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Invariant Natural Killer T Cells in Rheumatoid Arthritis and Other Inflammatory Arthritides

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1. Introduction

Like cells of the adaptive immune system, natural killer T (NKT) cells possess immune recognition receptors formed by germline DNA rearrangement. However, in common with cells of the innate immune system, the repertoire of NKT cell receptors is limited and NKT cells can mount a robust effector response with little capacity for immunological memory, sharing some of the characteristics of other innate-like lymphocytes such as γδ T cells, marginal zone B cells, B1 B cells and NK cells. NKT cells have been shown to play a staggering array of roles in infection, cancer and autoimmunity. Autoimmune diseases in which NKT cells have been implicated include type I diabetes, multiple sclerosis, systemic lupus erythematosus and graft-versus-host disease. In rheumatoid arthritis, much work has been done to characterise the frequency and phenotype of NKT cells. Animal models such as collagen-induced arthritis or the antibody-mediated arthritis in the K/BxN serum transfer model have provided valuable insight into the multi-faceted potential of these remarkable cells and in time, pharmacological manipulation of their immune function may provide us with the prospect of novel therapeutic tools.

2. Biology of natural killer T cells

Natural killer T cells were first recognised in 1990 as $CD4^{-}CD8^{-}$ double negative (DN) thymocytes bearing the murine natural killer (NK) cell marker NK1.1 or the orthologous CD161 in humans (Ballas & Rasmussen, 1990). Since their discovery, the unexpected ontological complexity, development, function and pathophysiological roles of NKT cells have begun to unravel. Here, we briefly review the biology of NKT cells and focus on a subset known as invariant NKT (iNKT) cells by virtue of their CD1d-restricted, semi-invariant T cell receptor in order to better understand their significance in autoimmune diseases such as rheumatoid arthritis.

2.1 CD1 molecules & the ontogeny of the NKT cell

The first mouse anti-human monoclonal antibody recognised an antigen found on human thymocytes and certain B cell lymphoma lines subsequently termed the first cluster of differentiation or CD1. Five CD1 protein isoforms encoded on chromosome 1 bear resemblance to the α chains of MHC class I encoded on chromosome 6. CD1 molecules associate non-covalently with β_2-microglobulin, but unlike MHC class I molecules they

show very limited polymorphism. Human CD1a, -1b and -1c (CD1 group 1) are widely expressed on dendritic cells and other professional antigen presenting cells (APC). CD1d (CD1 group 2) expression appears to be independent of group 1 isoforms and can be found on lymph node mantle zone B cells, cortical thymocytes, activated T cells, gut and liver tissues. CD1b was first shown to present microbial antigen in 1992 which was later identified as lipid antigen. Since then it has become clear that CD1 molecules bind diverse hydrophobic ligands, with the exception of CD1e which remains intracellular and is thought to be involved in lipid antigen processing and loading (Brigl & Brenner, 2004; Strominger, 2010).

After the discovery of $NK1.1^+$ $CD4^-CD8^-$ double negative (DN) thymocytes, further work led to iNKT cells bearing a TCR consisting of an invariant Vα14-Jα18 rearrangement and a limited repertoire of Vβ8.2, -7 or -2 chains in mice or the homologous Vα24-Vα18 and Vβ11 chains in humans. Later, the TCR of iNKT cells was shown to be CD1d-restricted and these cells were coined the type I NKT cells (Godfrey et al., 2004). Like iNKT cells, type II NKT cells are CD1d-restricted but possess a more diverse TCR repertoire that also displays bias. They have been shown to possess regulatory and pathogenic functions but have been less well studied than iNKT cells. Non-CD1d restricted T cells that possess NK cell markers also exist and are referred to as NKT-like or type III NKT cells. In mice, 20-80% of $CD3^+NK1.1^+$ T cells stain with α-GalCer/CD1d-tetramer. In humans, while 20-25% of T cells are $CD161^+$, less than 1% are α-GalCer/CD1d-tetramer$^+$ and many staining cells are $CD161^-$. These cells comprise CD1 group 1-restricted NKT cells that can be $\alpha\beta^+$, $\gamma\delta^+$, $CD4^+$, $CD8^+$, or $CD4^-CD8^-$ T cells, and may also include mucosa associated invariant T cells (MAIT) that express an invariant TCRα chain restricted to the MHC class I-like molecule MR1 (Godfrey et al., 2010).

2.2 iNKT cell development and homeostasis

iNKT cells develop in the thymus when invariant TCRα rearrangement and CD1d recognition initiate positive and negative selection, but unlike conventional T cells positive selection of iNKT cells involves double-positive $CD4^+CD8^+$ cortical thymocytes rather than cortical epithelial cells. TCR, SLAM and other co-stimulatory signals (CD28, ICOS, TGF-β) are required for maturation, expression of activation (CD44, CD69, CD122) and NK (KLRG1 and NK1.1) markers, and acquisition of innate effector functions dependent on the transcriptional regulator promyelocytic leukaemia zinc finger protein (PZLF). Maturation to $NK1.1^+$ or $CD161^+$ occurs in the periphery in mice and humans, or in the thymus in mice where a long-lived population of thymic mature iNKT cells may contribute to fine-tuning the negative selection of conventional T cells (D'Cruz et al., 2010).

Similar to naïve conventional T cells, peripheral iNKT cell are not expanded in unchallenged mice, with an average clonal size of 5-10 cells that do not require interaction with CD1d for homeostasis. Nevertheless, iNKT cell frequency is orders of magnitude higher than that of naïve MHC class I and II restricted T cells. Humans have fewer iNKT cells than mice although there is wide variation amongst individuals, varying between undetectable and 3% of peripheral lymphocytes. It is not known whether this is related to thymic development and migration, or peripheral proliferation and maintenance differences but the phenomenon appears to be a stable, genetically determined phenotype in both mice and humans (Van Kaer et al., 2011).

iNKT cells are most abundant in the liver and spleen in mice but exceed the number of antigen-specific T cells in the lymph nodes by 500-5000 fold. In keeping with their innate function, NKT cells also accumulate in inflammatory lesions where they can rapidly become

activated to release a diverse range of cytokines, proliferate and influence the subsequent adaptive immune response (Fox et al., 2010).

2.3 Antigen and antigen-independent activation pathways

Unlike conventional T cells, iNKT cells appear to be selected in the thymus for their ability to recognise both microbial lipid antigen and self-antigen. The direct pathway of activation involves recognition of microbial lipid antigen presented on CD1d. In the case of the lipoglycan α-GalCer, CD1d-restricted antigen presentation is sufficient for iNKT cell activation although co-stimulatory signalling mediated by constitutive CD28 expression can further augment the iNKT response. Alternatively, the indirect pathway activates iNKT cells in response to microbial organisms lacking cognate glycolipid antigens by recognition of self-antigen, together with co-stimulatory cytokine signals (IL-12, IL-18) from toll-like receptor (TLR) ligand-activated APCs. Finally, cytokine-mediated iNKT cell activation independently of CD1d involvement has also been observed in response to lipopolysaccharide (LPS) or viral CpG-activated APCs (Reilly et al., 2010).

By virtue of their semi-invariant TCR, all iNKT cells can recognise glycolipids consisting of a galactose or glucose moiety α-linked to the polar head of a lipid. The prototypical extrinsic iNKT antigen is the α-linked galactosylceramide α-GalCer, a glycosphingolipid extracted from the non-sterile marine sponge *Agelas mauritianus.* α-GalCer and related glycosphingolipids are not present in mammalian cells but can be found in the cell walls of *Novosphingobium*, previously known as *Sphingomonas* bacteria that colonise the marine sponge. Glycosylated diacylglycerol lipids in *Borrelia burgdorferi* are also recognised by a sub-population of murine iNKT cells and iNKT cell deficiency is associated with reduced spirochete clearance and chronic inflammation. Other microbial CD1d-restricted lipids are thought to be present in *Plasmodium falciparum*, *Trypanosoma* spp, *Leishmania* spp, *Ehrlichia* spp, *Streptococcus pneumoniae*, *Helicobacter pylori* and *M bovis*. The importance of these other microbial lipid antigens is however unclear as they are not strong TCR agonists (Brigl & Brenner, 2010).

The nature of relevant lipid self-antigen(s) has remained a matter of considerable debate. Phospholipids such as phosphatidylinositol, phosphatidylethanolamine, and phosphatidylglycerol can be eluted from CD1d but most however are not stimulatory or stimulate only a small fraction of iNKT cells. In contrast, sphingolipids such as the tumour-derived ganglioside GD3 or the lysosomal ϐ-linked glycosphingolipid isoglobotrihexosylceramide (iGb3) have been shown to be recognised by iNKT cells (Gapin, 2010). Lysophosphatidylcholine (LPC) is produced from membrane phosphatidylcholine by phospholipase-A2, an enzyme produced by myeloid APC and activated during the inflammatory response. Thus increased presentation of LPC to iNKT cells during inflammation has been proposed to lead to their enhanced activation and cytokine production (Fox et al., 2010). In addition to constitutive stimulatory self-antigens, neo-self-antigens have been proposed to arise following exposure of APC to microbial or viral danger signals resulting in increased iNKT cell sensitivity to existing self-lipids, generation of novel lipid entities that are not constitutively expressed or increased antigen presentation and co-stimulation (Reilly et al., 2010).

2.4 iNKT cell activation and effector functions

Stimulatory antigens such as α-GalCer rapidly activate iNKT cells within hours, up-regulating surface markers such as CD25 and CD69 and producing cytokines through

constitutive expression of mRNA for IL-4 and IFN-γ. iNKT cells proliferate and expand up to 10-fold in the spleen, 5-fold in blood, bone marrow and lymph nodes, and 2- to 3-fold in liver with a peak at 3-4 days after antigen exposure. Unlike conventional T cells, iNKT cells show not only a lack of secondary memory response but a hyporesponsive state of immunological anergy lasting up to 2 months (Van Kaer et al., 2011).

Upon activation, iNKT cells have been reported to be capable of producing a wide variety of both Th1 and Th2 cytokines (IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17, IL-21, IFN-γ, TNF-α, GM-CSF) (Coquet, 2008). The precise pattern of cytokines produced may depend on factors such as tissue distribution, iNKT cell subset, antigen processing, activation pathway, TCR signal strength and cytokine milieu. In addition, chemokines and chemokine receptors allow homing to inflammatory sites where iNKT cells form a bridge between the innate and adaptive immune systems by jump starting antigen-specific responses (Salio et al., 2010).

Activated iNKT cells can stimulate inflammatory myeloid APC, NK and B cell function by the production of CD40L and pro-inflammatory cytokines such as IFN-γ and TNF-α which can induce maturation with up-regulation of co-stimulatory molecules, perpetuation of cytokine and chemokine production, thus enhancing MHC-restricted T cell stimulation and ensuing adaptive immune responses. In contrast, iNKT cells have also been shown to potentiate antigen-specific immune tolerance in a number of animal models of autoimmunity, organ transplantation and therapeutic mucosal immune tolerance induction. The mechanisms by which iNKT cells induce or maintain tolerance may be mediated by a shift in secretion toward regulatory cytokines such as IL-10 and IL-4 although experimental data have not been consistent and cytokine-independent mechanisms such as generation of regulatory DCs may play a role (Hegde et al., 2010).

2.5 Pathophysiological roles of iNKT cells

In summary, iNKT cells are a unique subset of T cells that can help orchestrate both pro-inflammatory and regulatory immune responses. Despite their small population size, they can simultaneously promote resistance against microbial infection, participate in tumour immunosurveillance, maintain peripheral tolerance and prevent autoimmunity.

iNKT cells have long been known to react to self. Such autoreactivity is TCR and CD1d dependent in both mice and human iNKT cells. It has been suggested that the antigens responsible for autoreactivity are the same as those involved in thymic selection so that iNKT cells are autoreactive by design (Gapin, 2010). An immunosuppressive role for iNKT cells has now been shown in a number of animal models of autoimmunity including type I diabetes in non-obese diabetic (NOD) mice, experimental autoimmune encephalomyelitis as a model of multiple sclerosis in C57BL/6 and NOD mice, models of systemic lupus erythematosus (SLE) and graft-versus-host disease (GvHD). In some cases, iNKT cells have been shown to play a pathogenic rather than protective role. Despite such apparently conflicting results, iNKT function can be harnessed for tolerance induction, as demonstrated most notably in the prevention of GvHD. In this chapter, we will focus our discussion on the possible roles of iNKT cells in rheumatoid arthritis (RA) and other inflammatory arthritis.

3. iNKT cells in rheumatoid arthritis

RA is an inflammatory arthritis affecting small and large synovial joints, mediated by a destructive interplay between T cells, B cells, macrophage-like synoviocytes and fibroblast-like synoviocytes, ending with synovial cartilage invasion and ultimately joint destruction

(Scott et al., 2010). Given the dual pro-inflammatory and regulatory potential of iNKT cells, the study of their frequency and phenotype in RA, and their role in animal models of inflammatory arthritis, have been of significant interest to researchers in the field.

3.1 iNKT cell frequency

Until the advent of α-GalCer/CD1d-tetramers and invariant TCR chain-specific monoclonal antibodies, much of the earlier work on iNKT cells had been muddied by the lack of specific reagents for reliable iNKT cell identification. However, despite the limitations posed by older identification methods, most studies have consistently shown that NKT or iNKT cell absolute and relative frequencies are reduced in RA. Here we review studies examining the peripheral and synovial compartments, individual iNKT cell subsets and the relationship between iNKT cell frequency, disease activity and treatment response.

3.1.1 Peripheral blood compartment

In the earliest published study on NKT cells in RA, Yanagihara et al. (1999) looked at $CD3^+NKR\text{-}P1A^+(CD161^+)$ NKT cells in 60 patients with established RA compared with 36 healthy controls. They found a 5.8 fold difference in NKT cells but no difference in NK cells. Although patients and controls were mismatched for age, no correlation with age was found in either group. There was no apparent correlation with disease duration, clinical disease activity, inflammatory markers, RF status or drug treatment.

Recent studies of iNKT cell frequency using more specific detection reagents have confirmed results from earlier studies. Parietti et al. (2010) detected iNKT cells with a monoclonal antibody (mAb) against the canonical Vα24Jα18 invariant TCR chain in 36 RA, 43 SLE and 31 healthy subjects. The investigators confirmed the lower frequencies and percentages of iNKT in RA and SLE vs controls (0.09% and 0.01% vs 0.26%, respectively). They found no effect of age, gender or treatment on iNKT cell frequency.

Our own group analysed the frequency of $V\alpha24^+V\delta11^+$ NKT cells among 46 RA and 22 healthy controls, taking care to use a statistically robust minimum number of lymphocyte-gated events set at 500,000 in order to reliably measure the infrequent iNKT cells. Our results showed that RA patients have a 15-fold lower iNKT cell relative frequency compared to healthy controls (0.001% vs 0.21%, respectively), either before or after commencing immunosuppressive treatment (Tudhope et al., 2010).

3.1.2 Synovial compartment

In a comparative analysis of NKT cell frequency in different compartments, Spadaro et al. (2004) studied 29 patients with psoriasis and psoriatic arthritis (PsA), 27 patients with RA and 27 healthy controls. Blood and synovial fluid (SF) lymphocyte subsets, including $CD3^+CD16^+CD56^+$ NKT cells, were measured and compared. In peripheral blood, there was no statistically significant difference in NKT cell absolute or relative numbers between PsA, RA and healthy control subjects (61 cells/μL or 3.6%, 93 cells/μL or 5% and 89 cells/μL or 3.9%, respectively). SF NKT cells however were significantly reduced in both absolute and relative numbers as compared to peripheral blood in PsA and RA (2% vs 3.2% and 1.6% vs 4.1%, respectively) (Spadaro et al., 2004).

Linsen et al. (2005) studied 23 RA and 22 healthy control patients using peripheral blood and, when available, synovial fluid and tissue specimens. They found that $V\alpha24^+V\delta11^+CD3^+$ NKT cells were significantly reduced in relative frequency in RA as compared to control

peripheral blood (0.03% vs 0.11%, respectively) but unlike Spadaro et al. (2004), their synovial fluid samples showed an inconsistent trend toward higher percentage of NKT cells in SF from seven patients as compared to matched peripheral blood (0.08% vs 0.05%, respectively).

3.1.3 iNKT cell subsets

iNKT cell subsets include $CD4^-$ (DN), $CD4^+$ and $CD8^+$ cells. iNKT cells from these subsets have been shown to be functionally distinct and therefore individual subset frequency may be immunologically more relevant than global iNKT cell numbers.

In a study of patients with a range of autoimmune diseases including 20 patients with RA, $V\alpha24J\alpha18^+$ DN T cells were found to express polymorphic Vδ11 and CD161 almost universally, suggesting that these were likely to be true iNKT cells. The frequency of these DN NKT cells was markedly reduced in RA patients who had a mean of 48.8 cells/ml as compared to 290 cells/ml in healthy controls. Similar findings applied to patients with SLE, systemic sclerosis (SSc) and Sjogren's syndrome (SS) but not Behcet's disease (BD), and $CD4^+$ NKT cells were similarly reduced in frequency (Kojo et al., 2001).

$V\alpha24^+CD8^+$ NKT cells have been shown to consist mainly of $CD161^+$, CD1d-restricted NKT cells that have an immunoregulatory phenotype (Ho et al. 2004; Takahashi 2002). Mitsuo et al. (2006) examined the frequency of this NKT cell subset in patient with RA (n=24), SLE (n=54), SSc (n=14), mixed connective tissue disease (MCTD) (n=15) and polymyositis/ dermatomyositis (PM/DM) (n=13) compared to healthy controls (n=18). The absolute frequency of $CD161^+CD8^+$ T cells was reduced in all patients compared to healthy donors, although in RA patients the relative frequency was not statistically significantly lower. No correlation with age, gender or treatment was noted (Mitsuo et al., 2006).

3.1.4 Correlation with disease activity and treatment response

Whilst Yanagihara et al. (1999) found no correlation between iNKT cell frequency and clinical disease activity, Parietti et al. (2010) noted a trend toward lower iNKT cell numbers with higher disease activity (DAS28), ESR and CRP. Our group also found no correlation with disease activity although a moderate but statistically significant inverse relationship with CRP could be demonstrated (Tudhope et al., 2010).

Parietti et al. (2010) measured iNKT cell frequencies in seven patients before and after treatment with rituximab, an anti-CD20 monoclonal antibody targeting B cells. They found a significant increase in NKT frequency and percentage from baseline at days 45 and 120 post-infusion (1.7, 3.4 and 4.1 cells/μL, or 0.1%, 0.32% and 0.3%, respectively). There was also a clear correlation between clinical outcome and NKT cell frequency change with non-responders showing no change whilst responders saw a 600% increase in frequency. In our study, we measured iNKT cell frequencies in seven patients before and after initiating methotrexate therapy and found that iNKT frequency increased as early as two weeks after the start of treatment. Unlike Parietti et al.'s results however, we found no obvious link to clinical response (Tudhope et al., 2010).

3.2 iNKT cell phenotype

3.2.1 Response to α-GalCer

Kojo et al. (2001) examined the functional phenotype of $V\alpha24J\alpha18^+$ DN iNKT cells in patients with RA and other autoimmune diseases. Peripheral blood mononuclear cells

(PBMC) stimulated with α-GalCer for 10 days resulted in expansion of iNKT cells in just 3 out of 10 RA patients (from 0-10 to 61-1480 cells/10^5 lymphocytes) as compared to all 7 healthy controls (from 6-123 to 350-3169 cells/10^5 lymphocytes). The proportion of responders was 50% in patients with SLE (n=10) and SSc (n=8). No clear relationship was noted to disease activity.

In further experiments, Linsen et al. (2005) also characterised the phenotype of $V\alpha24^+V\delta11^+CD3^+$ iNKT cells in RA compared to healthy controls. The capacity of NKT cells to respond to α-GalCer was tested in peripheral blood of 7 healthy and 13 RA patients as well as the SF from 5 RA patients. PBMC or synovial mononuclear cells (SFMC) were stimulated with α-GalCer and re-stimulated at day 7 with pulsed, irradiated PBMCs then analysed by flow cytometry at day 14. The number of NKT cells from RA peripheral blood (PB) and SF remained lower than that of healthy controls (8.4 and 4.4 vs 15.8%, respectively). Like Kojo et al. (2001), the investigators noted two separate RA populations comprised of non-responders (6/13 patients) and responders (7/13 patients). In fact, RA responders showed stronger responses than healthy controls (294 vs 149 fold, respectively). They too did not find any correlation between response and clinical or treatment phenotype. SF iNKT cells however responded in all tested patients.

In keeping with these earlier results, our own experiments showed that peripheral iNKT cells stimulated with α-GalCer for 12 days display impaired expansion in RA patients compared to controls (31 vs 121 fold, respectively). Using the 25th percentile of fold-expansion in healthy controls, 75% of RA vs 20% of healthy controls are non-responders with no detectable differences between early and late RA (Tudhope et al., 2010).

While investigating the mechanism underlying non-response to α-GalCer in RA patients, Kojo et al. (2001) found that non-responder APCs could expand responder iNKT cells in the presence of α-GalCer and IL-2, albeit with a lower response than responder APCs. In contrast, non-responder iNKT cells failed to expand in the presence of responder APCs under the same culture conditions. This suggested that in non-responders, the defect lay within iNKT cells rather than APCs.

3.2.2 Cytokine production

In keeping with iNKT cell frequency as measured by flow cytometry, linsen et al. performed ELISPOT analysis of cytokine production by isolated PBMC stimulated with α-GalCer and found a decreased frequency of reactive cells producing IFN-γ (2.3 vs 24.3, respectively) and IL-4 (0.2 vs 3.9, respectively) in RA patients compared to healthy controls. The IL-4/IFN-γ ratio was also reduced (0.07 vs 0.30, respectively), suggesting a Th1-like phenotypic bias in RA which could not be explained by any differences in $CD4^+$ and $CD4^-$ subsets, or selective clonal expansion as shown by Vα24 and Vδ11 TCR CDR3 fragment length spectra analysis (Linsen et al., 2005).

Intracellular cytokine staining of iNKT cells in PB revealed marked differences between RA patients and healthy controls for IFN-γ^+ (92.5 vs 64.5%, respectively), IL-4^+ (1.4 vs 15.7%, respectively) and IFN-γ^+IL-4^+ iNKT cells (6.1 vs 19.7%, respectively), confirming the Th1 bias in PBMC-derived iNKT cells. In contrast, cytokine profiles for SF iNKT cells were more similar to that of healthy control PB iNKT cells, with smaller proportion of IL-4^+ and higher proportion of IFN-γ^+IL-4^+ iNKT cells (5.3 and 28.4%, respectively), suggesting a Th0-profile in SF-derived iNKT cells. No correlation with response to α-GalCer, or disease and treatment parameters was noted (Linsen et al., 2005).

3.2.3 Clonal heterogeneity

Linsen et al. (2005) also examined clonal heterogeneity by TCR CDR3 region fragment length analysis using primers for Vα24 and the TCR-α constant region on PBMC from 5 healthy controls and 7 paired PB-SF or PB-ST samples from RA patients. In PB, whilst healthy controls exhibited a polyclonal peak profile corresponding to the invariant NKT TCR α chain at 350 base pairs, RA patients exhibited a more restricted monoclonal (1 peak) or oligoclonal (2-4 peaks) pattern which included the invariant TCR-α chain in all patients. In SF or ST, Vα24 TCR usage was more variably skewed or polyclonal although all samples peaked corresponding to the invariant TCR-α chain thus confirming iNKT cells are present in SF and ST.

3.3 Why are iNKT cells defective in RA?

The above studies suggest that cross-subset iNKT cell frequency is reduced in RA as compared to healthy controls in peripheral blood, with conflicting reports on the synovial fluid compartment. This reduction is not specific for RA and at best would appear to correlate weakly with disease activity or systemic inflammation. Notably, iNKT cell frequency deficiency is reversible as shown by the response to treatment with methotrexate or rituximab. This is associated with a functional defect in a majority of RA patients, as shown by impaired proliferative and altered cytokine responses to α-GalCer.

So why are iNKT cells reduced in frequency and defective in peripheral blood of RA patients? Peripheral blood iNKT cell homeostasis depends on both thymic output, cell turnover and migration into extra-vascular compartments. It has previously been shown that recent thymic emigrants of conventional T cells, identified by the presence of T-cell receptor excision circles (TRECs), are reduced in number compared to age-matched controls (Koetz et al., 2000; Ponchel et al., 2002). Whether similar thymic dysregulation applies to iNKT cells remains yet to be investigated. Whether the survival of iNKT cells in the periphery is abnormally shortened in RA patients also remains undetermined, although it seems unlikely that the reduced frequency is due to a selective loss of a limited number of iNKT clones as shown by the polyclonal Vδ11 profile of expanded peripheral RA iNKT cells. In non-responders, impaired iNKT cell response to α-GalCer and potentially to self-lipid ligands could be a sign of an intrinsic defect that might lead to reduced physiological expansion or a shortened cellular lifespan, but this phenomenon was restricted to the PB compartment only. Abnormal CD1d-mediated ligand presentation by APCs could similarly also impact on iNKT cell homeostasis but experimental data do not support a major defect in APC function. In fact, CD1d expression on PBMC from RA patients is similar to that of healthy controls (Kojo et al., 2003). Intriguingly, a soluble form of CD1d with capacity to bind α-GalCer and stimulate iNKT cells is significantly reduced in RA as compared to healthy controls, and correlates with $V\alpha24^{+}V\delta11^{+}$ NKT cell frequency (Segawa et al., 2009).

It has been suggested by Kojo et al. (2001) that low iNKT cell number and functional defect could be due to chronic activation and overstimulation as has been shown for anti-CD3ε or IL-12 stimulated NKT cells (Eberl & MacDonald, 1998). In this respect, it would be interesting to analyse markers of iNKT cell activation to support or refute this theory. In the same vein, Linsen et al. (2005) proposed that the measured deficiency in iNKT cell numbers is in fact artefactual, stemming from down-regulation of the TCR as a result of chronic activation (Wilson et al., 2003). Further studies are also required to examine this latter proposition.

Finally, migration of iNKT cells into secondary lymphoid, liver or joint compartments might account for some of the apparent reduction in iNKT cell numbers in RA. Selective migration into joint synovial tissue or synovial fluid however is not supported by analysis of cell frequency and clonality in these compartments, but we cannot exclude migration into secondary lymphoid organs or liver as a possible factor. As most iNKT cells are found in the spleen or liver tissues, with relatively significantly fewer iNKT cells in peripheral blood, assessing the iNKT cell population size from peripheral blood frequency alone is therefore potentially misleading (Eberl et al., 1999; Matsuda et al., 2000).

3.4 Animal models of rheumatoid arthritis

Given the distribution of iNKT cells to organs which are not easily amenable to investigation in human subjects, animal models of RA-like inflammatory arthritis have provided invaluable insight into the possible pathogenic or protective roles played by iNKT cells, particularly with the help of CD1d$^{-/-}$ or invariant Jα281$^{-/-}$ knockout mice deficient in iNKT cells. Furthermore, these models have given investigators an opportunity to explore interventions to either harness or subdue iNKT cell functions for therapeutic benefit. In this section, we review the findings and attempt to make sense of the occasionally puzzling and conflicting data arising from experimental animal studies.

3.4.1 Collagen-induced arthritis

Collagen-induced arthritis (CIA) in mice was first demonstrated in 1981 by Wooley et al. as a promising animal model of inflammatory arthritis. The parenteral administration of heterologous chicken or bovine-derived type II collagen (CII) in complete Freund's adjuvant (CFA) containing heat-killed *Mycobacterium tuberculosis*, followed by a booster 21 days later to genetically susceptible mice results in a persistent inflammatory arthritis sharing many of the features of RA, including mononuclear synovial infiltrate, presence of rheumatoid factor and pannus formation, leading to cartilage destruction and bone erosion. CD4$^+$ T cells are thought to be the primary mediators of disease induction characterised by early Th1 polarisation, whilst complement-fixing anti-CII autoantibody are responsible for the chronic inflammation during the effector phase of the immune response (Luross & Williams, 2001). CIA has been extensively characterised and has proved useful not only in furthering our understanding of basic pathophysiological mechanisms involved in inflammatory arthritis, but also in testing the therapeutic efficacy of potential new treatments for RA (Hegen et al., 2008).

3.4.1.1 C57BL/6 mice

Unlike the susceptible DBA/1 mice normally used in collagen-induced arthritis models, C57BL/6 mice NKT cells express the NK1.1 marker but are also susceptible to CIA with a 70% arthritis incidence. Prior to the widespread availability of more specific detection reagents, Wang et al. (2003) first noted that C57BL/6 mice with higher clinical arthritis scores had lower NK1.1$^+$CD3$^+$ T cell spleen and LN frequency but greater synovial membrane infiltration in more severely affected joints. NK1.1$^+$ cell depletion before CIA induction however did not lead to disease amelioration.

In a further C57BL/6 model of CIA, Ohnishi et al. (2005) found significantly reduced arthritis incidence and severity in Jα281 and CD1d knockout mice. Serum anti-CII autoantibody levels were also significantly reduced. Splenocyte proliferation assays to CII at day 9 post-primary immunisation however revealed no significant difference and neither

did IL-4 and IFN-γ cytokine responses differ between Jα281 knockout and wild-type C57BL/6 mice. At 5 days after booster immunisation on day 21, and therefore during the disease development phase, there were fewer activated $CD69^+$ T and B cells in Jα281 knockout mice. This was associated with lower splenocyte mRNA for IL-4 and IL-1δ, and higher IFN-γ expression.

Using more specific detection reagents, Chiba et al. (2005) analysed the frequency of $Vα24^+$ iNKT cells stained with α-GalCer-loaded CD1d dimers in C57BL/6 murine models of CIA. Unlike human and earlier murine studies using less specific detection reagents, they found increased liver and peripheral blood, but not lymph node or spleen iNKT cell numbers which peaked with clinical disease severity in CIA compared with control mice. Anti-CD1d blocking antibody administered bi-weekly from day 21 resulted in disease amelioration but not susceptibility. Furthermore, Jα281 knockout mice showed an increased IgG1/IgG2a ratio of anti-CII antibodies in keeping with a Th2 deviation in iNKT cell deficient mice. Accordingly, draining lymph node (DLN) lymphoid cells stimulated with CII 10 days post-secondary immunisation produced much more IL-10 in Jα281 knockout mice than wild-type C57BL/6 mice, but less IL-2 and IFN-γ although levels of these two cytokines were already low in wild-type mice.

IL-17 produced by Th17 lymphocytes has been shown to mediate an IL-1-independent role in synovial inflammation and joint destruction in CIA (Lubberts et al., 2001). Studies of $IL17^{-/-}$ mice have shown a role in T cell priming, collagen-specific IgG2a production and a markedly suppressed CIA severity in the absence of this cytokine (Nakae et al., 2003). TGF-δ and IL-6 promote Th17 cell development (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006) whilst IL-23 has been shown to be essential to Th17 cell maintenance (Aggarwal et al., 2003; Langrish et al., 2005). Thus $IL\text{-}23^{-/-}$ knockout mice lack Th17 cells, and similarly fail to develop clinical or histological disease in CIA (Murphy et al., 2003). Neutralisation with anti-IL-17 after the onset of arthritis results in suppression of disease in keeping with a role in both induction and effector phases of CIA (Lubberts et al., 2004). Interestingly, IFN-γ suppresses IL-17 production by CII-specific T cells and may be relevant to the suppressive role of IFN-γ during the effector stage of CIA (Chu et al., 2007). This was subsequently demonstrated in experiments showing that the neutralisation of IFN-γ after day 10 post-primary immunisation results in IL-17 dependent exacerbation of CIA (Sarkar et al., 2009).

Yoshiga et al. (2008) confirmed the pathogenic role of iNKT cells in C57BL/6 CIA by noting the reduced disease incidence and severity in Jα281 knockout mice compared to wild-type mice. DLN cells from $Jα281^{-/-}$ mice stimulated with CII at day 11 post-primary immunisation produced similar amounts of IFN-γ and minimal IL-4, with increased IL-10 (not significantly) and significantly less IL-17. This was associated with a marked reduction in the percentage of IL-17 producing $CD4^+$ cells. Administration of α-GalCer in C57BL/6 but not $Jα281^{-/-}$ mice resulted in IL-17 production by splenocytes with detectable $IL\text{-}17^+$ iNKT cells distinct from the $IFN\text{-}γ^+$ and $IL\text{-}4^+$ population, as well as increased IL-17 production by non-iNKT cells as compared to $Jα281^{-/-}$ mice. Splenic naïve and stimulated iNKT cells were found to express IL-23R and RORγt mRNA similarly to Th17 cells, but only stimulated cells expressed IL-17. $NK1.1^+$ and $NK1.1^-$ iNKT cells splenocytes were incubated with mitomycin-C-treated $CD11c^+$ DCs with either IL-23 or α-GalCer. Results showed that IL-17 is only produced by the $NK1.1^-$ subset in response to α-GalCer and more weakly after IL-23 stimulation. Furthermore, α-GalCer-induced IL-17 could not be abrogated with IL-23 and IL-23 was not detectable in culture supernatant, suggesting that $NK1.1^-$ iNKT cells can produce IL-17 is both IL-23-dependent and independent (Yoshiga et al., 2008).

3.4.1.2 DBA1/J mice

The DBA/1 mouse model of CIA is more susceptible to CIA than C57BL/6 mice despite similar CD1d/α-GalCer-dimer$^+$ NKT cell frequency and in vivo or in vitro function. In a DBA/1 model of CIA, Jung et al. (2009) showed that CD1d$^{-/-}$ knockout mice display a reduced incidence and severity of CIA, IgG2a and IgG1 CII-specific antibody levels, CII-stimulated production of IFN-γ, IL-17, IL-1δ and IL-6, and increased production of IL-10 by splenocytes on day 35, as compared to CD1d$^{+/-}$ mice. In wild-type mice, iNKT cells producing IFN-γ were maintained throughout all disease stages whilst IL-17 producing iNKT cells steadily increased in frequency and number until the late stages of disease (Jung et al., 2009).

In a further DBA/1 model of CIA, Miellot-Gafsou et al. (2010) found that hepatic iNKT cells released IFN-γ, IL-4 and IL-17A in response to intra-peritoneal (i.p.) α-GalCer 2hr before euthanasia at day 6, i.e. early during the course of CIA. This was associated with increased expression of CD69 on CD1d/α-GalCer-tetramer+ hepatic iNKT cells without any difference in the proportion of iNKT cells as compared to non-immunized mice. In keeping with an early role in disease development, anti-CD1d administered early (days 0, 3 and 6 post-induction) but not late (days 27-39) delayed onset, reduced disease incidence and ameliorated clinical and histological severity. No difference in FoxP3$^+$ Treg frequency in spleen and lymph nodes or their suppressor function was noted. Splenic dendritic and macrophage APCs however showed reduced expression of CD40, CD80 and CD86 co-stimulatory molecules (Miellot-Gafsou et al., 2010).

In addition, Jung et al. (2010) have demonstrated a more subtle late regulatory role for NKT cells in tolerogenic DC (Tol-DC) mediated suppression of CIA. Thus TGF-δ induced, peritoneal exudate cell (PEC)-derived Tol-DC from CD1d$^{+/-}$ but not CD1d$^{-/-}$ mice could suppress the incidence, onset and severity of CIA when administered on day 28. This was associated with suppressed serum and joint tissue IFN-γ and IL-17 cytokine and mRNA production, respectively. Anti-CII specific IgG2a was reduced whilst IgG1 was higher in CD1d$^{+/-}$ compared to CD1d$^{-/-}$ Tol-DC. Similarly, CII-stimulated splenocytes from CD1d$^{+/-}$ but not CD1d$^{-/-}$ Tol-DC produced lower levels of IFN-γ and IL-17, and higher levels of IL-4 and IL-10 .

3.4.2 Antibody mediated arthritis

Although the initating event and processes involved in RA and animal models of autoimmune arthritis may differ, the final inflammatory pathways leading to joint damage are more likely to be common to both (Nandakumar & Holmdahl 2006). Antibody-mediated models of autoimmune arthritis give us a unique opportunity to study these terminal events independently of the initation processes involved, and thereby to evaluate more clearly the role of iNKT cells during the terminal effector stage of disease.

3.4.2.1 K/BxN serum transfer model

The K/BxN mouse model of spontaneous autoimmune arthritis is a cross between the KRN TCR C57BL/6 transgenic mouse that is specific for an I-A^k restricted peptide of bovine ribonuclease (RNase), and the non-obese diabetic (NOD) mouse (Kouskoff et al., 1996). The K/BxN mouse T cells recognise an I-Ag7-restricted peptide from glucose-6-phosphate isomerase (G6PI) and B cells produce G6PI-specific antibodies (Matsumoto et al., 1999). Serum transfer alone into C57BL/6 mice can also mediate disease after just several days (Ji

et al. 2001). Complement, neutrophils and mast cells play an essential role, and it is also apparent that Fcγ receptors (FcγRs) and IL-1 are also required in mediating joint inflammation and destruction (Ditzel, 2004).

In an earlier study of the K/BxN serum transfer model of inflammatory arthritis, Chiba et al. (2005) found an increased percentage of Vα24^{+}α-GalCer/CD1d-dimer^{+} iNKT cells in liver, peripheral blood, spleen and lymph nodes as compared to control mice given BxN serum. CD1d$^{-/-}$ and Jα281$^{-/-}$ knockout mice deficient in iNKT cells exhibited milder disease and reduced histological severity but not susceptibility, supporting a role for iNKT cells during the inflammatory effector phase of arthritis.

Subsequently, Kim et al. (2006) showed using in the K/BxN serum transfer model that CD1d$^{-/-}$ and Jα281$^{-/-}$ knockout mice have reduced clinical disease severity and neutrophil inflammatory infiltration, while transfer of liver NK1.1^{+}TCR-δ^{+} NKT cells from normal B6 mice into CD1d$^{-/-}$ knockout mice restored wild-type disease severity. Furthermore, splenocytes from Vα14Jα281 TCR transgenic (Tg) RAG$^{-/-}$ mice but not Jα281$^{-/-}$ knockout mice restored disease severity in CD1d$^{-/-}$ mice, confirming the specific role of iNKT cells and in addition, the injection of α-GalCer as an in vivo activator of iNKT cells resulted in exacerbation of disease in B6 but not CD1d$^{-/-}$ mice. These investigators went on to dissect the mechanisms by which iNKT cells play a pro-inflammatory role in the KxB/N mouse model. Joint tissue Vα24Jα281 TCR mRNA measured by RT-PCR was observed at days 3, 5 and 7 in wild-type B6 (C57BL/6) mice, but not on day 0 or in CD1d$^{-/-}$ knockout mice, suggesting *de novo* iNKT cell infiltration during the inflammatory response. Joint tissue mRNA level of TGF-δ1 was increased but IL-4 and IFN-γ were reduced in CD1d$^{-/-}$ mice compared to B6 mice, although similar levels were noted in splenocytes. Adoptive transfer of NKT cells reduced TGF-δ1 and restored IL-4 and IFN-γ mRNA in joint tissue without changing splenic transcript levels. Postulating a role for TGF-δ1 in resistance to joint inflammation, anti-TGF-δ mAb was administered i.p. three times a week before and after serum transfer in CD1d$^{-/-}$ and wild-type mice. Anti-TGF-δ increased disease severity to wild-type levels in CD1d$^{-/-}$ but had no effect in wild-type mice. Administration of recombinant TGF-δ1 to wild-type mice in turn reduced disease severity, suggesting that iNKT cells mediate their pathogenic role in a TGF-δ1-dependent fashion. But how do iNKT cells regulate TGF-δ1 production? To answer this, synovial cells from B6 mice were stimulated with concanavalin A (ConA) to induce TGF-δ1 production. This was suppressed by α-GalCer or anti-CD1d mAb in keeping with iNKT cell-mediated regulation. Similarly, injection of α-GalCer into treated B6 mice resulted in suppressed TGF-δ1 production in joint tissues, indicating the capacity of NKT cells to specifically suppress TGF-δ1 production. Blocking IL-4 and IFN-γ could restore TGF-δ1 production, while IL-4$^{-/-}$ or IFN-γ$^{-/-}$ mice NKT cells transferred into CD1d$^{-/-}$ serum-treated mice resulted in minimal joint swelling and TGF-δ1 transcriptional expression remained elevated. In contrast, transfer of NKT cells from B6 mice restored wild-type disease severity and TGF-δ1 suppression. Finally, anti-IL-4 or anti-IFN-γ resulted in resistance to joint inflammation and enhanced TGF-δ1 production in Vα14Jα281 TCR Tg RAG$^{-/-}$ mice (Kim, 2005).

Speculating that the observed NKT-mediated aggravation K/BxN serum transfer model of autoimmune arthritis may not be due to TCR-mediated recognition of CD1d-restricted self-lipid but instead could be due to Fcγ receptor engagement by immune complexes providing activating signals to NKT cells independently of TCR, Kim et al. (2006) showed that hepatic, splenic and thymic NK1.1^{+}TCR-δ^{+} NKT cells in B6 mice selectively expressed FcγRIII,

mainly on $CD4^+$ or $CD4^-$ cells. Of these, 62% stained with α-GalCer/CD1d tetramer, indicating a mixed invariant and non-invariant NKT cell population. Furthermore, TCR stimulation of liver mononuclear cells (LMNCs) by α-GalCer/CD1d upregulated FcγRIII expression. Aggregated IgG but not soluble IgG, could activate NKT cells from B6 mice but not T or NK cells or NKT cells from $Fc\gamma^{-/-}$ mice, and upregulate activation markers CD25 and CD69. NKT cell FcγRIII engagement by aggregated IgG could upregulate expression of T-bet and GATA-3, and induce production of IL-4, IL-10, IL-13 and IFN-γ independently of simultaneous TCR stimulation. Simultaneous TCR and FcγRIII engagement resulted in cytokine output greater than either signal alone. In mice treated with K/BxN serum, joint swelling was detected at 4-5 days and maximal at 8-9 days. $CD1d^{-/-}$ mice administered NKT cells from B6 mice showed similar disease severity to wild-type mice, but administration of NKT from $Fc\gamma R^{-/-}$ B6 mice showed minimal joint swelling and only mild inflammatory infiltrate. Joint tissue Vα14Jα281 TCR mRNA was similar in $CD1d^{-/-}$ administered B6 NKT cells and $CD1d^{-/-}$ administered $Fc\gamma R^{-/-}$ B6 NKT cells. Furthermore, there was no difference in hepatic NKT, NK, CD4 and CD8 T cell numbers in $Fc\gamma^{-/-}$ B6 mice and α-GalCer/CD1d complex-stimulated LMNC cells from $Fc\gamma R^{-/-}$ mice produced IL-4, IL-10, IL-13 and IFN-γ similarly to wild-type B6 mice. These findings suggested that FcγR engagement rather than TCR engagement mediated NKT activation in the serum transfer model. RT-PCR analysis confirmed elevated joint tissue but not liver levels of TGFϐ-1 and reduced levels of IL-4 and IFN-γ in $CD1d^{-/-}$ mice administered $Fc\gamma R^{-/-}$ NKT cells as compared to B6 NKT cells, indicating that FcγRIII engagement by joint tissue –deposited IgG enhances IL-4 and IFN-γ production and results in suppressed TFG-ϐ1 (Kim et al., 2006).

IL-12 has previously been shown both to attenuate and to exacerbate CIA in C57BL/6 (Murphy et al., 2003) and DBA/1 mice (Butler et al., 1999; Malfait et al., 1998; Matthys et al., 1998), respectively. To elucidate the role of IL-12 in the terminal effector stages of arthritis, Park et al. (2010) studied the K/BxN mice serum transfer model in $IL\text{-}12p35^{-/-}$ and $IL\text{-}12R\beta2^{-/-}$ mice. Knockout mice had slower onset and less severe disease associated with milder inflammatory synovial infiltrate as compared to B6 mice. IL-12 mRNA was detected in joint tissues in B6 mice and recombinant (rm) IL-12 could restore arthritis severity in knockout mice. Serum transfer resulted in infiltration of large numbers of $CD11b^+$ and $Gr1^+$ granulocytes and smaller numbers of B, T, NK and $CD11c^+$ cells. Using IL-12p40 cytokine reporter mice, the investigators showed that IL-12 is produced most prominently by $CD11c^+$ cells, but also by $CD11b^+$ cells and $Gr1^+$ granulocytes. Joint tissue TGF-ϐ transcript was higher and IFN-γ, IL-6 and IL-4 were lower in knockout mice. IL-17 and IL-23 were undetectable in wild-type and knockout mice, and joint inflammation and cytokine production were similar in B6 and $IL\text{-}17^{-/-}$ mice. Recombinant IL-12 restored cytokine levels to wild-type pattern. Both rmIFN-γ and anti-TGF-ϐ restored arthritis severity in $IL\text{-}12p35^{-/-}$ mice. In joint tissue only $NK1.1^+TCR\text{-}\beta^-$ NK and $NK1.1^+TCR\text{-}\beta^+$ NKT cells were found to express surface IL-12Rϐ2. Adoptive transfer of splenocytes from B6 and $Gr\text{-}1^+$ granulocyte-depleted B6 mice into $IL\text{-}12R\beta2^{-/-}$ mice restored arthritis severity and production of IFN-γ and IL-4, whereas transfer of splenocytes from iNKT-deficient $J\alpha281^{-/-}$ had no effect on arthritis severity and maintained higher levels of joint tissue TGF-ϐ transcript. The investigators therefore suggested that IL-12p35 produced by $CD11b^+$, $CD11c^+$ and $Gr\text{-}1^+$ cells promote antibody-induced inflammation by engaging IL-12 Rϐ2 expressed on iNKT cells in joint tissues (Park et al., 2010).

3.4.2.2 Other models of autoimmune arthritis

Chiba et al. (2005) administered a mixture of four anti-CII mAb to C57BL/6 mice, followed 72h later by LPS. Jα281$^{-/-}$ and CD1d$^{-/-}$ knockout mice also showed reduced disease severity without any difference in susceptibility, in keeping with a role for iNKT cells during the effector as well as adaptive stages of CIA.

Antigen-induced arthritis (AIA) occurs when bovine serum albumin (BSA) is injected into the knee joint of B6 mice after priming with subcutaneous BSA injection, resulting in a week-long acute inflammatory arthritis (van den Berg et al., 2007). Teige et al. (2010) showed that CD1d$^{-/-}$ knockout mice experience a more severe AIA associated with a stronger splenocyte antigen-specific IFN-γ response without any difference in cell proliferation. Notably, NK1.1-depletion resulted in more severe arthritis.

3.4.3 Lessons learnt from animal models of autoimmune arthritis

CIA models using either C57BL/6 or the more susceptible DBA/1 mouse strains have consistently shown that iNKT cell deficient animals experience a reduced incidence and/or severity of arthritis. This therefore suggests that in contrast to other models of autoimmune disease and despite the apparent reduction in frequency of peripheral iNKT cells in RA, iNKT cells play a pathogenic role in animal models of autoimmune arthritis.

The increased incidence is a clue for an early role for iNKT cells in disease initiation, and this is supported by the observation of DLN cytokine deviation by day 11 in C57BL/6 mice (Yoshiga et al., 2008) and a reduction in incidence and disease amelioration when anti-CD1d is administered early, but not late in DBA1/J mice (Miellot-Gafsou et al., 2010). Ohnishi et al. (2005) found however no evidence of an effect on splenocyte cytokine deviation until day 26 in C57BL/6 mice, but given that they used the same mouse strains and CIA induction protocol as Yoshiga et al. (2008), the conflicting results are likely to be due to site and time-specific differences between DLN and splenocyte populations.

There is also reasonable evidence that iNKT cells play a role in later, effector stages of CIA too. IL-17 producing iNKT cells have been noted to steadily increase in numbers until the very late stages of disease in DBA/1 mice (Jung et al., 2009) and anti-CD1d administered to C57BL/6 mice on day 21 results in disease amelioration (Chiba et al., 2005). In addition, Jung et al. demonstrated that CD1d$^{+/-}$ but not CD1d$^{-/-}$ Tol-DC could suppress CIA in DBA/1 mice when administered as late as day 28 (Jung et al., 2010).

So how do iNKT cells influence the immune pathogenesis of CIA? Experimental data suggest that early on during induction and continuing into the effector stage of disease, iNKT cells skew the immune response to CII by promoting a Th1 and/or Th17 phenotype. It seems likely that by altering the cytokine environment, iNKT cells also influence the corresponding observed shift in autoantibody isotype from IgG1 to complement-fixing IgG2a. Furthermore, iNKT cells enhance both T and B cell responses as evidenced by the expression of activation markers and levels of circulating anti-CII autoantibodies, no doubt mediated in part by increased APC co-stimulatory molecule expression.

Whilst providing remarkable insight into the immunological role of iNKT cells in autoimmunity, murine models of CIA have also raised new issues and left us with many unanswered questions. It is unclear which particular iNKT cell subset is responsible for exacerbating disease, whether the same subsets are pathogenic at different stages or even whether some subsets play a protective role as suggested by Jung et al.'s Tol-DC experimental model (Jung et al., 2010). Neither do we know how iNKT cells become

activated or the mechanism(s) whereby they skew the phenotypic immune response to CII. Given that iNKT cells are found in diseased joints, do they play any direct role in terminal joint inflammation and tissue destruction?

Models of antigen and antibody-mediated arthritis are useful in clearly dissecting the role of iNKT cells during the induction and effector stages of autoimmune arthritis and confirm that iNKT cells play a pathogenic role during the terminal effector phase of disease. The work of Kim et al. (2006) and Park et al. (2010) was a particularly elegant demonstration of how iNKT cells appear to be stimulated through their IL-12Rß2 and FcγRIII receptors, resulting in increased IL-4 and IFN-γ and suppressed TGF-ß production, and subsequent modulation of arthritis severity. The only caveat to their work is the use of relatively non-specific markers which did not distinguish iNKT from other NKT cell subsets. It also still remains unclear which iNKT cell subsets are important and whether invariant TCR-self-lipid-CD1d interaction plays any role in iNKT cell activation during the effector stage. Further work is required to define the source of seemingly critical IL-4, IFN-γ and TGF-ß, and ultimately, the extent to which these findings are applicable to rheumatoid arthritis in humans.

3.5 Clinical relevance of iNKT cells in RA

Despite the demonstrated pathogenicity of iNKT cells in animal models of autoimmune arthritis, their frequency and phenotype may yet serve as useful biomarkers, and their immune manipulation may serve a beneficial therapeutic purpose as shown in the context of infection, cancer immunity and other autoimmune diseases.

3.5.1 iNKT cells as diagnostic and prognostic tools

It seems that peripheral iNKT cell frequency is reduced in a range of autoimmune diseases including diabetes, multiple sclerosis and SLE. This may indicate a response to systemic inflammation or a generalised association with autoimmunity. As such, iNKT cells could in theory be used to distinguishing RA from other forms of arthritis. Furthermore, additional detailed analysis of iNKT subsets and function may provide prognostic information to guide individual treatment. Finally, it is tempting to speculate whether iNKT cells could serve as markers of self-tolerance to signal the safe withdrawal of long term immunosuppression in patients achieving clinical remission.

Our own work in this area showed that peripheral iNKT cell frequency was reduced among patients with RA, osteoarthritis (OA) and other inflammatory non-RA groups compared to patients with non-inflammatory, non-OA joint pain and healthy controls. This indicates that in practice iNKT cell frequency alone would prove of limited diagnostic or disease monitoring value. Whilst iNKT cell counts appear to improve with methotrexate therapy, we found no correlation with clinical response and therefore the significance of this observation remains unknown (Tudhope et al., 2010).

3.5.2 Manipulating iNKT cells for clinical benefit

OCH, a synthetic analogue of α-GalCer with a truncated sphingosine chain, has been shown to induce a Th2 bias in iNKT cytokine production and can efficiently inhibit EAE in C57BL/6 mice. Chiba et al. therefore studied the effects of intra-peritoneal α-GalCer and OCH administration, before and after induction of CIA in C57BL/6 mice. Unlike α-GalCer, OCH significantly reduced the clinical severity score of CIA from 13 to 4.6. When OCH was

administered to Jα281-knockout mice, there was no difference in disease severity score as compared with vehicle alone, confirming the requirement for iNKT cells for OCH-mediated suppression of CIA. αGalCer injection induced a rapid rise in serum IL-4 and IFN-γ levels, while OCH increased IL-4 levels with much less IFN-γ production. Consistent with an iNKT cell derived source of cytokines, Jα281 knockout mice did not exhibit a rise in either IL-4 or IFN-γ (Chiba et al., 2004).

Miellot et al. (2005) explored the therapeutic potential of α-GalCer in the autoimmune prone DBA/1 model of CIA. Mice immunised with intra-peritoneal α-GalCer both before, at or after induction showed reduced disease severity but no difference in susceptibility or timing of disease onset. The optimal effect was noted with a single injection on day 0 and correlated with reduced histological inflammation and tissue destruction without any difference in levels of IgG1, IgG2a or IgG1/IgG2a ratio. Cytokine production by draining lymph node (DLN) $CD4^+$ T cells in response to CII stimulation showed no significant differences for IFN-γ or IL-4, but a significant increase in IL-10 production in α-GalCer/CII/CFA as compared to CII/CFA-immunised mice. Accordingly, the protective effect of α-GalCer on CIA was completely abrogated by blocking with anti-IL10R mAb.

Administration of a single dose of α-GalCer in a DBA/1 model of CIA on day 5 post-immunisation by Coppieters et al. (2007) resulted in an attenuated course of clinical disease and histological parameters. Splenic T-bet expression was reduced whilst GATA-3 was unchanged, suggesting an altered balance in favour of Th2 differentiation associated with a reduced antigen-specific CII proliferative response in DLN cells. Similar disease attenuation occurred following administration of the α-C-GalCer analog, a C-glycoside analogue of α-GalCer. Serum cytokine analysis after α-C-GalCer administration however showed marked differences as compared to α-GalCer administration, with negligible production of IL-2, TNF-α, IL-4 and IL-10, reduced IL-5 and IFN-γ, and increased IL-12p70 and IL-6. α-GalCer, but not α-C-GalCer, induced increased IgG1 levels with no difference in IgG2a between groups. T cell cytokine profiles induced with in vivo administration of anti-CD3 on day 14 after arthritis onset confirmed that mice treated with α-GalCer exhibited increased IL-10 production whilst mice treated with α-C-GalCer revealed lower levels of IL-4, IL-5 and IFN-γ. Furthermore, IL-10 production at 10 days post-immunisation was increased in α-GalCer-treated wild-type but not $J\alpha281^{-/-}$ mice, suggesting that the increased IL-10 in α-GalCer treated mice is iNKT cell-dependent. The authors therefore proposed that α-GalCer protection may be mediated by the development of an IL-10 producing population of regulatory cells whilst α-C-GalCer may operate by inducing a general hyporesponsive state as evidence by lower cytokine production.

α-carba-GalCer is another analogue of α-GalCer known to stimulate Th1 responses in iNKT cells. In DBA/1 mice, α-carba-GalCer can strongly induce IFN-γ, IL-2 and IL-12 but unlike α-GalCer, does not induce IL-4. When administered with CII/CFA on day 0, α-carba-GalCer reduced the incidence and severity of arthritis in an IFN-γ-dependent manner. This was associated with reduced CII-specific IgG2a antibody levels and inhibition of IL-17 production by CII-stimulated DLN cells. No change in apoptotic or regulatory FoxP3+ T cell frequency in DLN was noted. As early as day 3, DLN cells expressed reduced IL-6 and IL-23p19 with unchanged TGF-ϐ while CD1d-$tetramer^+$ iNKT cells produced higher IFN-γ in spleen, liver and DLN. On day 10, DLN T cells stimulated with PMA and ionomycin showed polarisation towards Th1 and suppression of Th17 cell differentiation, in keeping with protective Th1 deviation during the initiation phase of CIA (Yoshiga et al., 2011).

Screening a panel of analogs of α-GalCer for the ability to suppress the development of arthritis in a B6 model of K/BxN serum transfer-induced arthritis, Kaieda et al. (2007) found SGL-S23, an analog with a 5-carbon longer sphingosine base that could inhibit clinical and histological disease severity to a greater degree than α-GalCer by i.p. administration on days 0, 3 and 7. Furthermore, they showed a therapeutic effect when administered to mice with established arthritis. By injecting Jα281$^{-/-}$ knockout mice, they confirmed that SGL-S23-mediated disease suppression requires iNKT cells. Neutralisation of IL-4, IL-10 or TGF-б1 did not abolish the protective effect of SGL-S23. Instead, SGL-S23 was able to stimulate weaker in vitro cell proliferation and IFN-γ production than α-GalCer. Similarly, in vivo injection of SGL-S23 resulted in weaker IFN-γ serum responses. In keeping with the known essential role played by mast cells in the development of arthritis in the K/BxN serum transfer model, Kaieda et al. (2007) showed an elevation in serum histamine level five minutes after serum injection, abolished by pre-treatment with low-dose IFN-γ. SGL-S23 also suppressed histamine release, and more effectively so than α-GalCer or high dose IFN-γ.

Finally, CIA suppression through NKT cell manipulation can be accomplished with non-glycolipid stimulation as shown by Liu et al. (2011). Immunisation with murine CII induces a weaker responsethan heterologous collagen, characterised by multiple epitopes sharing a common motif, the strongest located at position 707-721. B10.Q or B6 mice immunised with $mCII_{707-721}$ mount a strong and specific immune response. In fact murine $mCII_{707-721}$ can bind to CD1d and partially competes with α-GalCer. B10.Q immunisation with $mCII_{707-721}$ resulted in significant expansion of $CD4^+NK1.1^+$ NKT cells. Reactive cells showed skewed TCR usage toward Vα14Jα18 with a diverse range of Vб chains, with some skewing toward Vб8.2. NKT cell proliferation was dependent on CD40-CD40L and B7-CD28 co-stimulation, while activated NKT cells produced IFN-γ, IL-4 and TGF-б but less TNF-α, peaking at 72h and thus resembling conventional T cell kinetics. Vaccination with $mCII_{707-721}$ ameliorated CIA, reducing clinical severity, joint inflammation, CD4 synovial infiltration and joint tissue IFN-γ, IL-4 and TNF-α production.

In summary, both α-GalCer and its analogues have been shown to modulate the severity of autoimmune arthritis in animal models during both the initiation and effector stages of disease. The mechanism of iNKT cell-mediated protection appears to be complex as evidenced by conflicting reports of Th1/Th2/Th17 polarisation, variable immunoglobulin isotype profiles and anergy induction by some agonists but not others. Immune alteration however is not restricted to the adaptive system, as shown by effects on mast cell activation in a serum transfer model of autoimmune arthritis. Before glycolipid ligands can be considered for use in rheumatoid arthritis, we will need to gain a better understanding of the factors important in predicting how these ligands will modulate the immune response, including ligand structure, dose, route of administration and timing of treatment.

4. Conclusion

Invariant NKT cells are powerful immune protagonists that can be engaged during innate immune system activation to amplify and direct subsequent adaptive immune responses by cytokine and cell contact-mediated cross-talk with a wide range of other immune players. In both rheumatoid arthritis and other autoimmune disorders, iNKT cells appear to be deficient in both number and function. Nevertheless, animal models of autoimmune arthritis consistently tell us that iNKT cells play a pathogenic rather than protective role.

Despite this, manipulating iNKT cell responses through the use of glycolipid or even peptide ligands has been shown to be effective both prophylactically and therapeutically. At the present time however our knowledge of iNKT cell mass, phenotype, function and significance in human disease remains limited. Furthermore, it is unclear whether animal models are truly representative and can be relied on to characterise iNKT cells in rheumatoid arthritis. Whilst pharmacological manipulation is evidently achievable, the general immunomodulatory governing principles are far from established. Future research will therefore need to address these issues in order to safely harness the full therapeutic potential of iNKT cells for effective clinical use.

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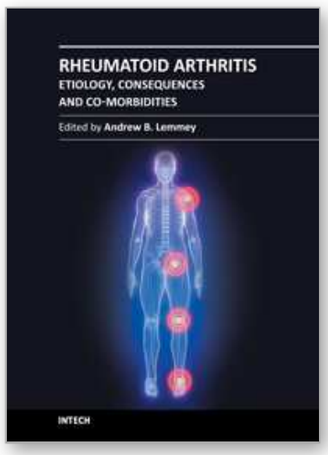

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The purpose of this book is to provide up-to-date, interesting, and thought-provoking perspectives on various aspects of research into current and potential treatments for rheumatoid arthritis (RA). This book features 16 chapters, with contributions from numerous countries (e.g. UK, USA, Japan, Sweden, Spain, Ireland, Poland, Norway), including chapters from internationally recognized leaders in rheumatology research. It is anticipated that Rheumatoid Arthritis - Etiology, Consequences and Co-Morbidities will provide both a useful reference and source of potential areas of investigation for research scientists working in the field of RA and other inflammatory arthropathies.

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