Review

NKT cells: manipulable managers of joint inflammation


The importance of T cell participation in the aetiology and pathogenesis of rheumatoid arthritis (RA) is now widely appreciated. The disease is mediated by activated pro-inflammatory, self-reactive T helper cells, instigating the chronic autoimmune response characteristic of rheumatoid inflammation. Natural killer T (NKT) cells are a distinctive population of T cells thought to protect self-tissues from damaging inflammatory immune responses, and are often recognized as a regulatory T cell subtype, regulating the magnitude or class of the immune response. Recently, a number of studies have provided insight concerning the role of NKT cells in different models of autoimmune joint inflammation, suggesting the involvement of this specialized T cell subset in controlling initiation and perpetuation of arthritic disease. The aim of this review is to provide rheumatologists with an introduction of the principal features of NKT cells, to give an overview of the data obtained in animal models of arthritis and to discuss the hypothesized mechanisms. Finally, we will speculate on future prospects with regard to NKT cell-targeted treatment of arthritic disease by use of glycolipids.

KEY WORDS: Rheumatoid arthritis, NKT cells, Cytokines, Inflammation, Mouse, Autoimmunity.

Introduction: NKT cells and autoimmunity

A link between iNKT cells and autoimmunity was initially suggested by the finding that several mouse strains that are genetically susceptible to autoimmunity [i.e. type-1 diabetes, experimental allergic encephalomyelitis and systemic lupus erythematosus (SLE)] as well as patients with type-1 diabetes, systemic sclerosis (SSc), multiple sclerosis, SLE and RA have reduced iNKT cell numbers and show functional iNKT cell deficiencies [1].

In contrast to conventional CD4⁺ T cells, which are activated by peptide antigens in the context of a specific MHC class II molecule expressed on the surface of an antigen-presenting cell (APC) [2]; NKT cells typically recognize glycolipid antigens bound by the MHC class I-like protein CD1d [3]. They represent a subset of innate-like lymphocytes that share receptor structures and functions with both conventional T cells and NKT cells [4]. Classical, so called ‘type-1’, NKT cells express an invariant TCRalpha chain encoded by a Valpha14-Jalpha18 rearrangement in mice [5] and Valpha24-Jalpha18 in humans [6, 7]. These cells recognize the glycosphingolipid alpha-galactosylceramide (alpha-GalCer) which is able to potently activate these type-1 NKT cells. However, non-classical, ‘type-2’, NKT cells lack this TCR repertoire bias, are not reactive to alpha-GalCer and, as a consequence, have been less well characterized [8, 9]. We will focus on the more widely studied type-1 NKT cells, generally termed ‘iNKT cells’ hereafter [10]. Some of the principal features of iNKT cells are depicted in Fig. 1.

Alpha-GalCer was originally isolated from a marine sponge by the Kirin Brewery Company (Japan) as an agent that inhibits metastatic tumor growth in mice [11, 12]. Alpha-GalCer was later found to be a potent stimulator of both mouse and human iNKT cells, indicating the striking conservation of this immune recognition system [13–18]. Although the discovery of a specific iNKT cell ligand substantially propagated their characterization, their precise physiological relevance remained elusive until very recently, a number of mammalian and bacterial antigens, structurally related to alpha-GalCer, were reported to stimulate iNKT cells [19–25]. A key property of iNKT cells is their capacity to rapidly produce a variety of cytokines, including Th1 cytokines such as IL-2 and IFN-gamma, and Th2 cytokines such as IL-4 and IL-10, upon TCR engagement [13, 26–28]. This ability to potently modulate adaptive immunity upon stimulation of a restricted set of antigen-specific receptors, together with their lack of immunological memory, closely resembles the properties of cell types belonging to the innate immune system [29]. As such, our current understanding suggests a straddling function between innate and adaptive immunity both in terms of initiation and modulation of adaptive responses [30]. Therefore, some authors have termed this as bridging immunity.

The relevance of the iNKT cell subset as a therapeutic target in autoimmunity was further demonstrated by several groups studying the response of animal models of autoimmune disease upon therapeutic treatment with alpha-GalCer and its analogues [31, 32]. In general, iNKT cell activation by repeated glycolipid administration conferred protection in a wide range of spontaneous and induced models of autoimmune inflammation, mostly mediated by so called ‘Th2 deviation’ of the immune response. Differences in site, dosage, frequency and timing of glycolipid administration most likely are responsible factors for differential outcomes upon treatment. Here, we will provide an overview of recent results obtained in animal models of arthritic disease and discuss the underlying mechanisms. Based on a better knowledge of iNKT cell biology, specific therapeutic intervention with glycolipids can be designed to suppress synovial inflammation and joint destruction in rheumatic diseases.

iNKT cells in collagen-induced arthritis: pointing Th cells the direction

Collagen-induced arthritis (CIA) is an animal model for RA that is broadly used to address questions of disease pathogenesis and is considered the model of choice in terms of validating therapeutic targets [33]. Arthritis is induced in susceptible mice by immunization with heterologous type-II collagen in adjuvant, leading to a robust T and B cell response to this articular antigen [34, 35]. Susceptibility is strongly associated with MHC types I-A and I-A', analogous to DR4 and DR1 association with RA. Additionally, the main histopathological features of the resulting joint inflammation closely resemble the pattern observed in RA,
including a proliferative synovitis, pannus formation, erosion of cartilage and bone and fibrosis. As outlined subsequently, two different strategies have been employed to study the role of iNKT cells in this model. While one approach entails specific iNKT cell activation by glycolipid administration, other groups use CD1d or iNKT cell knock-out animals to delineate the intrinsic roles of the iNKT cell subset.

CIA is characterized by the dominant activation of a particular T helper (Th) cell subtype expressing pro-inflammatory cytokines (Th1 cells), concordant with clinical and experimental findings in RA patients [35]. A considerable volume of work has been devoted to the modulation of the Th1/Th2 balance towards the latter [37]. The same group confirmed therapeutic potential of this compound in CIA, in WT but not in NKT cell deficient animals. In contrast, repeated administration of alpha-GalCer was shown to induce Th2 bias [39–42]. Furthermore, simultaneous in vivo neutralization of IL-4 and, even more pronounced, IL-10 abrogated the ability of OCH to suppress CIA. Based on these findings it is reasonable to assume that OCH exerts its therapeutic activity by an iNKT cell induced shift from a Th1 response in CIA towards protective Th2 immunity. Interestingly, lower levels of TNF and RANKL mRNA were found in iNKT cells upon in vivo treatment with OCH, as compared to alpha-GalCer, suggesting that iNKT cells may have, depending upon their mode of activation, the capacity to modulate major contributors to joint inflammation and destruction.

The physiological role of iNKT cells in the development of CIA was further examined in two similar studies by use of iNKT cell-deficient mutant mouse strains. In the first, the interaction of CD1d and iNKT cells was blocked in a semitherapeutic fashion by use of anti-CD1d mAbs, resulting in...
suppression of clinical arthritis scores [43]. The partially redundant involvement of a functional iNKT cell subset in CIA development was confirmed by the finding that Jalpha18<sup>-/-</sup> iNKT cell deficient knock-out mice developed less severe arthritis compared with wild type animals. The Jalpa18<sup>-/-</sup> mice also showed elevated collagen type-II (CII)-specific IgG1/IgG2a antibody ratios in the serum, which is generally considered representative of the current Th2/Th1 balance [36]. Indeed, IgG1 production is indicative for an ongoing Th2 bias, as Th2 cells orchestrate the immunoglobulin class switching to IgG isotypes that do not fix complement. Moreover, ex vivo restimulation of T cells with collagen type II led to higher production of IL-10, again indicating that skewing of the immune system towards the Th2 direction is associated with protection against CIA. In a second paper, analogous conclusions were drawn from Jalpa18<sup>-/-</sup> and CD1d<sup>-/-</sup> mice, as they were both shown to develop milder CIA upon induction as compared to wild type [44]. Remarkably, although CII-specific antibody levels as well as numbers of CD69<sup>+</sup>-activated T and B cells in the spleen were lowered in the mutant strains, no difference was found upon ex vivo splenic T cell restimulation with CII, neither with regard to proliferative response nor IFN-gamma and IL-4 production. Finally, the likely assessment of a Th2 shift to protection was reproduced, as assessed by altered splenic mRNA ratios of IFN-gamma and IL-4 in Jalpa18<sup>-/-</sup> mice.

Adding complexity to the situation, Miellot et al. [45] subsequently demonstrated attenuated clinical signs of arthritis upon repeated prophylactic and semi-therapeutic treatment with alpha-GalCer, in contradiction with previous reports, which failed to obtain significant results with alpha-GalCer in CIA. In addition, CII-specific T cell restimulation led to up-regulation of IL-10, but not IL-4 and IFN-gamma. This observation was virtually confirmed by the finding that IL-10R-blockade by use of neutralizing mAb treatment reversed clinical efficacy of alpha-GalCer, but the absence of a control group administered only anti-IL10R makes this finding somewhat difficult to interpret. However, the capability of alpha-GalCer to efficiently attenuate the progression of CIA, at least in part by functional Th2 polarization, was reproduced in our laboratory, as we demonstrated that a single dose administered during the early induction phase was sufficient to modulate clinical course and histopathology (unpublished results).

Taken together, these findings strongly suggest that the intrinsic capacity of iNKT cells to modulate CIA is clearly distinct from their suppressive role upon activation with glycolipid antigen.

**iNKT cells in antibody-induced arthritis: harmful by suppression**

Transfer of serum or immunoglobulins from K/BxN mice, which spontaneously develop progressive, joint-specific autoimmune inflammation, to healthy mice causes an inflammatory arthritis by deposition of auto-antibody in the joint space, inducing an inflammatory cascade by activation of complement and Fcgamma receptor pathways [46-48]. Since inflammation in antibody-induced arthritis (ABIA) occurs in the absence
of a primary cellular immune response, this model is considered useful in exploring the terminal effector mechanisms of RA.

Recently, the functionality of iNKT cells in the K/BxN model was elegantly unravelled by Kim et al. [49]. In a first effort, they explored whether susceptibility to ABIA was altered in Jα18−/− and CD1d−/− mice upon serum transfer of K/BxN mice, and found that the iNKT cell deficient mice were less prone to joint inflammation. The aforementioned publication by Chiba et al. [43] later also demonstrated disease promoting activity on iNKT cells in K/BxN mice, and in a similar model, which is established by the injection of a mixture of anti-CII mAbs, followed 72 h later by a single administration of LPS. Convincing evidence was provided by Kim et al. for the deteriorating role of iNKT cells during ABIA development, as upon adoptive transfer of sorted iNKT cells in CD1d−/− mice the arthritic disease progression became identical to susceptible wild type mice. Conversely, in vivo stimulation of iNKT cells by administration of alpha-GalCer in B6 mice moderately aggravated joint inflammation. Perhaps the most fascinating data, however, described the actual appearance of iNKT cells in the synovium upon ABIA induction, a site were they are usually absent, and the consequent induction of significant alterations in cytokine balance within the joint. Most notably, local mRNA levels of transforming growth factor-beta1 (TGFβ1) were markedly increased in CD1d−/− mice, whereas transcription of IFN-gamma and IL-4 was decreased. This modulation was determined to be joint-specific and iNKT cell dependent, since no differences were found in the spleen and induced changes could be partially restored upon adoptive transfer of sorted iNKT cells. The crucial involvement of TGFβ1 in mediating the protective effect was evidenced by in vivo administration of neutralizing anti-TGFβ1 mAb and recombinant TGFβ1, which abrogated amelioration of ABIA in CD1d−/− mice and conferred protection in wild-type mice, respectively. Finally, adoptive transfer experiments of IL4−/− and IFNgamma−/− iNKT cells demonstrated that iNKT cell-mediated secretion of IL-4 and IFN-gamma is causative and indispensable for suppression of TGFβ1 production in the joint. The detailed mechanisms behind this type of TGFβ suppression remain to be unravelled.

Very recently, the same group provided exciting novel insight into a previously unrecognized mode of iNKT cell stimulation [50]. Kim and co-workers found that FcgammaRIII (CD16) engagement, mediated by IgG immune complexes in joint tissues during ABIA, was capable of directly activating iNKT cells in the absence of simultaneous TCR stimulation. FcgammaRIII is expressed by neutrophils, NK cells, macrophages and NKT cells and specifically recognizes the Fc portion of immunoglobulins, thereby mediating phagocytosis and antibody-dependent cell-mediated cytotoxicity. Kim et al. showed that FcgammaRIII engagement by aggregated IgG specifically activates iNKT cells and not T cells or NK cells, as examined by upregulation of CD25/CD69 surface expression. Although lower levels of cytokine production by sorted iNKT cells were measured upon aggregated IgG treatment as compared to anti-CD3 TCR ligation, levels of IL-4, IFN-gamma, IL-10 and IL-13 were considerably decreased upon ex vivo aggregated IgG treatment in iNKT cells derived from FcgammaR−/− mice compared to wild type. Finally, in vivo restoration of full-blown ABIA in CD1d−/− mice was not obtained by adoptive transfer of FcgammaR−/− iNKT cells, concordant with the unaltered production of IL-4, IFN-gamma and TGFβ1. The ability of FcgammaR−/− iNKT cells to infiltrate joint tissues and to respond adequately to TCR stimulation by alpha-GalCer was, however, intact, indicating that the failure to induce joint inflammation in CD1d−/− mice was not due to a downstream functional defect of the transferred iNKT cells, but rather to the inability to stimulate these cells via the FcgammaR. Together, these results shed light on the localized involvement of iNKT cells in the joint space during ABIA development, and point out a crucial role for an activating Fc receptor on iNKT cells (Fig. 3).

**iNKT cells: from bench to bedside**

Accumulating data support the involvement of iNKT cells in the pathogenesis of rheumatic diseases in human patients. Some notable differences must be taken into account, however, when aiming to delineate parallels between results derived from animal models versus human subjects, in terms of iNKT cell biology. Significantly, humans have lower numbers of iNKT cells than mice and iNKT cell subsets from these phylogenetically different species most likely have distinct functional properties as well [29, 51]. In humans, for instance, functionally distinct iNKT cell subsets were described based on the differential expression of CD4: whereas CD4+ iNKT cells produced both Th1 and Th2 cytokines, CD4− iNKT cells were predominantly biased towards Th1 cytokine production [52, 53]. Analogously,
these different iNKT cell phenotypes have been observed in mice, and some evidence exists supporting a differential functionality in vitro and in vivo [8, 9, 54, 55]. Nevertheless, it remains to be elucidated as to what extent these corresponding iNKT cell phenotypes share the same physiological functions between species. This possible limitation is further exemplified by the finding that mouse iNKT cells generally do not express the CD8 molecule, whereas up to 50% of human iNKT cells express CDαα-αβ, although only a small portion expresses CDαβ-βα, and regulatory functions were ascribed to this human CD8αα-iNKT cell subset [5, 18, 53, 56, 57]. In addition, the consensus that experimental models do not always closely mimic the human disease under study, represents another obstacle for reliably predicting the outcome of iNKT cell activation in human autoimmune conditions [58].

There are several arguments favouring a role for iNKT cells in human rheumatic diseases as a consistent reduction of both the aforementioned CD4+ and CD4+ iNKT subsets in peripheral blood from RA, SLE, SSc, and sjögren syndrome patients have been reported [59]. Interestingly, the CD4+ iNKT cell fraction was also found to be decreased locally in rheumatoid synovium. Moreover, Kojo and co-workers defined two categories of patients according to their alpha-GalCer responsiveness. Co-culture experiments of sorted iNKT cells from healthy controls together with antigen-presenting cells (APCs) from non-responders or healthy controls, respectively, subsequently suggested that the non-responsiveness is not due to aberrant functionality of alpha-GalCer presenting APCs, but rather to an abnormality in the CD4+ iNKT cell subset. Another study by Linsen et al. confirmed the decreased frequency of iNKT cells in the blood of RA patients, the existence of alpha-GalCer responsive and unresponsive iNKT subsets, and additionally reports on their Th1-like phenotype in the blood but not in the synovial fluid [60].

Taken together, these studies provide attractive indications for a possible role of iNKT cells in human arthritic disease, encouraging further investigation of iNKT cell activation as a therapeutic strategy.

Lessons from animal models: context matters

The interpretation of data obtained in animal models inherently requires an integrated approach. While these models allow us to examine mechanistic hypotheses, which cannot be elucidated by use of human samples, they often yield conflicting results. Nevertheless, most contradictions can be explained by the different underlying etiology and pathology of the various models, differences in treatment protocols, mouse strains, nature of the therapeutic compound, etc.

ABIA, as induced in the K/BxN serum transfer model does not include an initial adaptive immune response as observed in CIA, and therefore only represents the final effector phase of established RA [48]. Kim et al. already proposed that iNKT cells have a dual functionality depending on the ongoing autoimmune process, being suppressive in the inductive phase, and becoming provocative during progression of the disease [49]. Likewise, our data support this kinetic, biphasic functionality in CIA (unpublished results), as do results from alpha-GalCer treatment in EAE and SLE models [32, 61]. Thus, variation in treatment protocol may be, at least in part, the underlying cause of the profound differences in clinical outcome upon alpha-GalCer treatment in CIA and between results obtained in CIA and ABIA models.

Given the apparent downmodulation of iNKT cell numbers in RA, it seems conceivable that glycolipid administration might be beneficial in autoimmunity by contributing to the restoration of iNKT cell numbers, as revealed in two phase 1 studies conducted in advanced cancer patients [62, 63]. However, results in CIA now clearly highlight the crucial importance of the type of glycolipid antigen with respect to the iNKT cell response, induced cytokine profile, and downstream cellular interactions in the eventual clinical outcome. Structurally modified forms of alpha-GalCer, such as OCH and alpha-C-GalCer, stimulate qualitatively altered responses from iNKT cells [37, 64]. Systematic alteration of the length and extent of unsaturation of the N-acyl substituents changes the outcome of iNKT cell activation, in some cases leading to an anti-inflammatory Th2 cytokine bias [65]. The molecular mechanism of preferential Th2 cytokine production upon iNKT cell stimulation has been elegantly studied for OCH [66]. In conclusion, a better understanding of the relationship between structure and functionality of iNKT-stimulating glycolipids will be important in order to design analogues with superior immunotherapeutic properties, ultimately leading to tailored therapeutic manipulation of iNKT cells in RA.

Significant interference with results in CIA can also be expected from differences in genetic background between mouse strains. Highly susceptible male DBA/1 mice were used by Miellot et al. [45] and the induction of CIA was achieved by a standard immunization protocol. The other studies mentioned all utilize B6 mice, which are normally less predisposed to arthritis development, and which require modified immunization conditions. These deviations may be held accountable for some of the observed variation in results. The actual presence of iNKT cells within the joint has been demonstrated in both mice and humans, and some evidence has been gained suggesting distinct functional characteristics of this local population as compared to iNKT cells from peripheral blood [49, 60]. Furthermore, an organ-dependent diversity of the resident iNKT cell subpopulations has been clearly shown in mice and humans, both in terms of frequency and functionality [8, 67]. An attractive hypothesis is that local, i.e. intra-articular, glycolipid treatment could modulate severity of arthritis.

Finally, the variety of iNKT cell responses induced upon glycolipid treatment goes far beyond the concept of prototypic Th2 bias. Complex mechanisms, including cellular interplay with dendritic cells, NK cells, and conventional T cells, have been found to orchestrate the resulting immune response after iNKT cell activation [4]. Of interest, a subset of IL-17-producing T cells, called Th17 cells, was recently described as a novel effector CD4+ T cell arm [68, 69], and IL-17 was repeatedly shown to be of critical importance in experimental autoimmune arthritis [70–74] and RA [75, 76]. It has been suggested that iNKT cells promptly produce IL-17 upon activation, which may account for yet another pathway by which iNKT cells influence ongoing inflammatory processes [77]. Depending on the type of antigen, duration of stimulation and various other parameters, iNKT cells can confer to processes such as anergy and tolerization or, conversely, display adjuvant properties [78–81]. Indeed, a single injection of alpha-GalCer was shown to induce long-term NK cell hyporesponsiveness in mice, reminiscent of the anergic state that is induced in conventional T cells by strong stimuli such as superantigens [79]. This observation raises the issue of the avoidance of anergy induction in the design of treatment regimens that use alpha-GalCer as a specific activator of iNKT cells. Given this broad range of potential mechanisms, it is therefore reasonable to assume that future work, propagated by the development of novel glycolipids, will reveal yet other modes of action of iNKT cells in arthritic disease, different from Th2 immune deviation.

Concluding remarks

The involvement of iNKT cells in RA has now been sufficiently documented to support further investigation of the application of alpha-GalCer and its analogues in human disease. Some obstacles, however, should be taken into account when translating preclinical studies to the clinic. Indeed, immunological properties of human iNKT cells as compared to mouse iNKT cells,
and in particular how they may differentially respond to glycolipid activation, should be examined in depth. A better understanding of the structural basis for the induction of alternate iNKT cell responses upon stimulation with different glycolipid analogues will enhance future tailored design of iNKT cell antigens. In addition, novel methods of administration, such as repeated systemic and local glycolipid injection, may be appropriate in order to optimize clinical outcome of iNKT cell activation. Nevertheless, several concerns regarding the safety of therapeutic iNKT cell stimulation in humans remain to be tackled. Although short-term treatment of human subjects with alpha-GalCer appears to be safe, the induction of allergic airway inflammation, liver toxicity and abortions in mice, demand cautiousness when aiming to implicate this glycolipid in future therapies [82]. On the other hand, appropriate glycolipid design may overcome this hurdle, possibly by the presence of lower iNKT cell numbers in humans, which may evoke less pronounced side effects. Altogether, the exploitation of the iNKT cell subset as a flexible, yet powerful system holds great promise for therapeutic purposes in RA.

**Rhematology key messages**

- iNKT cells influence autoimmune disease in mice and men by various mechanisms.
- iNKT cells can be activated in vivo by administration of glycolipid ligand.
- iNKT cells may be suitable targets for therapy of arthritis.

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Role of iNKT cell in experimental arthritis


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