large amount of cytokines in response to cytokine signals independently of TCR signals. It has been shown that NKT cells can be activated by Escherichia coli LPS (via Toll Like Receptor 4), and produce IFN?, but not IL-4. Nagarajan and Kronenberg have shown that the production of IFN? was dependent upon LPS-induced IL-12 and IL-18 from APC, but did not require CD1d-mediated presentation of an endogenous Ag. Furthermore, they showed that exposure to a combination of IL-12 and IL-18 sufficiently activated the NKTcells (Nagarajan and Kronenberg, 2007). TCR-independent production of Th1 cytokine strongly indicates the innate NK property of NKT cells.

Beaudoin *et al* have recently shown that without applying any exogenous glycolipids, NKT cells could prevent a T cell-transfer model of diabetes by

inducing an anergic state of the pathogenic, islet-specific T cells. In contrast to other related works, this suppression did not require Th2 cytokines but was dependent upon direct cell-cell contact (Beaudoin *et al*., 2002). Subsequent studies showed that the cellular interaction does not involve CD1d recognition by NKT cells (Kent *et al*., 2005; Novak *et al*., 2007). The mechanism of NKT cell mediated regulation remains unclear, although a newly recognized NKT cells (MR1-restricted Va19 NKT cells) have been reported mediating immune regulation via direct contact with B cells independently from TCR recognition (Croxford *et al*., 2006).

While NKT cells can also exhibit cytotoxic activity similar to CD8+ T cells, it is still unclear whether all NKT cells have this capability (Kawano *et al*., 1998).

At least one study in humans demonstrated that the NKT cell pool can be divided into cytotoxic and noncytotoxic subsets, although all NKT cell subsets examined thus far constitutively express FasL and have the potential for cytotoxic activity via Fas (Ho *et al*., 2004). Interestingly, again in humans, a subset of cells that appears to be responsible for cytotoxic activity lacks CD4 expression, express CD8aa homodimers, are CD1d reactive and express the Va24 T-cell receptor (Ho *et al*., 2004).

Antigen presentation and activation of NKT cells by non-professional APCs

Stimulating NKT cells with aGalCer presented by professional APCs leads to production of both pro- and anti-inflammatory cytokines. However, when non-professional APCs such as Schwann cells (Im *et al*., 2006) are used, aGalCer could induce a preferential production of Th2 cytokines from NKT cells.

Gober *et al* studied antigen presentation by CD1d-bearing KCs as well as CD1d bonemarrow-derived cells. NKT cells incubated with KCs and aGalCer did not secrete either IFN? or IL-4 as measured by a sensitive ELISA. In contrast, THP-1, a monocyte-derived cell line, triggered robust IFN? secretion as well as significant IL-4 secretion. To confirm the ELISA data, in parallel, they extracted total cellular RNA from NKT cells and assayed IFN? and IL-4 gene expression using real-time PCR. The PCR data confirmed that NKT cells were triggered to activate IFN? and IL-4 gene expression in the presence of CD1d+ monocyte cell line and aGalCer, but not by CD1d-bearing KCs (Gober *et al*., 2007). Bonish *et al* studied the relative induction of IFN? and IL-4 production of three different NKT cells clones combined with either untreated or IFN? pretreated and extensively washed KCs in the presence of the lipid 12-O-tetradecanoyl phorbol-13-acetate (TPA). They reported that when untreated KCs were combined with these NK-T cell clones, virtually no IFN? was produced, but when KCs were pretreated with IFN?, the NK-T cell clones were stimulated to secrete IFN?. In contrast, the production of IL-4 was relatively low and was not consistently enhanced by combining any of the NK-T cell clones with either untreated or IFN? pretreated KCs. They therefore postulate that a positive feedback loop could be established in skin due to the presence of NK-T cells being activated to produce IFN? upon contact with CD1d-positive KCs, leading to further CD1d expression and subsequent NK-T cell release of more IFN? (Bonish *et al*., 2000).

NKT and cytotoxicity

Previous investigations have focused on the activation and proliferation of NKT cells and shown this to be dependent on Va24 TCR recognition of a-GalCer and similar glycolipids presented by CD1d on antigen-presenting cells. The effector phase and functions of the DN NKT cells require further clarification. Nicol *et al* described that one effector function of human DN NKT cells is cytotoxicity and that some human tumours are susceptible to this cytotoxic activity. This implies that activation of cytotoxic activities of DN NKT cells may be partly or wholly responsible for the anti-tumour effects of a-GalCer in humans.

CD1d expression increased sensitivity to cytotoxic activity of DN NKT cells. All normal and tumour cells expressing CD1d including normal cells (DC and Mo-DC), tumour cell lines and CD1dtransfected cells were susceptible to DN NKT cell cytotoxicity, with the exception of the Daudi cell line. The Daudi cell line expresses a b2-microglobulin-free form of CD1d and this could explain why they are not sensitive to killing. Monocytes, which do not express CD1d, were not susceptible to DN NKT cell cytotoxicity until CD1d expression was induced with differentiation into the Mo-DC phenotype by GM-CSF and IL-4.

Cell lines were only susceptible to cytotoxicity after induction of CD1d expression by transfection and the resulting cytotoxic killing was inhibited by antibodies against CD1d. The a-GalCer further increased CD1d-mediated killing but cytotoxic activity resulting from CD1d expression on the target cells, without added a-GalCer implies the presence of a natural ligand present in the culture systems. An explanation is that Va24TCRs recognize CD1d without glycolipid presentation but that the affinity of CD1d ±TCR interaction is increased by the

glycolipid a-GalCer. Perforin (prf) appears to be a major component required for the induction of CD1d-independent cell lysis by DN NKT cells. This is indicated by the 80% inhibition of lytic activity resulting from the use of CMA which inhibits perforin-mediated killing. The finding that DN NKT cells have cytoplasmic perforin strengthens the case for a role for prf (Nicol *et al*., 2000). Killer lymphocytes release prf and granzymes from cytotoxic granules into the immunological synapse to destroy target cells as a critical mechanism in the defense against viruses and cancer.

Prf, a Ca2+-dependent pore-forming protein that multimerizes in membranes, delivers granzymes into the target cell cytosol

Once released from the cytotoxic lymphocytes, granzyme B (grzB) binds its receptor, the mannose-6-phosphate/insulin-like growth factor II receptor, and is endocytosed but remains arrested in endocytic vesicles until released by perforin. Once in the cytosol, grzB targets caspase-3 directly or indirectly through the mitochondria, initiating the caspase cascade to DNA fragmentation and apoptosis. Granzyme A (grzA), a serine protease in the cytotoxic granules of natural killer cells and cytotoxic T lymphocytes, induces caspase-independent cell death when introduced into target cells by prf. GrzA induces single-stranded DNA damage as well as rapid loss of cell membrane integrity and mitochondrial transmembrane potential through unknown mechanisms.

GrzA is the most abundant protease in the cytotoxic granules of NK cells and cytotoxic T lymphocytes. Similar to grzB and prf, grzA is stored in cytotoxic

granules, which are specialized secretory lysosomes, and is released into the immunological synapse upon triggering by engagement with a target cell.

GrzA and GrzB have been extensively studied, little is known about granzyme K. Flow cytometric analysis of peripheral blood lymphocytes revealed that grzA, grzM and prf show a similar distribution. They are expressed in almost all CD16CD56 NK cells, CD3CD56 NKT cells and CD T cells as well as in 20–30% of all CD3CD8 TC cells. Surprisingly, grzK was not detected in the highly cytotoxic CD16CD56 NK cells, but was preferentially expressed in lymphocytes of the T cell lineage, staining 20% of CD3CD8 TC cells, 50% of CD3CD56 NKT cells and 40% of CDT cells, as well as 60% of the small sub-population of CD56bright NK cells suggesting that human granzymes are differentially expressed in distinct sub-populations of peripheral blood lymphocytes.

Cell death induced by grzK involves the late release of DNA from target cells in a fashion similar to grzA. This is accompanied by a loss of the mitochondrial transmembrane potential and the generation of reactive oxygen species but does not involve caspase activation or chromatin condensation.

	NKT cells	NK cells	T cells	CD25+CD4+ Treg
CD4, CD8	DN - CD4+ - CD8+	X	CD4+ - CD8+	CD4+
Markers	CD94 – CD161	CD94 – CD161	X	CD25
Ligands	glycolipids	peptides lipids (via “missing self”)	peptides	peptides
Receptors	TCR	KIR KLR (NKG2D)	TCR KLR (NKG2D)	TCR
Restriction elements	CD1d	X	MHC class I (CD8) MHC class II (CD4)	MHC class II

Table 1. Comparison between NKT cells, NK cells, T cells and CD25+CD4+ Tregs.

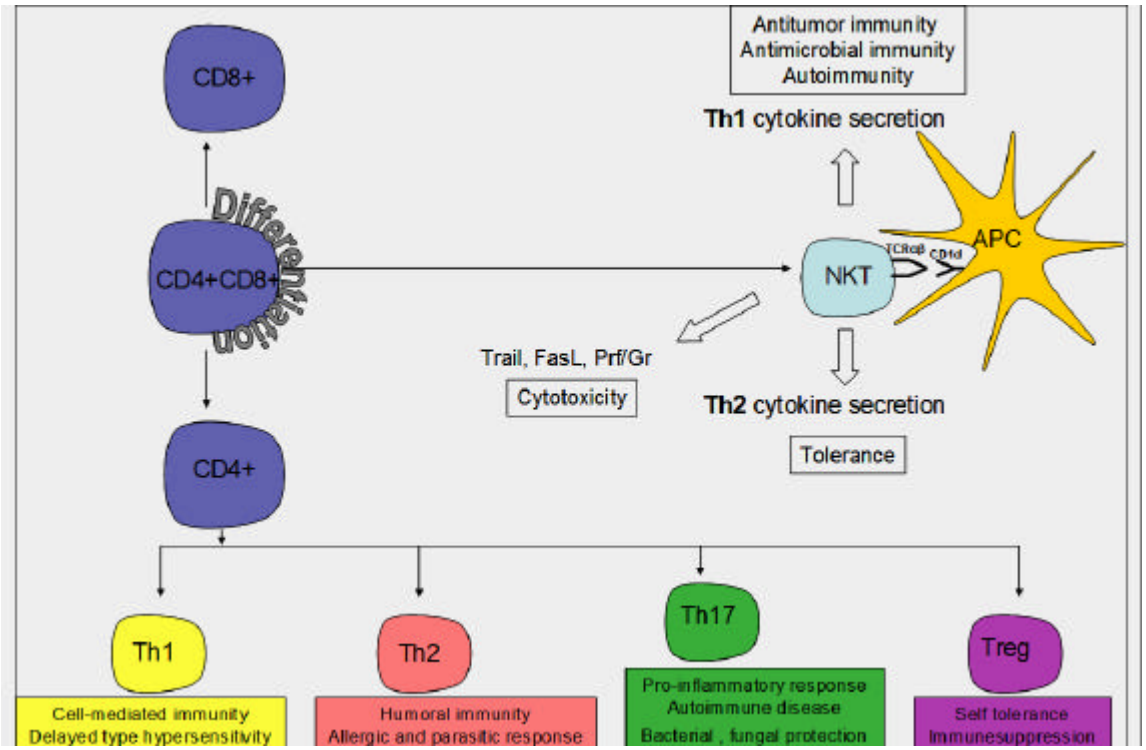


Figure 1. Differentiation of CD4+CD8+ T-cells and activation of NKT cells.

CD4+CD8+ T-cells can differentiate in CD4+, CD8+ and NKT cells (that can be double negative for CD4/CD8 or may be single positive for CD4 or CD8). CD4+ T-cells differentiate in Th1, Th2, Th17 and Treg. Each type of these cells is required for a specific immune response. NKT cells are stimulated by APC via CD1d-TCRaß interaction and rapidly produce Th1 or Th2 cytokines. They also have cytotoxicity activity through TRAIL, FasL and prf/Gr.

Materials and Methods

Human subjects

The criteria for enrollment of subjects in this study was the presence of a 1–2+ score of the patch test allergic reaction at 48 or 96 hours clinical readings (Belsito, 2004). Informed consent was obtained, and skin biopsy specimens were obtained from paired clinically normal skin and the patch test reaction sites. This study was approved by the University of Maryland Baltimore Institutional review board, and adhered to the Declaration of Helsinki Principles. The patients, allergens, patch test scoring, timing of obtaining the skin biopsy specimens from the patch test skin reactions are summarized in Table 2. One patient with plaque-type psoriasis was studied as a disease control. A skin biopsy specimen was taken from uninvolved, clinically normal skin as well as the edge of a psoriatic plaque.

RNA extraction, cDNA synthesis, and real-time PCR (RNeasy Mini Protocol, Qiagen, Valencia, CA)

Skin biopsy specimens from paired clinically normal skin and elicitation sites of ACD were snap-frozen in liquid nitrogen and stored at -70°C until RNA extraction. RNA was extracted and cDNA was prepared, using previously published methods (Fishelevich et al., 2006). Real-time PCR (LightCycler, Roche, Indianapolis, IN) was used to confirm differences in the levels of expression of selected mRNA. The primers, PCR protocol, and product quantification for 18S ribosomal RNA were exactly as reported previously

(SYBER green detection) (Schmittgen and Zakrajsek, 2000). All other primers were designed and prepared by TIB Molbiol (Adelphia, NJ). Amplification (40–50 cycles) of a single PCR product was confirmed by gel electrophoresis and melting curve analyses. The cDNAs were assayed in duplicate, and mean±SD values are depicted in the graphics for the quantitative PCR.

PCR Detection of Perforin and granzymes mRNA was performed using RT-PCR. Sequences of the primers were as follows:

- prf -sense, 5'GCCTCGGTGAAGAGAGGATA3'
  -antisense, 5'AGCAGCAGGAGAAGGATGC3'
- grzA -sense, 5'CAGTTGTCGTTTCTCTCCTGC3'
  –antisense, 5'GGACCATGTAGGGTCTTGAA3'
- grzB –sense, 5'AACCAATCCTGCTTCTGCTG3'
  –antisense, 5'AGATAAGCCATGTAGGGGCG3'
- grzK –sense, 5'CGTTTGTGGAGGTGTTCTGA3'
  –antisense, 5'GAGAGTGTGCGCCTAAAACC3'

The PCR products were run on an agarose gel and photographed.

Reagents and antibodies

Anti-prf, anti-grzA, anti-grzB mouse monoclonal antibodies and anti-grzK rabbit polyclonal Ab were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); Anti-TCR Va24 and anti-Vb11 mAbs were from Immunotech (Fullerton, CA); goat anti-mouse IgG1 and IgG2a biotinylated antibodies, were purchased from

BD Pharmingen (San Diego, CA). Calcein-AM was purchased from Molecular Probes (Eugene, Oreg.) as a 1-mg/ml solution in dry dimethyl sulfoxide.

Immunohistochemical procedures

Frozen ACD samples were cut into 5-mm sections and fixed with cold acetone for 10 minutes. Perforin, Granzymes A and B were stained with mouse monoclonal antibody, whereas Granzyme K with rabbit polyclonal antibody by indirect immunofluorescence for frozen sections, followed by rhodamine or FITC conjugated secondary antibodies. NKT cells were detected for expression of both Va24 (IgG1) and Vb11 (IgG2a) using rhodamineconjugated goat anti-mouse IgG and goat-anti-mouse IgG subclass antibodies and streptavidin–FITC. For double immunofluorescence of NKT cells, frozen sections were stained with primary antibody to TCR Va24 or Vb11 by indirect immunofluorescence, followed by treatment with anti-prf/grzA/grzB/grzK Abs. The staining was observed with Nikon Eclipse E600 epifluorescence microscope equipped with a digital camera. The images were documented using SpotTM imaging system (Diagnostic Instruments, Sterling Heights, MI).

Primary KC, HaCaT, THP-1 and K562 cell lines

Single-cell suspension of KCs for primary culture was prepared by trypsinization of the epidermal sheets after separation from the dermis by dispase digestion of newborn foreskins, with approval of the local institutional review board. KCs were cultured in the

presence of low concentration of CaCl2 (0.05mM) in Epi-Life growth medium supplemented with epidermal growth factor and pituitary extracts (Cascade Biologics, Portland, OR) at 37 °C with 5% CO2 in air. The human KC cell line HaCaT was cultured in DMEM with 10% fetal calf serum and antibiotics. THP-1 and K562 cell lines were cultured in RPMI with 10% fetal calf serum and antibiotics.

NKT-cell assay

A highly enriched cell line (>98% Va24 positive) was developed using previously published methods (Motsinger et al., 2002).

Cultured KCs were derived from foreskins and cultured in serumfree medium containing low (0.05mM) or high (1.20mM) CaCl2. KC culture was performed as published previously (Fishelevich et al., 2006). Twenty-four hours before co-culture with NKT cells, the KC monolayers were treated with IFN-g (500U ml-1) to increase CD1d cell-surface expression. The KCs were trypsinized into single-cell suspensions and co-cultured with NKT cells in a 96-well, round-bottomed plate in a 5% CO2 atmosphere at 37°C for 24 hours in RPMI 1,640 with 10% fetal calf serum. After 24 hours, RNA was extracted to evaluate gene expression of prf and grzs (Pickering et al., 2002). Alpha-galactosylceramide was incubated with NKT cells for 15 minutes at 37°C.

Cytotoxic assays

Cytotoxicity assays were performed according to the Calcein-release method, as previously described (Neri et al., 2001).

After trypsinization, target cells (HaCat and primary KC) were resuspended in complete medium at a final concentration of 10^6/ml and incubated with 15 µM calcein-AM for 30 min at 37°C with occasional shaking. Afterwards they were plated at 5000 cells/well by using 96-well V-bottomed microtiter plates.

The effector (NKT cells) was adjusted to a 10, 20, 30, 40:1 E/T ratio and applied in triplicate. After 4 hours of culture, plates were spun, and 100 mL of supernatant was removed from each well for calcein release measurement.

Samples were measured using a Spectramax Gemini dual-scanning microplate spectrofluorimeter (Molecular Devices, Sunnyvale, Calif.) (excitation filter: 485± 9 nm; band-pass filter: 530±9 nm).

The percentage of specific lysis was calculated as 100 X (experimental release – spontaneous release)/(maximum release – spontaneous release).

Statistical analyses

Quantitative data were analyzed for statistically significant differences between control and treatment groups using the GraphPad Instat Software Program (GraphPad Software, San Diego, CA). Because multiple comparisons were examined, a one-way ANOVA (Tukey Kramer multiple analyses) or unpaired Students' t-test for a simple analysis of two means were applied to the quantitative data ($P<0.05$ were considered significant).

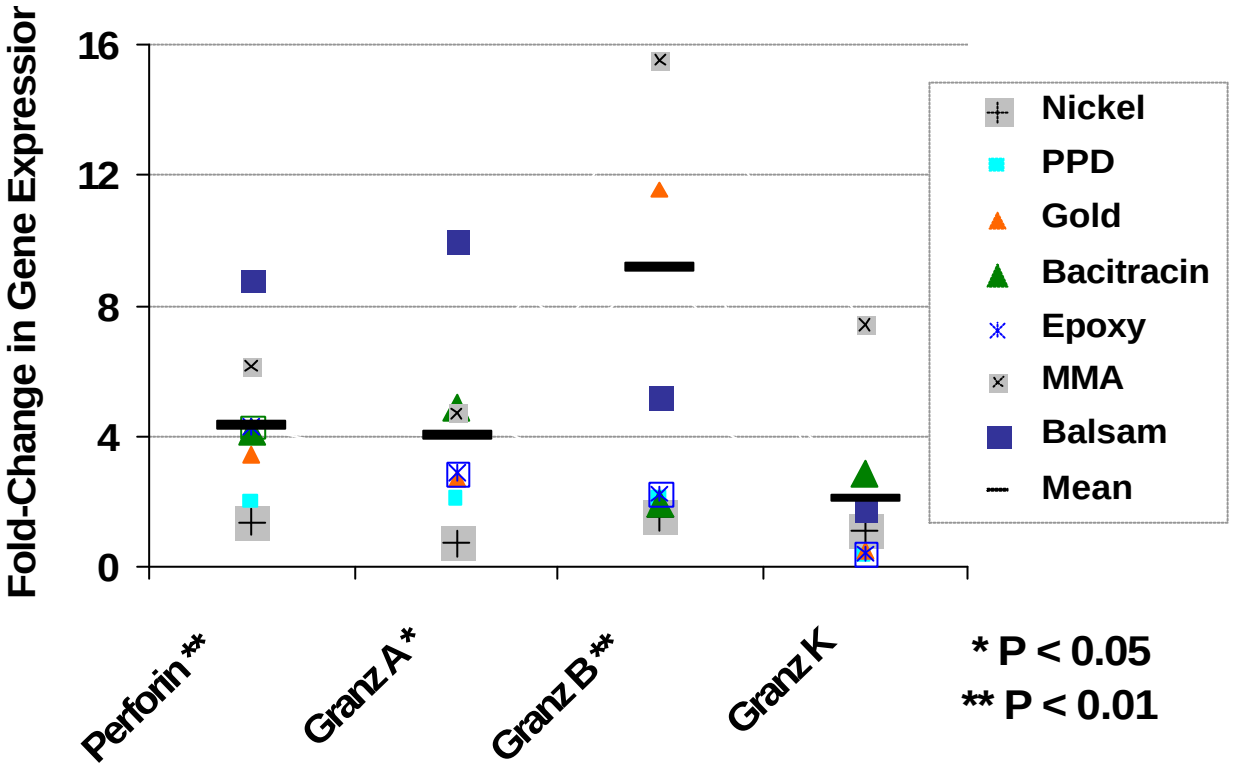
Patient#	Age/race/gender	Allergen/scoring/duration
CD#1	36yrs/white/male	Gold/2+/96hrs
CD#2	49yrs/black/female	Paraphenylendiamine/2+/48hrs
CD#3	40yrs/white/male	Balsam of Peru/1+/48hrs
CD#4	53yrs/white/male	Epoxy resin/1+/48hrs
CD#5	71yrs/white/female	Nickel sulphate/2+/96hrs
CD#6	26yrs/black/male	Bacitracin/2+/48hrs
CD#7	42yrs/white/male	methylmethoxylate/1+/72 hrs

Table 2. Patients' characteristics, allergens, patch test scoring and timing of obtaining the skin biopsy specimens.

Results

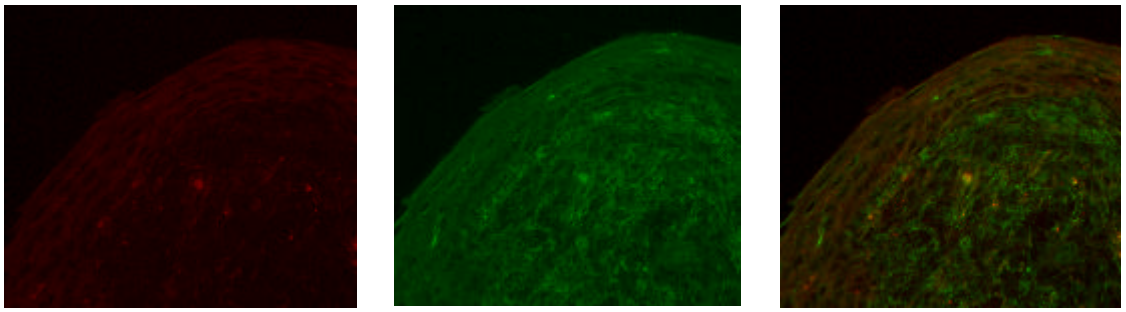
Cytotoxic molecules gene expression in ACD

All the cytotoxic molecules were overexpressed in positive patche test reaction respect to normal skin. The fold-change increase is expressed as mean. GrzA increase was of 3.97 ($p<0.01$), whereas prf and grzB were respectively 4.29 and 9.16 ($p<0.05$). A tendency to overexpression was also registered for grzK (2.04), even if there was no statistical significance.

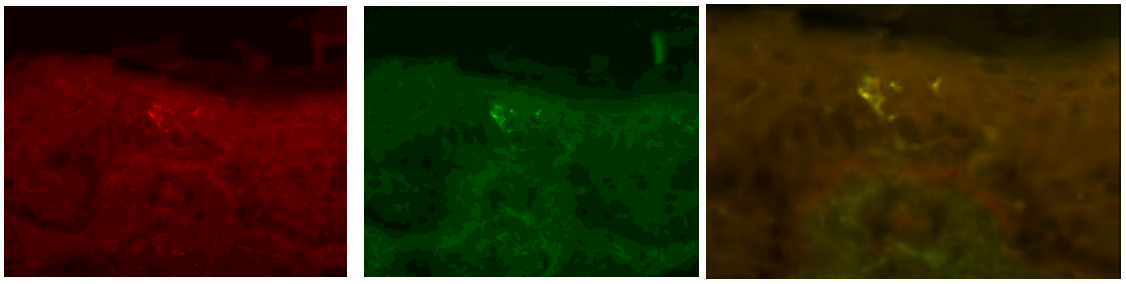


Co-localization of NKT cells and cytotoxic molecules in ACD.

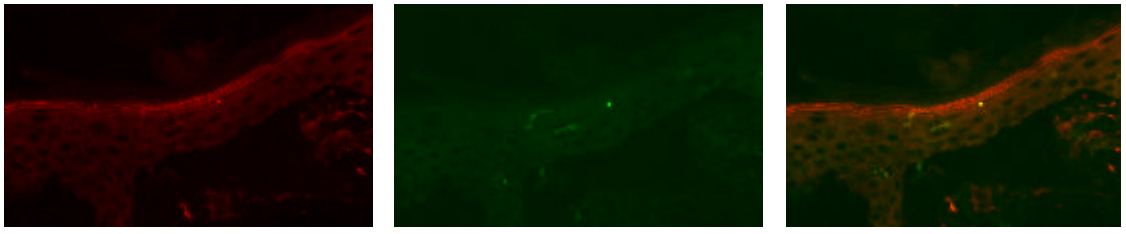
All the cytotoxic molecules were present in positive patch test reaction and co-localized with NKT cells.



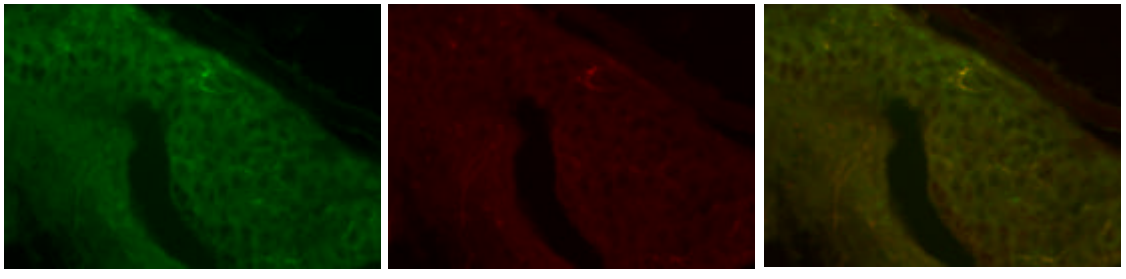
PRF **Vb11** **Merged**



GrzB **Va24** **Merged**



GrzA **Vb11** **Merged**

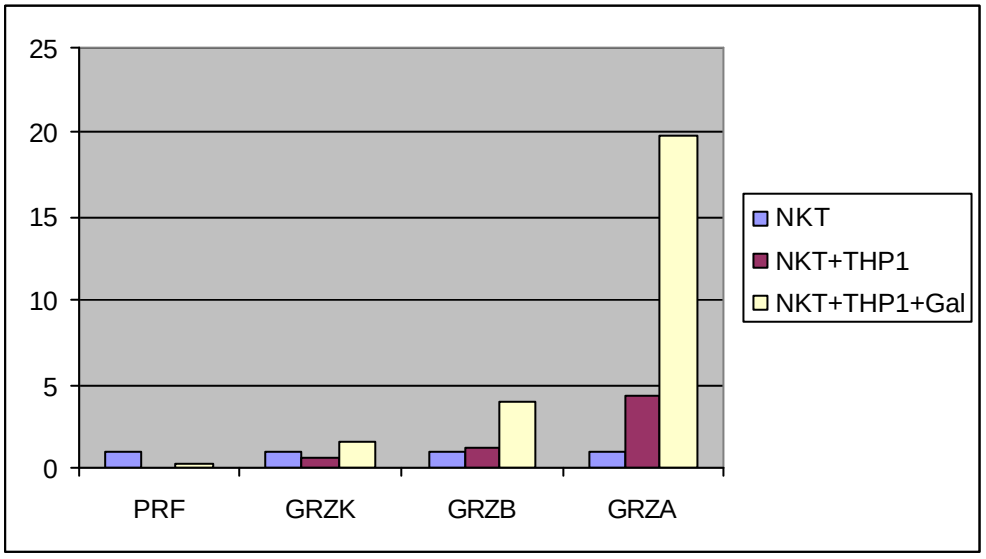


GrzK **Va24** **Merged**

Gene expression during antigen presentation

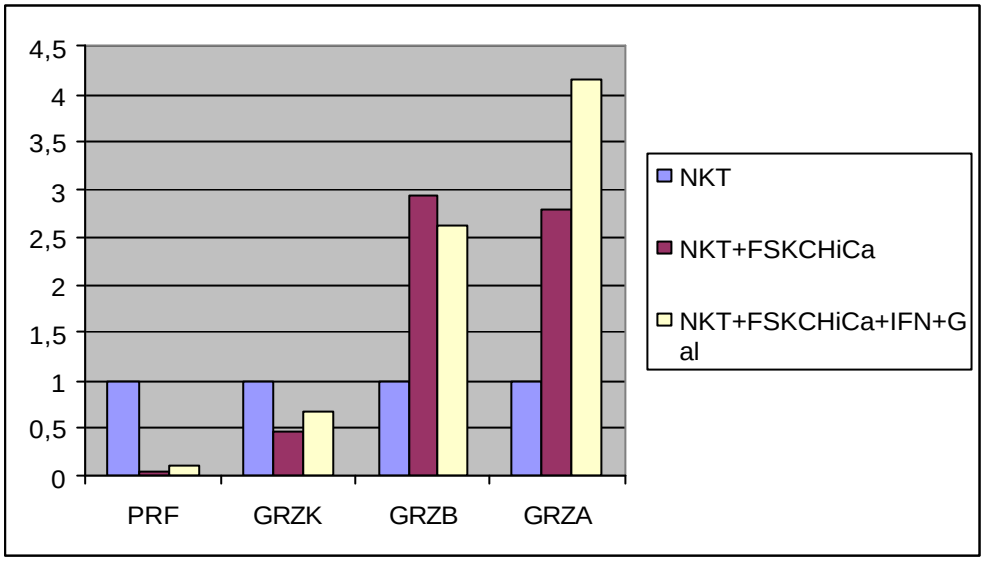
Granzymes were overexpressed during antigen presentation by THP-1 cells, especially when alpha-galactosylceramide was added.

An increase of 4.40 was detected for grzA in the co-culture of NKT and THP-1 cells, whereas an increase of 19.80 was registered when alpha-galactosylceramide was added. There was an increase also for GrzB expression in the co-culture system (1.21), but with the adding of alpha-galactosylceramide it was of 3.99. We found a light increase of the expression of grzK (1.56) in the presence of alpha-galactosylceramide, but no increase in the co-culture system without the stimulus. No increase was registered for prf expression in both conditions.



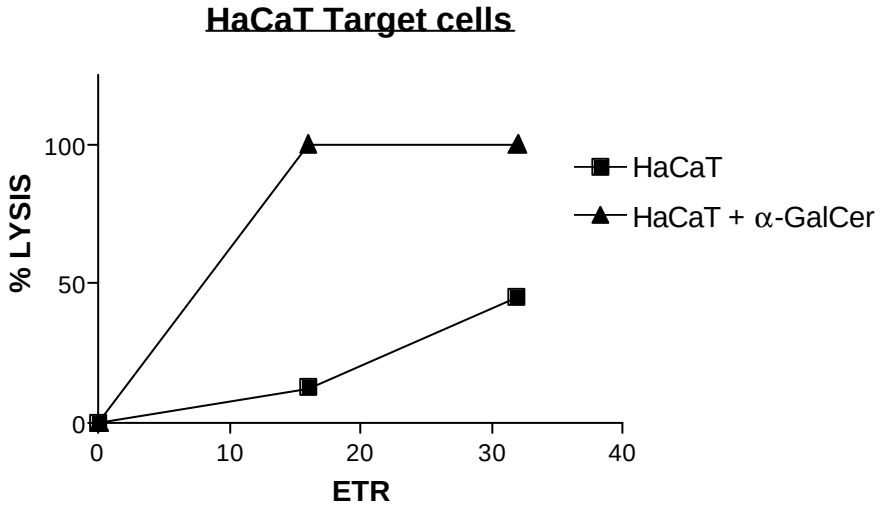
Granzymes A and B were overexpressed during antigen presentation by primary keratinocytes. An increase of 2.78 was detected for grzA in the co-culture of NKT cells and KCs, whereas an increase of 4.14 was registered when alpha-galactosylceramide was added. There was an increase also for GrzB expression

in the co-culture system (2.92), but with the adding of alpha-galactosylceramide it was of 2.62; in this case there was no difference in adding the stimulus. No increase was detected for prf and grzK expression in both conditions.



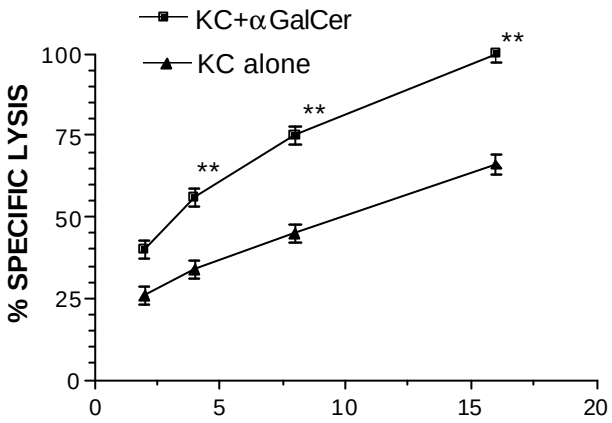
NKT cell cyototoxic activity vs keratinocytes

Cytotoxic activity of NKT cells is increased versus HaCat cells. The percentage of lysis was almost 50%, whereas the adding of alpha-galactosylceramide enabled NKT cells to kill 100% of HaCat cells.



Similar results were registered when the same experiment was repeated with primary KCs. Furthermore in this case the effector : target ratio needed to reach these results was the half.

LYSIS OF KC BY NKT-CELLS



Discussion

The skin represents a unique immunologic organ poised to protect the host from invading organisms and environmental antigens. The skin is also an important target for a variety of allergic and autoimmune responses.

NKT cells have been reported to play an active role in skin diseases such as contact sensitivity, have been implicated in atopic dermatitis, UV-induced immunosuppression and are present in psoriasis skin lesions.

Delayed-type hypersensitivity in the form of contact hypersensitivity (CHS) to haptens is a clinically important syndrome known as allergic contact dermatitis

(ACD) (Belsito, 1999). ACD is an important dermatologic disease with considerable morbidity and economic impact; CD from both irritant and allergic sources comprise 6% to 10% of all dermatology clinic visits (Thyssen *et al*., 2007). Some studies have focused on the role of NKT cells in CHS in experimental mouse models (Askenase, 2001; Campos *et al*., 2003; Stein-Streilein, 2003; Nieuwenhuis *et al*., 2005).

Campos *et al* indicated a role for this cell type in the early molecular events of CHS, in which NKT cells respond to hapten application and interact with B-1 lymphocytes, resulting in production of hapten-specific IgM (Campos *et al*., 2003) These findings were confirmed by an in vitro system too (Campos *et al*., 2006). Askenase *et al*. assessed that elicitation of CHS begins with an initiation process that results in T-cell recruitment. In fact, there are two starting points, both involving cells of the innate immune system. The first of these is induction of the initiation process. The other is actual initiation of the late elicited effector phase due to recruitment of the T-cells. Induction of the initiating process, which subsequently elicits T-cell recruitment, begins within minutes of immunization by postulated release of endogenous glycolipids from the sensitizing site. These glycolipids bind to CD1d on APCs, leading to the activation of NKTcells; these release IL-4 to coactivate B-1 cells.

In the second of the two mentioned starting points, initiation of the effector phase occurs within minutes of secondary local Ag challenge, when B-1 cell derived IgM antibodies trigger an elicitation cascade of innate responses. This starts with classical complement activation, generating C5a, which in turn activates mast

cells and platelets to release TNFa and serotonin; these then activate endothelial cells, leading to local T-cell recruitment (Askenase *et al*., 2004). Gober *et al* reported that CD1d gene expression was increased in elicitation sites of ACD relative to normal human skin. Similarly, both CD161 gene expression and variable region of TCR Va24, JaQ region of TCR gene expression in skin lesions of ACD were increased relative to normal skin, indicating that invariant NKT cells are commonly present in the T-lymphocyte infiltrate of ACD. It was demonstrated that human invariant NKT cells regularly appear in the lymphocytic infiltrate that occurs during the elicitation phase of human ACD, either at the 48 or 96 hours time frame of clinical readings of this skin reaction by immunohistochemistry, real-time PCR, as well as nested PCR (Gober *et al*., 2007). Lee *et al*. reported that in healthy adults, NKT cells are present at a very low frequency in the peripheral blood, approximately 0.1% of the total population of peripheral blood leukocytes (Lee *et al*., 2002). Based on this they demonstrated the frequency of NKT cells in the ACD lesions to range from 1.72 to as high as 33% of the total CD2+ infiltrating T lymphocytes, this representing a relative enrichment of NKT cells ranging from a 17- to 330-fold enrichment above the normally very low frequency found in the peripheral blood. This suggests that NKT cells may be specifically recruited into ACD sites, rather than a passive extravasation event, in which relative enrichment of frequency relative to other cell types would be expected not to occur. Additionally, immunohistochemical studies of ACD demonstrated that there was abundant KC apoptosis in this condition as well as atopic dermatitis, suggesting cell mediated cytotoxicity is active in both condtions

(Trautman *et al*., 2001). Our results show that cytotoxic molecules expression is increased in ACD and that cytotoxic molecules are co-localized with NKT cells in ACD. Furthermore prf and granzymes expression is increased during the process of antigen presentation; The increase is higher when THP-1 are the APC cells, especially when aGalCer is added. Also KC are able to behave as APC cells to NKT cells, especially when they are treated with IFN? and aGalCer. Another finding is represented by the cytotoxic activity of NKT cells versus keratinocytes. NKT cells are able to kill keratinocytes and this killing is increased by aGalCer.

From our data we can assess that NKT cells are in an activated state in the elicitation phase of ACD. The importance of NKT cells, at least, in the priming phase of CS is reinforced by numerous experiments, even if further work is needed to be performed to better understand the role of NKT cells in the elicitation phase too, and in ACD in general.

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