Antibodies **2015**, *4*, 279-294; doi:10.3390/antib4040279

OPEN ACCESS

antibodies

ISSN 2073-4468

www.mdpi.com/journal/antibodies

Review

B Cell Help by CD1d-Rectricted NKT Cells

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Academic Editor: Alexander Dent

Received: 25 August 2015 / Accepted: 24 September 2015 / Published: 9 October 2015

Abstract: B cell activation and antibody production against foreign antigens is a central step of host defense. This is achieved via highly regulated multi-phase processes that involve a variety of cells of both innate and adaptive arms of the immune system. MHC class II-restricted $CD4^+$ T cells specific for peptide antigens, which acquire professional follicular B cell helper functions, have been long recognized as key players in this process. Recent data, however, challenge this paradigm by showing the existence of other helper cell types. CD1d restricted NKT cells specific for lipid antigens are one such new player and can coopt bona fide follicular helper phenotypes. Their role in helping antigen-specific B cell response to protein antigens, as well as to the so called "help-less" antigens that cannot be recognized by T follicular helper cells, is being increasingly elucidated, highlighting their potential pathophysiological impact on the immune response, as well as on the design of improved vaccine formulations.

Keywords: B cell response; NKT cells; T cell help; follicular helper cells; CD1; lipids

1. Introduction

$CD4^+$ T cells that are specific for peptides presented by MHC class II (MHC I molecules have long been known to provide specialized help for B cell responses that involve protein antigens [1,2]. Recently, however, it became clear that CD1d-restricted natural killer T (NKT) cells, a subset of lipid-specific T lymphocytes that display innate-effector functions, can also help B cell responses [3]. Both $CD4^+$ T and

NKT cells coopt the follicular helper pathway that is critical to determining the T cell contribution; however, this results in distinct outcomes for antibody responses and implies complementary functions relevant both for host protection and vaccine design.

2. T Dependent Antibody Responses

T dependent (TD) responses are elicited by protein antigens recognized by B cells in the presence of a specialized subset of $CD4^+$ T cells, called T follicular helper (T_{FH}) cells [2]. TD responses are adaptive, because Ab titers develop slowly and lead, through the formation of Germinal Centers (GCs), to the generation of both effector plasma cells, which produce the protective Abs, and long lasting memory B cells, which maintain life-long protection and give rise to rapid Ab boosts upon Ag recall [4]. GCs are dynamic anatomical structures of the secondary lymphoid organs that derive from follicles and contain GC B cells, GC T_{FH} cells, follicular dendritic cells, macrophages and stroma [5,6]. In GCs, B cells undergo clonal expansion, somatic hypermutation and affinity maturation during immune responses [5,6]. GCs reach their maximal size within two weeks from the initiation of B cell responses, then slowly involute and within several weeks they disappear [5,6]. The initiation of the GCs reaction relies on the interaction between antigen-activated B cells and T_{FH} cells [5–7]. T_{FH} cells are characterized by the high and persistent expression of B cell lymphoma 6 (Bcl-6) transcription factor, Inducible T-cell COS stimulator (ICOS), CXC-chemokine receptor 5 (CXCR5), CXCR4, CD40 ligand (CD40L), interleukine-21 (IL-21), cytoplasmic adaptor protein SLAM-associated protein (SAP) and programmed cell death protein 1 (PD-1) [8–10]. Most human and mouse T_{FH} cell markers directly correlate [11,12]. Canonical T_{FH} cell differentiation is a multi-stage, multi-signal process initially driven by the naïve $CD4^+$ T cell recognition of DCs displaying cognate peptide-MHC complexes in the T cell zone [8,11]. This induces the upregulation of Bcl-6 and CXCR5 and the downregulation of CCR7 by the activated $CD4^+$ T cells, thereby allowing their relocation to the B cell follicle border and their subsequent interaction with cognate Ag-specific B cells [8]. This second stage is critical for the generation of the so called "GC T_{FH}" cells, which expresses higher levels of Bcl6 and CXCR5 than T_{FH} cells and migrate inside the GCs, completing the helper functions for the responding B cells [11]. GC T_{FH} secrete CXCL13, IL-21 and IL-4 that are critical helper molecules to sustain B cell activation [11].

The coordinated interplay between numerous transcription factors, costimulatory molecules and cytokines is required for T_{FH} cells development. Among them, Bcl-6 has a prominent role, as its expression is critically required for T_{FH} differentiation [13–15]. Indeed, T_{FH} cells generation does not occur in Bcl-6-deficient mice [13–15]. Bcl-6 promotes PD-1, ICOS, CXCR4, CXCR5 upregulation and CCR7 downregulation by functioning as a transcriptional repressor that antagonizes Blimp-1 or the master transcription factors (Tbet, GATA3, Rorγt) responsible for T_H1, T_H2 and T_H17 effector differentiation [13–15], therefore enforcing the T_{FH} pathway. CD80/CD86/CD28 and ICOS/ICOSL interactions are required for T_{FH} differentiation at the time of DC priming [16,17]. ICOS/ICOSL interactions are also important for T_{FH} migration towards B cells [18]. Missense mutation in the Roquin gene, a negative regulator of ICOS, results in spontaneous autoantibody production, lupus-like autoimmunity and increased number of T_{FH} cells [19], underscoring the central role played by this receptor in the biology of TD responses. PD-1, an inhibitory receptor expressed on the surface of activated T cells, might provide inhibitory signal for GC T_{FH} upon binding PD-L-1, PD-L-2 and CD80

ligands expressed by GC B cells, preventing massive T cells proliferation in GCs [20]. PD-1 and PD-1-L deficient mice show an increased frequency of T_{FH} cells [21,22], although endowed with a reduced capacity to secrete IL-4 or IL-21, resulting in worsened B cells responses [21]. The SAP protein is essential for GC formation [23] and plays a critical role in the stabilization of the GC T_{FH}-GC B cell interactions by competing out the binding of the inhibitory tyrosine phosphatase SHP-1 to the adhesion molecule SLAMF6, thus preventing interruption of intercellular adhesions [24]. The cytokines IL-2, IL-6, IL-21, IL-12 and IL-27 are also implicated in the T_{FH} cell differentiation [11,25]. IL-2 inhibits T_{FH} cell differentiation [26,27]. IL-6 and IL-21 are required for mouse T_{FH} cell differentiation *in vitro* by upregulating Bcl6, while their single or combined loss *in vivo* has led to contrasting results regarding their actual requirement for the generation T_{FH} cells [11,28]. In the human system, IL-12 produced by DCs promotes T_{FH} cell differentiation *in vitro* [29]. IL-27 promotes T_{FH} development indirectly, via the induction of IL-21 production [30].

3. T Independent Antibody Responses

T independent (TI) antibody responses are elicited by non-protein antigens recognized by innate-like B cells [31]. These responses can be subdivided into TI type 1 and TI type 2. TI type 1 responses are elicited by microbial ligands via Toll like receptors (TLRs), whereas TI type 2 responses occur when multivalent antigens, such as sugar or chemical haptens, strongly engage B cell antigen receptor (BCR) [31]. TI responses induce a robust primary Abs production that develops faster than the one elicited by TD responses. They are characterized by the formation of extrafollicular foci of plasma cells, abortive GCs, low levels of somatic hypermutation and limited isotype switching [31]. TI responses account for host's protection in the early phases of infections and fail to induce typical memory responses [32]. Recent findings suggest that TI response are not completely helper-less, but receive forms of innate-like help [33–35]. Splenic B helper neutrophils, defined as N_{BH} cells [34], and RORγt^+ ILCs [35] cooperate in delivering direct and indirect antibody-inducing signals, including BAFF, APRIL, CD40L, IL-21 and Notch ligand Delta-like 1 to MZ B cells, resulting into antibody class switching, plasma cells differentiation and secretion of IgM, IgG and IgA. N_{BH} cell help to MZ B cells not only may occur in the absence of $CD4^+$ T cells, but can also suppress $CD4^+$ T cells activation, at least *in vitro*, suggesting that innate-like help might skew antibody responses to MZ-based TI pathway at the expense of the follicle-based TD one [34].

4. CD1d-Restricted Natural Killer T Cells

Natural killer (iNKT) cells are T lymphocytes that co-express the TCR together with NK1.1 ($NKPR1_{a-c}$, CD161), a typical receptor of mouse NK cells [36–38]. NKT lymphocytes are restricted for MHC or for CD1d, a non-MHC encoded, non-polymorphic antigen presenting molecules that exhibit MHC-class I-like structures [36,39]. CD1d-restricted NKT cells are traditionally divided into two distinct populations: type I and type II [36,39]. Both cell types display constitutive effector/memory phenotype without prior antigen stimulation and are therefore considered innate-like T lymphocytes, as opposed to the adaptive-like MHC-restricted T cells that follow a naïve-effector/memory differentiation pathway that depends on antigen-exposure in the periphery [36,40].

Type I NKT cells express the same TCR V α region 14 invariably rearranged to J α- region 18 (Vα14-Jα18; TRAV11-TRAJ18) in mice, or the highly conserved Vα24-Jα18 (TRAV10-TRAJ18) chain in humans [41,42]. For this reason, they are called invariant (i) NKT cells. The invariant Vα chain pairs with a limited set of variable TCR Vβ chains, utilizing Vβ8, Vβ7 and Vβ2 in mice and Vβ11 in humans [41,42]. All iNKT cells recognize the prototypical glycolipid antigen α-galactosylceramide (αGalCer) [43], together with a range of bacterial and fungal glycosphingo and glycoglycero lipids with similar α-anomeric sugar configuration [38]. In addition, iNKT cells are self-reactive and recognize cell-endogenous phospholipids or α-linked glycosylceramides induced upon stress [44,45]. Owed to the development of iTCR specific mAbs [42,46] and to CD1d-αGalCer tetramers [47,48], iNKT cells can be traced unequivocally in mice and humans rendering them the best characterized NKT cell subset. Upon activation, iNKT cells upregulate several co-stimulatory molecules and produce a wide range of cytokines and chemokines, which have a strong impact on immune cells, such as dendritic cells (DC), monocytes/macrophages, granulocytes, natural killer (NK) cells, T and B cells [38]. As a result of this, iNKT cells have been involved in the control of cancer, infections and autoimmunity [36,38].

Type II NKT cells are also present in mice and humans and express a range of more variable TCRs than iNKT cells, although they are enriched for TCRs that utilize Vα3.2-Jα9 and Vα8 [49,50]. They do not recognize αGalCer but are specific for distinct glycolipid antigens, such as several endogenous mammalian lipids (sulfatide, phosphatidylglycerol, lysopholipids), M tuberculosis-derived and Listeria-derived phosphatidylglycerols and small non-lipidic aromatic molecules [50]. Type II NKT cells are difficult to detect by CD1d-tetramers and have been mainly characterized in mice selectively lacking iNKT cells ($J\alpha18^{-/-}$), or in humans by excluding $V\alpha24^{+}$iNKT cells [50]. In mice, they have the ability to modulate immune responses, including the suppression of autoimmunity, the control of viral, bacterial or parasite infections and the induction or inhibition of tumor rejection, depending on the model [50].

Several studies have shown that iNKT cells provide help to B cells, either directly or indirectly, with different outcomes [3]. Therefore, iNKT cells are considered as a bridge between innate and adaptive immune responses. Furthermore, recent data suggest also a helper role for type 2 NKT cells. This review will address the helper role of type 1 and 2 NKT cells for antibody responses.

5. Strategic iNKT Cell Localization for B Cell Help

iNKT cells can be localized with some precision in secondary lymphoid organs, unlike type 2 iNKT cells. At the steady state, iNKT cells are located in the MZ of the spleen, a position strategic for interacting with APCs that capture blood-borne Ags flowing through the red pulp, with a scattered distribution in the splenic white and red pulp [51–53]. Once activated after systemic αGalCer administration, iNKT cells accumulate rapidly at the MZ and bridging channels of the spleen, where they upregulate costimulatory molecules and produce cytokines [52,53]. This results in the maturation of DCs that upregulate CCR7 and relocate into the T-cell zone of the white pulp [53]. Licensed DCs can then enhance the induction of T_{FH} cells specific for any concomitant protein Ag, which provide help to cognate B cells [54]. Following activation, a fraction of iNKT cells acquire follicular helper phenotype and functions, and can be detected inside GCs, suggesting an influx from the MZ [55]. iNKT cells are also detected in lymphnodes, where they localize in the interfollicular regions and in the medulla, which are also areas of intense interactions with APCs, B and T cells [56,57].

6. Cognate iNKT Cell Help for B Cells

Lipid Ags' presentation by CD1d molecules expressed on B cells prompts cognate interactions with iNKT cells [58–61]. Human iNKT cells provide CD1d-dependent cognate help to both naive and memory B cells *in vitro*, inducing both B cell expansion and Ab secretion even without addition of αGalCer, suggesting the recognition of endogenous agonist lipid antigens [58]. This requires the expression of IL-4 and IL-13 by iNKT cells, while CD40L/CD40 engagement is partially involved *in vitro* [58]. Cognate iNKT cell help to B cells eliciting antigen-specific Ab response has been formally demonstrated in mice using either artificial conjugates of B cell protein antigens and αGalCer on the same nanoparticles [61], or chemical haptens directly conjugated with αGalCer as a carrier [60] (Figure 1). The physical linkage between Ags and αGalCer is of crucial importance for promoting efficient contact between iNKT and B cells [61]. The BCRs specific for the protein Ag or the hapten component of the complex internalize also the αGalCer carrier, which is then presented to iNKT cells in the context of the CD1d molecule. In this way, any Ag- or hapten-specific B cell has the highest chance to receive the help from iNKT cells, resulting in an efficient activation. To obtain a productive iNKT-B cell cognate interaction, the expression of CD1d by B cells is critical. The immunization of mice bearing $CD1d^{-/-}$ B lymphocytes with Ag-αGalCer conjugates does not result in Abs responses [60,61]. Furthermore, provision of CD80/CD86 dependent co-stimulation by B cells to iNKT cells, CD40 triggering on B cells by CD40L expressed by iNKT cells, and production of IFNγ are also required, otherwise the Ab response is reduced or abrogated [60]. Remarkably, $CD4^+$ T cells are not required, underscoring the autonomous helper function of iNKT cells and their potential to provide help for "helper-less" Ags [61].

iNKT cells that directly help B cells adopt a phenotype similar to the T_{FH} cells and are therefore defined as iNKT follicular helper ($iNKT_{FH}$) cells [55,62]. Upon immunization of mice with αGalCer-containing antigens, iNKT cells interact with CD1d-expressing DCs and upregulate PD-1, ICOS, CXCR5, Bcl6, CD40L [55,62]. These $iNKT_{FH}$ cells are not simply recently activated effector cells and persist in secondary lymphoid organs for three to six days [55,62]. $iNKT_{FH}$ cells secrete diverse helper cytokines, such as IL-4, IL-21 and IFN-γ, and localize at the T:B border and within GCs [55,63]. Moreover, primary $iNKT_{FH}$ cells expressing high levels of CXCR5 have been detected in human tonsils [55], implying that they are spontaneously induced by environmental Ags. The differentiation of $iNKT_{FH}$ and T_{FH} cells share similar molecular requirements, as $iNKT_{FH}$ cells' formation and cognate help to B cells are abrogated in the absence of Bcl-6 or SAP expression, respectively [55,64]. Consistent with the requirement of CD28 mediated co-stimulation for T_{FH} cells formation, also αGalCer immunization of $Cd28^{-/-}$ mice does not result in $iNKT_{FH}$ cell differentiation [55]. The sustained cognate interaction between B cells and primed T cells is critical for the maturation of T_{FH} cells because, in the absence of B cells, they are not maintained unless Ag presentation is enhanced [8]. Again, in line with T_{FH} cells, $iNKT_{FH}$ cell differentiation is impaired in B cell deficient mice [55]. Moreover, B cells must express CD1d and CD40 in order to present lipids and solicit cognate help by $iNKT_{FH}$ cells [55,62]. IL-21 is dispensable for formation of $iNKT_{FH}$ cells upon αGalCer immunization *in vivo*, although it remains essential for their cognate helper function [55,60]. Interestingly, however, $iNKT_{FH}$ cells expansion is neither enhanced or suppressed by exogenous IL-2 administration [65] that, by contrast, impairs T_{FH} differentiation, suggesting some difference in the molecular cues regulating the follicular helper differentiation between the two cell subsets.

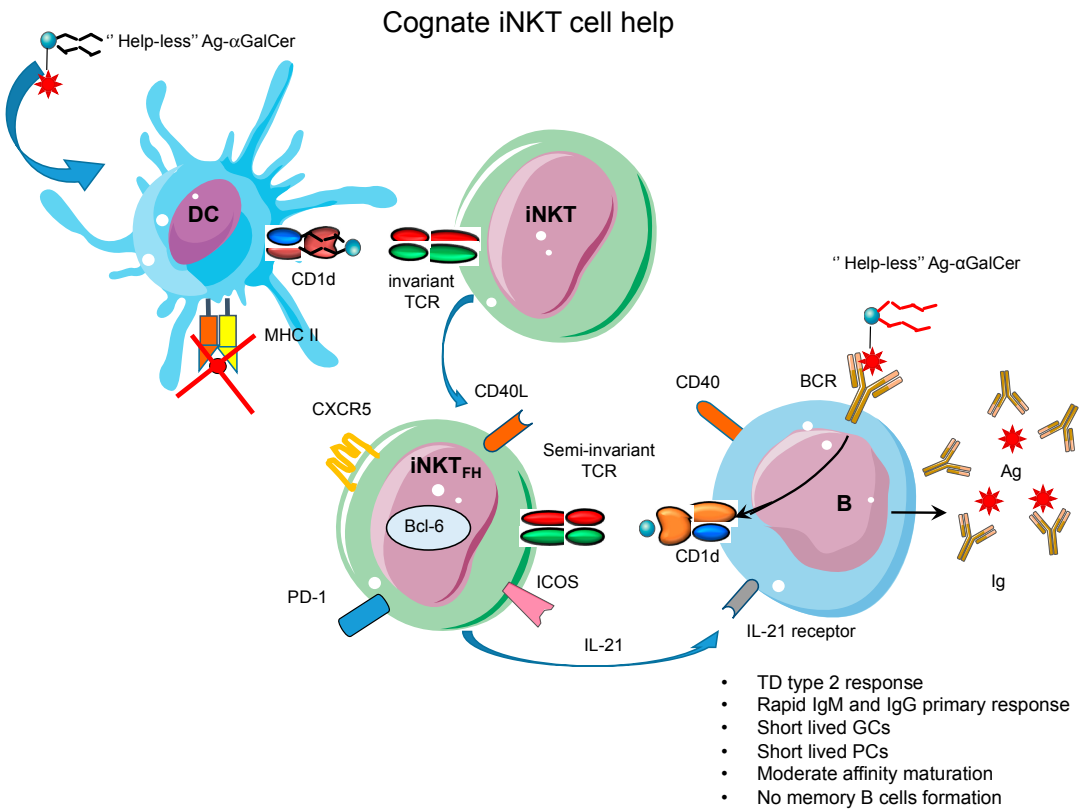

Figure 1. Cognate iNKT cell help for B-cell responses. iNKT cells provide direct cognate help to B cells in case of encounter with help-less antigens, which are constituted by non-protein haptens (sugars, lipids, chemicals) that are either chemically linked to αGalCer, or conjugate with it on the same nanoparticles. These structures cannot generate peptides that can be recognized by MHC II-restricted $CD4^+$ T_{FH} cells. Help-less Ags-αGalCer conjugates are non-selectively uptaken by $CD8\alpha^+$ DCs, which activate iNKT cells by presenting αGalCer on CD1d. These iNKT cells co-opt the follicular helper pathway ($iNKT_{FH}$) and migrate to the B zone, where they provide help to B cells that have internalized Ag-αGalCer complexes via their specific BCRs and present αGalCer on CD1d molecules. This interaction triggers the provision of CD1d-, CD40L- and IL-21-dependent help by $iNKT_{FH}$ cells, which leads to the so called TD type 2 B-cell response.

Unlike the mouse iNKT cell subsets that display bona-fide T_H1, T_H2 and T_H17 effector phenotypes, which are developmentally acquired in the thymus, $iNKT_{FH}$ cells are not a pre-formed thymic subset and result from the combined proliferation and differentiation in the periphery of apparently uncommitted precursors [65].

The direct iNKT cell-dependent help to B cells impacts also on the persistence of the Ab titers and PC survival. This function entails the provision of BAFF e APRIL by iNKT cells to Ag-activated plasmablasts [66], implying a likely role for iNKT cells in directly sustaining the effector B cells' response. Upon mice immunization with αGalCer, $iNKT_{FH}$ cells expand more in $CD4^+$ T cell-deficient than in WT mice [62], suggesting that in physiological conditions, the presence of T_{FH} cells might somewhat compete with $iNKT_{FH}$ cells.

7. Distinct Dynamics of B Cell Response Helped by iNKT$_{FH}$ Cells

Although iNKT$_{FH}$ cells show molecular and phenotypical features apparently indistinguishable from T$_{FH}$ cells and are able to promote GCs formation and primary Abs response, they are however unable to maintain persistent GCs and to induce affinity maturation and long lasting secondary Abs responses [55,62,63]. Extrafollicular plasmablasts foci and GCs form as early as three days upon immunization of mice with Ag-αGalCer conjugates and lead to a rapid raise in Ag-specific IgM and IgG, faster than that induced by protein Ag immunization [55,63]. GCs remain small throughout the response, while the differentiation of long-lived plasma cells and memory B cells is negligible [55,62,63]. Somatic mutations and affinity maturation of the NP-specific Igs induced by iNKT$_{FH}$ cell help can be detected, although they remain markedly reduced compared to those induced by T$_{FH}$ help upon NP-OVA immunization [55,63]. Because of these characteristics, King *et al.* [63] gave the humoral response ensuing cognate iNKT$_{FH}$-B cell interactions the new definition of "TD type II response", to distinguish it from the conventional TD responses that recruit peptide-specific T$_{FH}$ cells and result in substantial GC formation, affinity maturation and memory response. These results have suggested that iNKT$_{FH}$ cells may be less efficient than the T$_{FH}$ ones in promoting GCs and B cell memory formation. Recent studies, however, challenge this idea. In one case, mice were immunized with liposomal nanoparticles displaying synthetic lipid and capsular polysaccharide antigens from *Streptococcus pneumonia* [67]. Strong and prolonged antibody responses with isotype switch, affinity maturation, and long-lasting B-cell memory were observed, even though iNKT$_{FH}$ differentiation was modest or absent. Conditional ablation of CD1d revealed the requirement for a first iNKT cell/DC cognate interaction to activate iNKT cells, followed by subsequent iNKT/B cell interaction to induce isotype switch and memory [67]. In a second study [68], mice were immunized with a vaccine based on *S. pneumoniae* capsule polysaccharides directly conjugated to αGalCer, generating a typical hapten-carrier system. Also, this lipid-carbohydrate conjugate vaccine elicited germinal center formation, high-affinity IgG antibodies specific for pneumococcal polysaccharides and the differentiation of long-lived specific memory B cells. This direct chemical conjugation was able to induce iNKT$_{FH}$ cells [68].

It is unclear why, in some cases, TD type II responses result in short lived primary antibody response, whereas in others it can give rise to long lived protection and memory. One proposed possibility relies on the strength of BCR cross-linking provided by the nominal antigen that is physically associated with αGalCer. Different BCR crosslinking would result in sub-optimal *vs.* optimal B cell activation and, in turn, in sustained iNKT cell help, Ab titer and memory cell differentiation [4,8]. Alternatively, different Ag doses employed in the immunization could play a role, and only a direct comparison of the various immunogens in a side-by-side experiment could address this issue. It is also unclear why not all conjugate vaccine formulations can induce iNKT$_{FH}$ cells. The detection of iNKT$_{FH}$ cells by flow cytometry can be tricky due to their low frequency and they can be easily missed or underestimated. Specific optimized flow cytometry protocols may improve the detection of these cells [69].

Nevertheless, these types of conjugated vaccines that target iNKT cell help show remarkable efficacy in animal models. The vaccine formulation utilizing *S. pneumoniae* serotype 14 polysaccharide and αGalCer elicits superior responses compared to that given by clinically used vaccine (Prevnar) [70] and induces potent antipolysaccharide immunity that protected mice against pneumococcal disease [68].

iNKT help to B cells, hence, represents a promising target for B-cell vaccines directed against "T helper-less antigens"; that is to say antigens that are not MHC-restricted and cannot elicit conventional T_{FH} cells.

8. Non-Cognate iNKT Cell Help for B Cells

A number of experiments in mice also show that immunization of protein Ags simply admixed with αGalCer results is robust, long lasting and protective serological responses [71] (Figure 2). Surprisingly, with this specific Ag formulation, cognate iNKT-B cell interactions are not required for the antibody response [54], as also confirmed with airborne Ag in allergic airway inflammation model [72]. iNKT cells, instead, license antigen presenting cells (APCs) that co-expressed both CD1d and MHC II that, in turn, activate $CD4^+$ T helper cells specific for the protein Ags co-injected with αGalCer, therefore, optimizing their helper functions for B cells specific for the same Ag [54]. This non-cognate iNKT cell help requires CD40 expression on B cells [54], likely to receive CD40L stimulation by the conventional T_{FH} cells, whereas IL-4 and IFN-γ expression are necessary only to direct Ig class switch, but not for the development of the overall antibody response [54]. Non-cognate iNKT cell help elicits B cell responses also in splenectomized mice, ruling out the need for innate-like B cells that primarily reside in the spleen [54]. Interestingly, immunization of mice with protein antigens admixed with αGalCer elicits antibody responses also in the absence of $CD4^+$ T cells [62]. In this case, however, a cognate form of iNKT cell help is induced with the appearance of $iNKT_{FH}$ cells, which results in a typical short-lived T D type II response [62].

All the data mentioned above were obtained by immunizing mice with mixtures of protein Ags and αGalCer, hence eliciting classic T D type I responses. However, administration of αGalCer was also shown to enhance the production of antibodies reactive with T I antigen, such as NP-Ficoll [73], suggesting the possibility that the activation of iNKT cell effector functions may impinge on the innate helper mechanism that sustain innate-like B cell responses.

Thus, the αGalCer mediated help recapitulates the effect of classical adjuvants that stimulate the innate system in order to support the adaptive immune responses.

9. Spontaneous iNKT Cell Help to B Cells

iNKT cell help to B cells may occur also in a spontaneous manner, that is to say in the absence of αGalCer, as it was demonstrated by investigating the role of iNKT cells in HSV-1-induced B cell responses [74]. The amount of virus-specific IgM and IgG is significantly reduced in iNKT cell-deficient mice compared to their wild-type counterparts [74]. Furthermore, iNKT cells are relevant in shaping the IgG subtype profile of HSV-1-specific Abs, as IgG2c and IgG3 Abs are barely detectable in $J\alpha18^{-/-}$ mice compared to WT animals [74]. This deficit results from insufficient IFNγ production that promotes the switch to IgG2c and IgG3, suggesting that iNKT cells are required for HSV-1-induced IFNγ production [74].

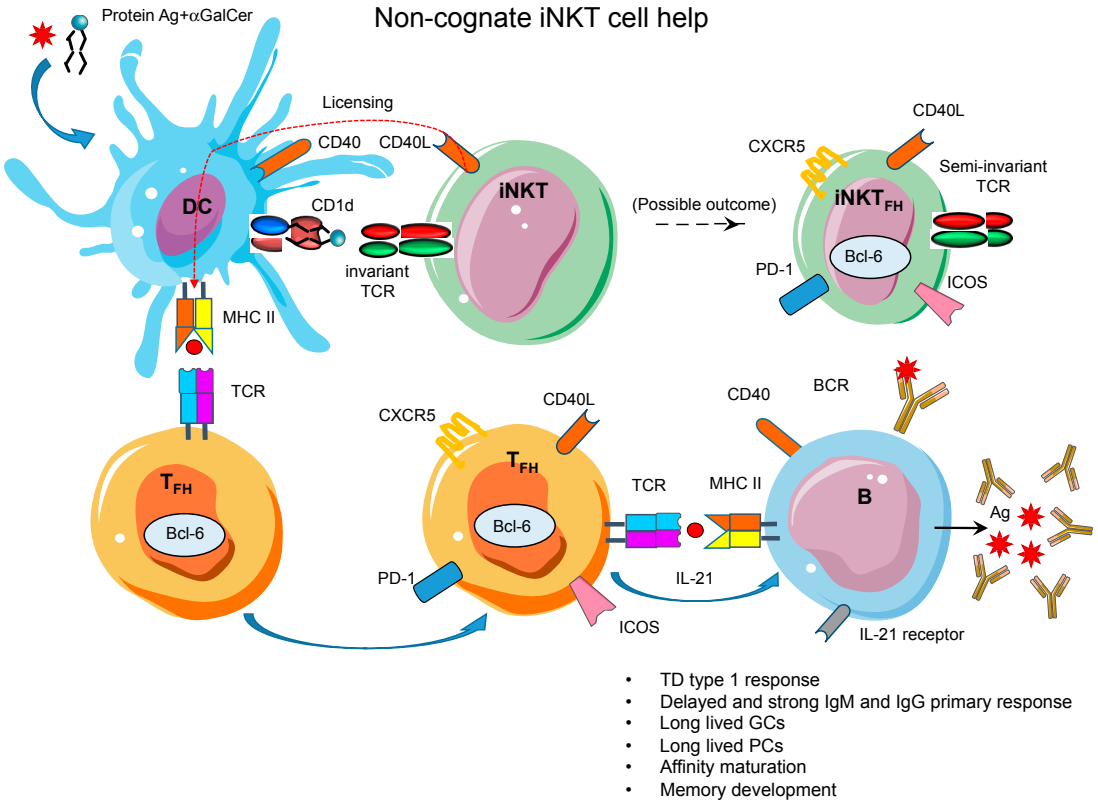

Figure 2. Non-cognate iNKT cell help for B-cell responses. iNKT cells provide indirect, non-cognate help to B cells in case of immune responses elicited by protein Ags admixed with αGalCer. In this case, proteins generate peptides that can be recognized by MHC II-restricted $CD4^+$ T_{FH} cells. Protein Ags and αGalCer mixtures get in contact with $CD8\alpha^+$ DCs, which internalize both and present the lipid and the protein-derived peptides into CD1d and MHC II, respectively. Recognition of αGalCer-CD1d complexes activates iNKT cells, which activate and license antigen-presenting functions in DC via CD40L-CD40 interaction. In turn, licensed DCs efficiently prime $CD4^+$ T helper cells that are specific for the co-administered protein antigen, which acquire T_{FH}-cell functions, migrate to the border of the T–B zone and provide efficient help to cognate B cells. This occurs independently of CD1d expression on B cells. T_{FH} cell–B cell interactions result in a typical TD response. Following activation by DCs, iNKT cells acquire a follicular help phenotype also in the case of immunization with protein Ags mixed with αGalCer. This, however, does not apparently impact the ensuing immune response, as indirect iNKT cell help elicits similar antibody titers irrespective of the expression of CD1d by B cells.

Another example of spontaneous iNKT cell help to B cells, which has relevant pathophysiological implications, occurs in a mouse model of primary biliary cirrhosis elicited by the infection of mice with the hepatotropic spingomonas *N. aromaticivorans* [75]. The bacterium contains the CD1d-restricted glycosphingolipid analog α-glycuronilceramide and the B-cell antigen pyruvate dehydrogenases complex E2 (PDC-E2) enzyme. iNKT cells, upon infection with the bacterium, can directly help CD1d-expressing B cells bearing BCRs specific for PDC-E2, implying a mechanism whereby the same B cells acquire both enzyme and α-glycuronilceramide contained in the same microbial cell wall

fragment, and present the glycosphingolipid to iNKT cells in a CD1d-dependent manner [75]. As a result of the iNKT cell help, B cells produce high-affinity and class-switched antibodies against bacterial PDC-E2, which cross-react with the homologous mammalian mitochondrial enzyme expressed in small bile ducts, thereby initiating organ damage that resembles human primary biliary cirrhosis [75]. Although iNKT cells are required to initiate the cellular and humoral components of the disease, they are not necessary for its chronic phase, as demonstrated with the adoptive transfer of the disease into naïve mice using pathogenic T cells from sick animals depleted of iNKT cells [75]. Therefore, iNKT cells mainly contribute to the innate phase of the response, by provoking the breakdown of tolerance.

10. Type 2 NKT Cell Help to B Cells

Recent data have also suggested that type II NKT cells help induction of humoral immunity. Type II NKT cells are required for antigen-specific IgG1 and IgG3 production because, in their absence ($CD1d^{-/-}$ mice), immunization with Alum-adsorbed TD antigens results in lower levels of these Ig subclasses than control mice [76]. $CD1d^{-/-}$ have intact development of the B cell compartment, suggesting that the lower antibody titers and plasma cells number in $CD1d^{-/-}$ mice are due to a poor response to Alum rather than a consequence of defects in the B cell compartment. Once primed after Alum immunization, type II iNKT cells transactivate T cells to secrete IL-4. This demonstrates that type II NKT cells are required for full expression of the Alum-stimulated cytokine response [76]. CD1d-blocking mAb significantly reduces cytokine production and IgG1 secretion suggesting that type II NKT cell help depends on CD1d expression by APC [76].

Another possible example of type II NKT cell help to B cells is inferred from the investigation of Gaucher disease (GD), which is an inherited deficiency of the acidic β-glucosidase enzyme that results in lysosomal accumulation of sphingolipids such as β-glucosylceramide (βGL1) and glucosylsphingosine (LGL1). This lipid accumulation leads to chronic inflammation and B-cell activation, often resulting in monoclonal and polyclonal gammopathy [77]. βGL1 and LGL1 loaded CD1d tetramers clearly demonstrate the existence of lipid specific CD1d-restricted NKT cells displaying type II characteristics, in terms of diverse TCR expression, and different genomic and cytokine profile compared to type I NKT cells [78]. Interestingly, the primary type II NKT cells found in GD patients exhibit a constitutive T_{FH} phenotype. Upon injection of βGL1 or LGL1 into WT mice, type II NKT cells can provide help to B cells and lead to lipid-specific antibodies production in a CD1d-dependent manner [78]. In parallel, βGL1- and LGL1-specific type II NKT cell coculture with autologous B cells results in plasmablasts differentiation [78]. Significant increase in type II NKT cells, and reduced iNKT cells, are also detected in GD patients [78] and in mice that recapitulate the phenotype of human GD [79]. Collectively, these results suggest that type II NKT cells might contribute to the B cell activation arising in GD by providing help/activation signals.

11. Concluding Remarks

Exploiting both direct and indirect helper functions of NKT cells for vaccine formulation is very attractive. A theoretical drawback of the indirect approach, *i.e.*, the case of vaccine formulated with protein Ags mixed with αGalCer, could pose the risk of breaking B cell tolerance and triggering autoimmunity, because B cells specific for self-Ags could acquire αGalCer and be helped by activated

iNKT$_{FH}$ cells. However, pre-clinical data obtained in mice suggest that αGalCer administration results in the suppression of autoreactive B cell response [80], while its administration in cancer patients for immunotherapy does not elicit any overt autoimmunity and has been proven to be safe [81]. Indeed, a phase I/II study of vaccination of healthy volunteers with the αGalCer analogue ABX196 mixed with the poorly immunogenic HBs' antigen of the hepatitis B virus is safe and induces the activation of circulating iNKT cells and protective anti-HBs antibody responses in the majority of individuals, demonstrating the efficacy of iNKT cell targeting vaccines in humans [82]. Also, vaccine formulations containing "helper-less" antigens, such as bacterial polysaccharides directly conjugated with glycosphingolipid agonists, hold promise for the induction of a protective response against capsulated bacteria. The more rapid rise in Ab titers elicited by NKT cell-targeting vaccines compared to those induced by conventional ones could also be explored in situations where the infectious pathogen is already present in the population that needs protection. The approach warrants further investigation for full clinical development.

Acknowledgments

The authors acknowledge the support of the Italian Ministry of Health, Associazione Italiana per la Ricerca sul Cancro (AIRC IG-15517 to G.C, IG-15466 to P.D.)

Author Contributions

Livia Clerici, Giulia Casorati and Paolo Dellabona wrote the review.

Conflicts of Interest

The authors declare no conflict of interest.

Abbreviations

TD, T dependent; TI, T Independent; GC, Germinal Centers; Ag, antigen; NKT, Natural Killer T cells; TCR, T cell Receptor; αGalCer, αgalactosyl ceramide; MZ B, marginal zone B cells; DCs, dendritic cells; PC, plasma cells; NP hapten, 4-Hydroxy-3-nitrophenyl acetyl hapten; BCR, B Cell Receptor.

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