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NKT cells are not the predominant T cell in asthma and likely modulate, not cause, asthma

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Abstract

Asthma is a multifactorial disease of the airways characterized by airway inflammation, mucus hypersecretion, and airway hyperresponsiveness. Conventional MHC-class II restricted $CD4^+$ T cells are considered a key cell in asthma pathogenesis as they have a broad T cell receptor repertoire, providing specificity and reactivity to diverse protein allergens. This notion was challenged when a study found that invariant (i) NKT cells were the predominant T cell in the lung and BAL of all asthmatic subjects studied. This finding was provocative because iNKT cells have a very limited T cell receptor repertoire and are specific for a restricted set of lipid antigens that bind to CD1d, a non-polymorphic MHC-like molecule. However, multiple subsequent studies failed to replicate the initial study and instead found that iNKT cells are present as a small fraction of the total T cells in the asthmatic lung. Thus, we believe that while CD1d-restricted NKT cells may play a role in modulating the asthmatic phenotype, they are not the critical drivers of the asthmatic response, a role we believe is still held by conventional MHC-class II restricted $CD4^+$ T cells.

Keywords

NKT; asthma; CD1d; Th2 cells; AHR

Introduction

iNKT cells burst onto the asthma scene in 2006 when the *NEJM* published a potentially paradigm shifting paper describing that invariant (i) NKT, and not conventional MHC class II restricted CD4 T cells, were the predominant T cell in the lung and BAL of all patients studied with allergic asthma.[1] In the ensuing 4 years, the weight of evidence strongly suggests that iNKT cells are not the predominant T cell in the asthmatic lung and, in fact, represent a small fraction of T cells in the asthmatic lung.[2-7] This coupled with the fact that iNKT cells have limited antigen specificity and reactivity to lipid and not protein antigens, leads us to believe that iNKT are not the major T cell that drives asthma pathogenesis and at most play a modulatory role.

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What are iNKT Cells and how are they identified?

iNKT cells (also referred to as Type I NKT cells) exhibit a very limited pattern of gene segment utilization in their T-cell antigen receptors. This is characterized by a semi-invariant T cell receptor alpha chain rearrangement (Vα24-Jα18 in humans and Vα14-Jα18 in mice), which is most commonly paired with Vβ11 in humans[8] and with Vβ8.2, Vβ7, or Vβ2 in mice.[9] The limited diversity of T cell receptors on iNKT cells is associated with a very narrow profile of antigenic specificity that is restricted to lipid antigen presented in the context of the β2-microglobulin-associated antigen presenting molecule, CD1d. Unlike the polymorphic MHC Class I and II antigen presenting molecules, which present peptide antigens to conventional CD8 and CD4 T cells, respectively, CD1d is non-polymorphic and, thus, engages a more limited repertoire of CD1d-restricted NKT cells. These cells can be identified by their recognition of CD1d loaded with the non-physiological glycolipid ligand, α-galactosylceramide (α–GalCer), derived from sea sponges.

There is also thought to be a more diverse pool of Type II NKT cells that are restricted by CD1d but that do not contain the canonical semi invariant TCR rearrangement. Some of these CD1d-restricted diverse NKT cells can also respond to α-GalCer, but others are restricted to specific lipid antigens.[10] The identification of type II CD1d-restricted diverse NKT cells is technically complicated by the fact that individual lipid antigens must be characterized and loaded in CD1d tetramers to recognize and enumerate corresponding type II NKT cells.

“Type III” NKT cells are not truly CD1d-restricted NKT cells but, rather, conventional T cells that express NK antigens, such as NK1.1 (CD161) and DX5 (CD49b) in the mouse or CD56 in humans. This is an important distinction since the term “Type III NKT cells” has been inappropriately used to suggest that conventional T cells expressing NK-like markers are CD1d restricted or recognize lipid antigen.

How are iNKT cells identified?

As mentioned above, Type I iNKT cells have been identified *in vivo* using CD1d tetramer loaded with a glycolipid derived from a marine sponge, α-galactosylceramide (α-GalCer) in both humans and mice. Although α-GalCer is neither an endogenous or relevent exogenous glycolipid in mammals, Type I iNKT cells bind and respond strongly to CD1d-mediated α-GalCer stimulation by rapidly producing of a number of cytokines.[11] In addition, human iNKT cells can be recognized by co-staining with 6B11 and Vα24 antibodies, which recognize the semi-invariant rearrangement, Vα24-Jα18. The 6B11 antibody was raised against the CDR3 loop of the Vα24-Jα18 rearrangement.[12, 13] However, since this epitope can be also be expressed on non-CD1d restricted T cell receptors or B cell receptors it must be used in combination with Vα24 or Vβ11 antibodies to verify specific iNKT staining. Failure to use 6B11 in conjunction with Vα24 or Vβ11 antibodies can lead to an overestimate of the apparent number of iNKT cells in a specimen and has generated confusion in the literature. Care must be taken to also exclude non-specific staining of tetramer and/or antibodies to cells other than lymphocytes by appropriate gating in flow cytomemtry analysis, especially in BAL samples that contain alveolar macrophages, which are notorious for being both highly autofluorescent and binding proteins non-specifically.[6, 7] In fact, we and others believe that the large number of iNKT cells identified the initial study by Umetsu's group include alveolar macrophages rather than lymphocytes alone, based on forward versus side scatter analysis in flow cytometry.[6,7]

How could iNKT cells be activated in asthma?

CD1d-restricted iNKT cells are thought to respond to either self-glycolipid or phospholipid antigens or closely related bacterial glycolipid antigens. Several endogenous glycolipid or phospholipid antigens have been identified that can stimulate subsets of Type I semi-invariant

iNKT and/or Type II diverse NKT cells.[10, 14, 15] In addition to endogenous lipid stimulation of CD1d-restricted iNKT cells, exogenous glycolipids have been suggested to play a role in CD1d-restricted iNKT cell activation. Glycosphingolipids isolated from the cell wall of the gram-negative LPS-negative bacterium *Sphingomonas* have been shown to stimulate iNKT cells.[16, 17] Sphingomonas is a ubiquitous organism that can be easily aerosolized and stimulates an iNKT response.[18] Other bacterial products, especially glycolipids from bacterial cell walls, also have the capacity to stimulate iNKT cells.[19] In addition, a glycolipid found in cedar pollen can stimulate iNKT cells.[20] However, no other associations of specific lipid antigens capable of activating iNKT cells have been made with other allergens. Mechanistically, it seems implausible that lipid activation of iNKT cells is likely to do more than augment a protein-allergen driven MHC class II CD4 T cell response and even this has yet to be shown in human asthma.

During bacterial infection, iNKT cells have been shown to be activated by the synergistic stimulation of self-lipids and pro-inflammatory cytokines.[21] While this has not been shown to occur in the asthmatic lung, it remains a possibility that iNKT cells could be activated by such a mechanism in asthma. However, this self antigen hypothesis still suggests that another cell type would be responsible for producing the initial allergen-specific signal in asthma, and therefore favors the argument that iNKT cells play a modulatory and not primary role in asthma pathogenesis.

Are iNKT cells found in human asthma?

iNKT cells were first suggested to play a critical role in the human asthmatic response in a 2006 *NEJM* study by the Umestsu group.[1] In that study, CD1d-restricted iNKT cells were found to comprise >60% of $CD3^+$ BAL T cells by flow cytometry analysis and a majority of $CD4^+$ lymphocytes in asthmatic airway biopsies by immunofluorescent histopathological staining. Since CD1d-restricted iNKT cells generally make up <0.2% of human peripheral blood T cells, this would indeed be a >300-fold increase in CD1d-restricted iNKT cells found in the BAL as compared to peripheral blood. We and all others[2-7] have observed significantly lower percentages of CD1d-restricted iNKT cells in the BAL or sputum of human asthmatic subjects (<2.7% of $CD3^+$ T cells) by flow cytometry using CD1d tetramers and the 6B11 antibody in combination with Vα24 or Vβ11 antibodies. This large discrepancy between the initial findings of the Umestsu group[1] and these other studies is likely accounted for by artifactually high staining in the original report due to non-specific binding of tetramer and antibodies to alveolar macrophages, which were not appropriately gated out in this original study.[6, 7]

The significantly lower numbers of iNKT cells observed in later flow cytometry studies were confirmed using a different method by Djukanovic's group who also used quantitative (real-time) PCR to detect the messenger RNA (mRNA) for the genes of the T-cell–receptor family, Vα24 or Vβ11, on BAL cells obtained from patients with asthma.[7] BAL cells from subjects with asthma did not express mRNA for either the Vα24 or Vβ11 gene, even though mRNA for the constant chain of the T-cell receptor was readily detected. This finding confirmed the presence of T cells in the samples, but it also ruled out the possibility that iNKT cells were numerous in BAL from subjects with asthma. In further experiments, real-time PCR for mRNA expression of the iNKT-cell receptor (Vβ11) relative to the expression of the constant chain of the T-cell receptor established that the proportion of T cells expressing T-cell receptor Vβ11-encoding mRNA was less than 2% in all samples obtained by BAL and sputum induction.

A follow-up study by the Umetsu group where the gating was now more appropriately performed failed to replicate their earlier study.[22] While an increase in CD1d-restricted iNKT cells in asthmatic subjects compared to non-asthmatic subjects was reported, the absolute magnitude of the percentage of iNKT cells in most of these patients was low and more than

10-fold less than the levels initially reported by this group[1]. Indeed, the percentage of iNKT cells among $CD3^+$ T cells in the BAL exceeded 10% in only a single patient. In contradistinction to the original report, this follow up study[22] and all other reported studies[2-7] agree that the vast majority of $CD3^+$ T cells in the BAL of asthma patients are therefore not iNKT cells.

In this follow up study, the Umestsu group also noted a trend towards an increase in percentages of iNKT cells in the lungs of patients with severe asthma as compared to patients with well-controlled asthma.[22] However, this observation failed to reach statistical significance and, moreover, this trend has not yet been observed by other investigators, making this an unsubstantiated hypothesis at present.

In addition to studies examining BAL and sputum for the presence of iNKT cells, several studies have examined bronchial biopsies obtained from asthma patients for the presence of iNKT cells. The original study from the Umetsu group reporting an abundance of iNKT cells in the BAL of asthma patients also noted that most $CD4^+$ cells in the bronchial mucosa stained positive for the iNKT cell epitope, 6B11.[1] In this study, the 6B11 antibody was used in histopathological sections in conjunction with a CD4 antibody and not with a Vα24 chain antibody. Since CD4 is also expressed on macrophages, this may have led to an overestimate in the number of iNKT cells in the tissue. In fact, this finding was contradicted a year later by the results of Djukanovic's group.[7] This study rigorously assessed the number of iNKT cells in bronchial biopsies from asthma patients through multiple approaches, including staining with antibody against Vα24, CD1d tetramers loaded with αGalCer, and the 6B11 monoclonal antibody. Regardless of the staining method employed, the counts of iNKT cells were very low and did not exceed 1.7% of the $CD3^+$ T cells in the bronchial mucosa. Moreover, in this study, the number of iNKT cells remained extremely low in patients with moderately severe asthma as well as in mild asthma, indicating that asthma severity did not affect the frequency of iNKT cells observed in the lung tissue.

Thus, the evidence in favor for a critical role of iNKT cells in human asthma remains limited to the demonstration that they are present in very low numbers in BAL and bronchial biopsies of patients with asthma, and, to date there is no association between iNKT cell numbers and a specific asthma phenotype. In contrast, there exists an abundance of evidence strongly implicating conventional MHC class II restricted T cell subsets in the pathogenesis of human asthma.[23-28] As these cells represent a far greater percentage of $CD3^+$ cells in the BAL, it would be difficult at this time to conclude that iNKT cells play a more critical role in asthma than conventional T cells.

What role might iNKT cells play in asthma?

The development of the allergic asthmatic response can be divided into sensitization, allergic inflammation and exacerbation phases. Environmental antigens are constantly encountered in the airway during the breathing process, yet only selected agonists stimulate an allergic response. Airway antigen presenting cells must distinguish between innocuous and potentially harmful stimuli, including viral and bacterial pathogens. Allergic asthma results from a dysregulation of the immune response to generate allergen-specific, rather than pathogen-specific, IgE and Th2 cells.

The initial process of allergen sensitization likely begins with a combined encounter of allergen along with an adjuvant signal. Epithelial cells line the conducting airways forming a barrier between inhaled antigens and mucosal DCs.[29] During the sensitization process, disruption of the epithelial cell layer can occur by intrinsic protease activity of certain allergens[30] or increased vascular permeability through VEGF induction[31] as well as through environmental factors, such as smoking[32] and ozone exposure.[33] In conjunction with adjuvant signals from

pattern recognition receptors and protease activated receptors, epithelial cells are triggered to produce TSLP and GM-CSF, which aid in the maturation and polarization of airway DCs towards a Th2-promoting phenotype. iNKT cells could facilitate sensitization in the initial allergic asthmatic response with endogenous or exogenous lipid antigen as an adjuvant, in place of or together with pattern recognition receptors on DCs.

Following sensitization to an allergen, triggering of the allergic asthmatic response is initiated by re-exposure to the allergen and binding of the antigen to pre-formed IgE antibodies. The early allergic response (EAR) is initiated by crosslinking of allergen-specific IgE on mast cells leading to mast cell degranulation and release of inflammatory mediators, including histamine, leukotrienes, and prostaglandins, as well as both inflammatory and Th2-associated cytokines and chemokines.[34] Airway DCs also bind the inhaled allergens, transport them to the draining lymph node, and present the allergen to both naïve and memory conventional T cells.[35] iNKT cells are unlikely to play a role in these well described allergen-specific adaptive responses. However, iNKT cells may contribute to asthma exacerbations by secreting cytokines and chemokines rapidly, which may serve to modulate T cell polarization and/or the recruitment of T cells, eosinophils, basophils, and neutrophils during the late allergic response (LAR).

Other significant features of the allergic asthmatic response occurring during the late phase include mucus hypersecretion, smooth muscle cell hyperreactivity, and airway remodeling, in large part felt to occur under the influence of Th2 cell derived cytokines. A contribution to the cytokine milieu from iNKT cells is possible. We found that while iNKT cells do not increase as a percentage of overall T cells, the total number of iNKT cells increased ~15-fold in the BAL following segmental allergen challenge in proportion with conventional T cells.[36] A recent study by another group detected a 2-fold increase in the number of iNKT cells in the bronchial mucosa following allergen challenge.[37] The increase in the number of iNKT cells during the late phase of the asthmatic response could allow iNKT cells to modulate and propagate the type and severity of the asthmatic response, although this has not been demonstrated in human subjects.

In addition to the “classical” allergic asthmatic response, it is important to consider the cell types that might be important in other phenotypes of human asthma, including so called “non-allergic” and severe asthma. The non-allergic asthmatic response may result from innate stimuli directly triggering the inflammatory cascade typical of allergic asthma in genetically susceptible individuals or through previously unrecognized allergens.[25, 38] The severe allergic asthmatic response is associated with more airway neutrophils and a cytokine profile that is suggestive of a Th1- and Th17-type response.[39, 40] Since iNKT cells can respond to lipid antigen and secrete a variety of cytokines rapidly, it is important to consider the ability of these cells to modulate the asthmatic response to create such a cytokine milieu. However, since CD1d is non-polymorphic and presents a similar lipid antigen among individuals, iNKT cells cannot easily account for the diversity of cellular responses to allergic and non-allergic stimuli or for differences in reactive and non-reactive individuals.

Have murine models helped clarify the role of iNKT cells in asthma?

iNKT cells were first implicated in the allergic asthmatic response using the OVA-alum mouse model of allergic asthma.[41, 42] In Balb/c and C57BL/6 Jα18$^{-/-}$ and/or CD1d$^{-/-}$ mice, airway hyperreactivity (AHR) was eliminated or decreased and airway inflammation (as measured by eosinophilia, anti-OVA IgE, and IL-4, IL-5 and IL-13 levels) was reduced as compared to wild-type mice. In these studies, AHR could be restored by transfer of wild-type iNKT, but not IL-4$^{-/-}$ IL13$^{-/-}$ double-deficient, iNKT cells.

However, studies by other groups have indicated that mice missing CD1d-restricted NKT cells (CD1d-deficient mice) developed the full phenotype of allergic airway inflammation, including

eosinophilia and Th2 cytokine production, which was indistinguishable from wild-type mice.[43, 44] Das *et al* further showed that Th2 airway inflammation response was dependent on MHC Class II restriction, not CD1d.[44] The Umetsu group has countered that AHR rather than airway inflammation is the primary end point affected by iNKT cells and this was not evaluated in these later studies. While this is an intriguing hypothesis, such a finding has yet to be reproduced by other groups. Moreover, even if this observation was confirmed, this would support a more limited role for iNKT cells in asthma than initially proposed.

Other mice that are missing iNKT cells (β2-microglobulin-deficient mice) display wild-type levels of AHR and airway inflammation in mouse models of asthma.[44-47] While the Umetsu group suggests that Type II CD1d-restricted diverse NKT cells can substitute for iNKT in this setting,[46] these cells cannot be identified by the αGalCer-CD1d tetramer, making this a theoretical argument. Moreover, even if this assertion was true, it would invalidate the hypothesis that iNKT are required for AHR.

In other mouse models of allergic asthma, CD1d glyocolipid has been used as a substitute for alum in the sensitization phase,[48] and as a direct inducer of AHR in unsensitized naive mice.[49] These data suggest that CD1d glycolipids can act as adjuvants via iNKT cell activation in the development of an asthmatic response. They also suggest that CD1d lipids can induce AHR directly when delivered into lung. However, the physiological relevance of these studies that deliver high concentrations of α-GalCer directly into the lung is of questionable significance. Futhermore, others have shown that the use of α-GalCer in an OVA-driven model during the challenge phase prevented both allergic airway inflammation and AHR, likely through increased production of IFN-γ.[50, 51] Based on these combined data, it is likely that the cytokine activity of CD1d-restricted iNKT cells can differentially modulate the allergic asthmatic response depending on the specific experimental conditions.

The role of iNKT cells in murine models of non-allergic ozone- and viral-induced asthma have also been explored. In a model of ozone-induced asthma, $CD1d^{-/-}$ and $J\alpha18^{-/-}$ mice did not develop AHR or neutrophilia compared to wild-type mice.[52] This effect was IL-17 dependent, which highlights the ability of CD1d-restricted iNKT cells to skew the cytokine milieu during the acute asthmatic response. In addition, respiratory virus infection is an important trigger of asthma symptoms in both allergic and non-allergic asthmatic subjects.[53] Using a chronic Sendai virus model, Kim *et al* found that that virally-induced chronic lung response characterized by increased mucus production and AHR, similar to asthma and COPD did not occur in $CD1d^{-/-}$ and $J\alpha18^{-/-}$ mice and was dependent on IL-13 produced by macrophages,[54]. Although the mechanism of iNKT cell activation in this model was not elucidated, it suggests that following viral infection, CD1d-restricted iNKT cells can stimulate a macrophage driven IL-13 lung inflammatory response. How this model relates to non-allergic or viral-induced exacerbations of human asthma awaits further investigation, but it does suggest that iNKT cells can alter the cytokine milieu during chronic lung inflammation in certain experimental settings.

Thus, data derived from mouse models have been somewhat confusing and certainly not uniformly positive but suggest that in certain experimental settings iNKT cells can be shown to contribute to asthma pathogenesis. However, some of these murine models are quite contrived and have certain artificial and idiosyncratic features that make it difficult to ascertain the physiological relevance to human asthma. It is therefore of vital importance to examine the data available from the study of human subjects before drawing any definitive conclusions about a role of iNKT cells in human asthma.

Conclusion

Initial excitement surrounding the finding that iNKT cells represented the majority of T cells in the lungs of asthma patients has dissipated upon realization that this observation was due to a technical artifact. Subsequent studies from multiple groups, including the investigators of the initial study, have consistently demonstrated that only a small proportion of T cells in the lung are represented by iNKT cells. There are no other lines of evidence from human studies pointing towards an important role of iNKT cells in asthma. Although the absence of evidence certainly does not disprove the hypothesis, it indicates that the notion that iNKT cells serve a critical function in the pathogenesis of asthma is entirely speculative at this juncture. It has been postulated that iNKT cells may play a niche role in the pathogenesis of severe asthma, but again, until there is more conclusive data in that regard, such a concept remains in the theoretical realm.

We certainly do not dismiss a possible role for iNKT cells in modulating the asthmatic disease process, and we agree that future studies of these cells would be worthwhile to pursue. However, at this time, there is clearly insufficient evidence to believe that iNKT cells play a critical role in human asthma.

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Abbreviations

iNKT	invariant natural killer T
AHR	airway hyperresponsivness
MHC	major histocompatibility complex
α-GalCer	alpha-galactosylceramide
CDR	complementarity determining region
LPS	lipopolysaccharide
QPCR	quantitative real-time PCR
mRNA	messenger RNA
BAL	bronchoalveolar lavage
DC	dendritic cell
VEGF	Vascular endothelial growth factor
TSLP	Thymic stromal lymphopoietin
GM-CSF	Granulocyte macrophage colony-stimulating factor
EAR	early allergic response
LAR	late allergic response
Th	T helper