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Invariant NKT cells provide innate and adaptive help for B cells

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Abstract

B cells rely on CD4⁺ T cells helper signals to optimize their responses to T-dependent antigens. Recently another subset of T cells has been identified which provides help for B cells, invariant natural killer T (iNKT) cells. iNKT cells are unique because they provide both innate and adaptive forms of help to B cells, with divergent outcomes. iNKT cells are widely distributed throughout the spleen at rest, consolidate in the marginal zone of the spleen early after activation, and are later found in germinal centers. Understanding the activation requirements for iNKT cells has led to the development of glycolipid containing nanoparticles which efficiently activate iNKT cells, enhance their cooperation with B cells, and which hold promise for vaccine development.

Introduction

Peptide-specific helper CD4⁺ T cells are critical contributors to the development of high affinity, class-switched, B cell responses. In the past few years, it has become evident that B cells can also receive help from a diverse community of adaptive and innate cells. Invariant natural killer (iNK) T cells are one of the innate-like cells which is capable of providing help for B cell proliferation and antibody production [1, 2]. iNKT cells recognize glycolipids presented by the non-polymorphic CD1d antigen presenting molecule and are capable of rapidly producing Th1, Th2, and Th17 cytokines upon activation. They represent 1–2% of the lymphocyte population in the murine spleen [3] and average 0.1–0.2% of T cells in peripheral blood of humans [4, 5], but express a T cell receptor with limited diversity and can respond en masse as a population. iNKT cells have the flexibility to respond to specific glycolipid antigens presented in the context of CD1d or to a combination of self-glycolipid plus dendritic cell (DC)-derived cytokines induced by pattern recognition receptor engagement [6]. This means that depending on the activation signal, B cells can receive purely innate help from iNKT cells restricted to a single unique pathogen-derived glycolipid, or they can receive a more adaptive form of help from innate iNKT cells via pathogen-engaged DCs and conventional CD4⁺ T cells. As a consequence, B cells could receive either of two different types of help from iNKT cells- direct, innate cognate help or indirect,

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adaptive non-cognate help, or both at once. As one might predict, these two forms of iNKT help drive different outcomes for the helped B cells. Once again, this reveals another point at which the immune system is finely tuned to appreciate the difference between a limited innate response or a more comprehensive adaptive response.

I. Innate cognate iNKT cell help for B cells

CD4⁺ T cells that help B cells during a thymus-dependent (T-D) antibody response have been defined as T follicular helper (T_{FH}) cells. They can be identified by their surface expression of programmed cell death protein 1 (PD-1) and CXC-chemokine receptor 5 (CXCR5), intracellular preference for B cell lymphoma 6 (bcl6) over PR domain zinc finger protein 1 (blimp1), and their localization to the germinal centers (GC)s of the spleen [7]. Within the GC, T_{FH} cells deliver developmental signals through several pathways, including interleukin (IL)-4, interferon (IFN)- γ , CD40 ligand (CD40L), IL-21, and B cell activating factor (BAFF) to GC B cells [7]. The T_{FH} cells also benefit from reciprocal SLAM-associated protein (SAP)-dependent and PD-1 mediated signals for maintenance and enhanced helper functions from the B cells they are helping [8]. Interestingly, iNKT cells that help B cells adapt a similar helper phenotype. This subset of iNKT cells, termed iNKT follicular helper (iNKT_{FH}) cells, express PD-1 and CXCR5; upregulate bcl6; downregulate blimp1; increase surface expression of CD40L; secrete cytokines and chemokines IL-4, IFN γ , IL-21, and BAFF; and localize in GCs when activated by the glycolipid α -Galactosylceramide (α GalCer) alone or α GalCer plus peptide [9].

As depicted in Figure 1, iNKT cells can provide direct, cognate help by interfacing with CD1d on antigen presenting B cells, in much the same way that peptide-specific CD4⁺ T cells interact with MHC class II⁺ B cells. Additionally, iNKT cells can provide non-cognate help by engaging CD1d on DCs, which indirectly enhances conventional CD4⁺T cell help for B cells by licensing DCs to be better antigen presenters [10]. Interestingly, the B cell outcome following innate cognate iNKT cell help is notably different than the B cell outcome after non-cognate iNKT cell help or adaptive CD4⁺ T cell help [9, 11, 12]. Cognate iNKT cell help can lead to GC formation, however non-cognate help induced by α GalCer plus protein leads to a greater number of protein-specific GC B cells [9, 12]. Cognate iNKT cell help is able to induce a robust primary IgG antibody response that requires IL-21 and IFN γ from iNKT cells, and co-stimulatory molecules CD40/CD40L, and CD28/CD80 or CD86, but not IL-4 [2, 12]. Another mediator of B cell–CD4⁺ T cell interactions which is required for proper GC formation and T_{FH} cell terminal differentiation is SAP [8]. SAP expression is also required for iNKT cell development in the thymus, but by using a unique inducible SAP knock out mouse, Detre and colleagues are able to bypass the developmental defects in SAP^{−/−} iNKT cells to show that iNKT cells with an induced SAP deficiency cannot provide cognate help for B cells [7, 9, 13]. Affinity maturation after cognate iNKT cell help is modest compared to noncognate help and the plasmablast population peaks early at day 5 after cognate help but disappears quickly and is gone by day 28 [9, 12]. Ultimately, only noncognate iNKT cell help leads to plasma cell (PC) development and a humoral memory response whereas cognate iNKT cell help leads to very little humoral memory or antigen-specific memory cells [9, 12]. Given that iNKT cells respond to any pathogen which drives a CD1d-bearing DC to produce IL-12, it might be best for them to drive a fully

mature memory response only with contributions from other antigen-specific T cells. As such, glycolipid antigens which recruit only innate cognate iNKT cell help have earned the designation as T-dependent type II antigens, to set them apart from T-dependent type I antigens which recruit conventional peptide-specific T cell help and go on to develop affinity maturation, long lived GCs, and eventually plasma cells and humoral memory [14].

II. iNKTs provide adaptive help for B cells via DCs and CD4⁺ T_{FH} cells

When α GalCer is used as an adjuvant with protein antigens, driving iNKT cells to engage α GalCer-presenting DCs and license them to recruit and activate peptide-specific CD4⁺ T cells it is termed noncognate help [Figure 1][15, 16]. Licensed DCs activate naïve CD4⁺ T cells, which will clonally expand, differentiate, and provide help to B cells as T_{FH} cells. Numerous investigators have clearly demonstrated that this type of non-cognate help by iNKT cells promotes GC formation and enhances antigen-specific primary and memory antibody production [12, 13, 17]. Most recently, Bai and colleagues have harnessed this form of adaptive, non-cognate help to drive robust class-switched, primary antibody responses specific for a polysaccharide from *Streptococcus pneumoniae* [18]. There is recurring evidence for iNKT cells to be drivers of the early phase of the antibody response, but they do not help maintain antibody titers or promote memory or plasma cells [19]. Peptide plus α GalCer immunized MHC class II^{-/-} mice, which lack CD4⁺ T_{FH} cells but still possess iNKT_{FH} cells, are able to form GCs and initiate a primary antibody response, but they are unable to maintain long lasting secondary antibody [20]. Furthermore, Detre and colleagues used inducible SAP^{-/-} mice to show that adoptively transferred SAP⁺ MHC class II-restricted T cells can complement ineffective B cell help from SAP^{-/-} iNKT cells to rescue antibody production [13]. This is consistent with the idea that the CD4 T_{FH} cells are the cells primarily responsible for the memory component of the non-cognate response.

Our work and that of others has identified a clear role for DCs in driving initial activation of iNKT cells [21, 22]. This initial activation step is required for the subsequent early B cell response because when DCs are depleted in CD11c-DTR bone marrow chimeras immunized with protein plus α GalCer, there is a delay in the primary IgG1 antibody response [23]. In addition to licensing DCs, iNKT cells may enhance the contributions of CD4⁺ T cells more directly. Crotty and colleagues have recently demonstrated that T_{FH} cells evolve into T memory (T_{mem}) cells as the immune response matures [24], and iNKT cells may enhance the development of T_{mem} cells via the production of IL-2, IL-4, BAFF, or a proliferation-inducing ligand (APRIL) [25] [26]. Collectively, these data demonstrate that iNKT cells mediate the more adaptive aspects of their helper functions by way of DCs and CD4⁺ T cells; the help provided by iNKT cells alone is not sufficient to develop a humoral memory response.

III. Visualizing iNKT help for B cells

Localization of T_{FH} cells within the germinal center is critical for delivery of their help to B cells, but until recently there was limited data on the localization of iNKT cells. iNKT cells pose a unique challenge for identification in situ because they are most definitively identified by binding to CD1d-tetramer rather than traditional lineage-specific surface

markers. However, early studies paired engineered *4get* mice, which produce GFP to faithfully report IL-4 expression, with IFN γ immuno-histochemical labeling to show cytokine positive effector iNKT cells localizing at the edge of the periarteriolar lymphoid sheath (PALS) 90 minutes after systemic anti-CD3 [27]. Geissman and colleagues took advantage of the high level of expression of CXCR6 on iNKT cells to use CXCR6-gfp reporter mice as another means to track iNKT localization [28]. They found that iNKT cells arrested in place in the sinusoids of the liver 40 minutes after systemic anti-CD3, α GalCer [28], or IL-12 plus IL-18 [29]. On the other hand, Kubes and colleagues saw CD1d-dependent arrest and consolidation of iNKT cells at the site of spirochete-containing Kupffer cells in the liver 8–12 hours after systemic administration of *Borellia burgdorferi* [30]. Barral and colleagues used adoptive transfer to document CD1d-dependent arrest of iNKT cells paired with CD169+macrophages in the subcapsular sinus region of the lymph node 2–16 hours after particulate glycolipid antigen administration, similar to the Kubes studies. They also identified the localization of transferred, but unstimulated, iNKT cells into the T cell and interfollicular regions, but not the B cell follicles, of the subcapsular sinus of the LN [31]. Collectively, these were the first studies to suggest that antigen recognition may drive the localization of activated iNKT cells, but they were restricted by the caveat that iNKT cells were identified indirectly using chemokine receptor reporter mice or adoptive transfer approaches.

Using a novel method of *in situ* mCD1d tetramer staining, in combination with cytokine reporter mice, we have now unambiguously identified a restricted localization and cytokine production profile for endogenous splenic iNKT cells. In partial agreement with previously published reports, we find that endogenous iNKT cells are widely distributed throughout the parenchyma of the spleen, including B and T cell follicles in the PALS, the marginal zone (MZ), and red pulp [21]. Importantly, this distribution changes to a restricted MZ localization within 4–8 hours of cognate activation by model and bacterially-derived glycolipids or *S. pneumonia* infection, presumably as a result of arrest in proximity to antigen loaded APCs, as suggested by previous reports [30, 31]. However, in contrast to data from the Velazquez studies [29], these experiments also show that iNKT cells remain evenly distributed throughout the spleen following activation by systemic IL-12 plus IL-18 [21]. The contradiction is likely explained by the different subsets of iNKT cells or antigen presenting cells which populate the spleen vs. liver, different integrin or chemokine trafficking requirements, or both. This localization profile is consistent with recent studies by Barral et al [32] using adoptive transfer and fluorophore diffusion approaches to localize iNKT cells recently activated by cognate glycolipid to the red pulp and MZ, but not the T cell zone. After a few days, Chang and colleagues find iNKT cells localized in germinal centers in the B cell follicle, consistent with the notion that they are functioning as iNKT_{FH} cells and directly helping antigen-specific B cells [6]. The localization pattern of iNKT cells during the course of immune responses to various antigenic stimuli is summarized in Figure 2.

IV. Harnessing iNKT cell help for humoral immunity using nano-vaccines

One of the most important advantages of understanding the mechanism of iNKT and B cell cooperation is the potential to harness this interaction for improved immune protection.

iNKT cells provide a rapid, innate form of help which can precede and/or complement protein-specific CD4⁺ T cell help. As such, iNKT-activating glycolipids represent potential adjuvants for vaccines against infectious agents. AlphaGalCer has already been repeatedly tested as a vaccine for cancer immunotherapy and has proven to be overwhelmingly safe in humans [33]. Currently, the best approach to efficiently harness iNKT cells for enhancing humoral memory is to deliver α GalCer in combination with protective B cell antigen, thereby driving a non-cognate, adaptive form of iNKT cell help. As described in sections I and II, soluble α GalCer can enhance class-switched, humoral memory responses against co-administered proteins, peptides, and parasites [17, 19, 34–37].

An exciting and novel approach to efficiently deliver α GalCer is in the context of a nanoparticle. Nanoparticles are increasingly well studied as a vaccine platform [38] and can be biodegradable, safe for humans, and small enough for delivery through even the most restrictive administrative routes. AlphaGalCer can be incorporated in many different forms of small particles including liposomes, exosomes, or solid polymers, that efficiently activate iNKT cells both *in vitro* and *in vivo* [18, 39–41]. Particulate delivery of α GalCer has already been shown to enhance the production of specific antibodies to co-administered protein antigens [1, 18, 41, 42]. An elegant study by Barral et al. demonstrated efficient, specific delivery of α GalCer and target protein to antigen-specific B cells when administered via liposomal particles [1]. The investigators used a sophisticated allotype disparate BM chimera system to demonstrate that the liposomes enhanced CD1d-dependent proliferation of only antigen-specific B cells, and induced a systemic class-switched antibody response *in vivo*. Furthermore, Bershteyn and colleagues reported an increase in antigen-specific IgG in the serum of mice immunized with protein and α GalCer complexed within a poly(lactide-coglycolide) nanoparticle vs. a comparable soluble dose [42]. The authors found the nanoparticle immunization approach to be dose-sparing, where at least 100-fold lower amounts of antigen and α GalCer than usual achieved robust antibody titers. Using a related approach, Gehrmann et al. developed a system of DC-derived α GalCer-loaded CD1d-bearing exosomes that also present MHC-loaded peptides [41], activate iNKT cells, promote CD4 T helper responses, and drive peptide-specific antibody production. Finally, an exciting study by Bai et al. employed a targeted nanoparticle immunization system where polysaccharide (PS) antigen from *Streptococcus pneumoniae* was incorporated into α GalCer-containing liposomes [18]. Immunization of mice with these targeted particles resulted in long-lasting, high-affinity, class-switched, PS-specific antibody titers. It remains to be seen whether liposome-elicited PS-specific response was protective against lethal *S. pneumoniae* infection, but this line of work does provide tantalizing suggestions that nanoparticle delivery of α GalCer and B cell antigens recruits non-cognate adaptive help which produces long term class-switched humoral memory and could be the basis for an exciting new vaccine platform.

Conclusion

It is fitting that innate iNKT cell help would drive a different B cell outcome than adaptive T cell help, given that they are likely to be recruited at two different stages of a response. Innate iNKT cell help will be called upon during the earliest hours of infection, and because of the broad potential for non-antigen restricted activation of iNKT cells, will be a

promiscuous response to a myriad of different pathogens. iNKT cells rapidly release large amounts of cytokines and equally quickly curtail their response. This intense but short-lived state of activation supports a B cell response which depends on T cells but looks more similar to a T-independent response in the long term. As a result, glycolipids which recruit innate iNKT cell help can be categorized as a new subset of T-dependent antigen, of the T-dependent Type II class [14]. At the same time, iNKT cells are an important source of early antigen recognition in the immune system, and can also enhance protein-specific B cell responses to recruit adaptive help. Adaptive iNKT cell help is evident later in an immune response after CD4⁺ T cells have consented to a more thorough immune response and provides a more prolonged outcome, nearly identical to T-D type I responses.

Early localization studies suggested that iNKT cells arrest when engaged by a relevant antigen presenting cell in the liver and spleen. Subsequent investigators showed a preference for localization of glycolipid activated iNKT cells to in the marginal zone of the spleen. This has now been confirmed using lineage specific tetramer labeling in situ in the spleen. Work remains to be done to identify the full trafficking pattern of iNKT cells, but a few days after activation, iNKT cells can be found in germinal centers, where they are likely providing help to B cells in the capacity of an iNKT_{FH} cell. Human tonsillar iNKT cells with an iNKT_{FH} phenotype have been described [9], but their localization and specific role in facilitating B cell responses have not yet been demonstrated.

iNKT cells are a logical target for nanoparticle vaccine formulations which deliver glycolipid adjuvants very efficiently to relevant antigen presenting cells. Numerous approaches have demonstrated efficient nanoparticle mediated activation of iNKT cells and some have shown the enhancement of long term humoral memory responses. Harnessing iNKT cells to provide help for humoral memory is an excellent translational application of the combined efforts of many investigators. It remains to be seen if this approach can protect against live infection, but this is certainly the subject of promising future studies.

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***Highlights**

1. iNKT cells provide innate, cognate help for T-dependent type II antigens.
2. iNKT cells provide adaptive, noncognate help for T-D type 1 and T-I antigens.
3. Cognate antigen drives splenic iNKT cells to arrest in the MZ then help GC B cells.
4. Nanoparticles containing glycolipids are highly efficient activators of iNKT cells.
5. Glycolipid and antigen containing nanoparticles are promising vaccine candidates.

Innate (Cognate) Help

Adaptive (Noncognate) Help

Figure 1. iNKT cells provide innate, cognate help as well as adaptive, noncognate help for antigen-specific B cells

Innate iNKT_{FH} cells recognize glycolipid-loaded CD1d on B cells and directly provide helper signals including CD40L, CD28, IL-21, and IFN γ which initiate plasmablast (PB) expansion, early germinal center B cell (GC B) development, modest affinity maturation, and primary class-switched antibody production. This direct form of help constitutes innate help and is specific for T-dependent type II antigens. Alternatively, iNKT_{FH} cells can recruit non-cognate or adaptive help by engaging DCs presenting glycolipids in the context of CD1d and licensing the DC to enhance MHC class II antigen presenting capabilities and upregulate costimulatory molecules such as CD40 to engage and activate CD4⁺ T cells. The peptide-specific CD4⁺ T cells then go on to provide help for protein-specific B cells which initiates development of plasmablasts, mature germinal centers, robust affinity maturation, primary class-switched antibody production by plasma cells (PC) and ultimately memory cells (B_{mem}). CD4⁺ T cells also benefit from iNKT cell- secreted IL-2, IL-4, BAFF, and APRIL. This adaptive form of iNKT cell help is capable of generating humoral memory and is elicited in response to T-dependent type I and T-independent antigens paired with a glycolipid adjuvant.

Figure 2. iNKT cell localization varies during the course of an immune response

Endogenous, resting iNKT cells are localized widely throughout the parenchyma of the spleen. At points of peak effector cytokine secretion induced by cognate glycolipid (4 hours) or systemic infection with *Streptococcus pneumoniae* (8 hours) the iNKT cells consolidate in the marginal zone (MZ). Alternatively, iNKT cells become activated to secrete effector cytokine in place without relocating 2 hours after stimulation by systemic IL-12 and IL-18. Finally, 3 days after activation with cognate glycolipid iNKT cells can be found in germinal centers (GCs). (Periaarteriolar lymphoid sheath, PALS)