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A subset of AID-dependent B-1a cells initiates hypersensitivity and pneumococcal pneumonia resistance

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We propose that there is a special B-1a B cell subset ("sB-1a" cells) that mediates linked processes very early after immunization to initiate cutaneous contact sensitivity (CS), delayed-type hypersensitivity (DTH), and immune resistance to pneumococcal pneumonia. Our published data indicate that in CS and DTH, these initiating processes are required for elicitation of the delayed onset and late-occurring classical T cell–mediated responses. sB-1a cells resemble memory B2 cells, as they are stimulated within 1 h of immunization and depend on T helper cytokines—uniquely IL-4 from hepatic iNKT cells—for activation and rapid migration from the peritoneal cavity to the spleen to secrete IgM antibody (Ab) and Ab-derived free light chains (FLCs) by only 1 day after immunization. Unlike conventional B-1a (cB-1a) cell–produced IgM natural Ab, IgM Ab produced by sB-1a cells has high Ag affinity owing to immunoglobulin V-region mutations induced by activation-induced cytidine deaminase (AID). The dominant cB-1a cells are increased in immunized AID-deficient mice but do not mediate initiation, CS, or pneumonia resistance because natural Ab has relatively low Ag affinity because of unmutated germ-line V regions. In CS and DTH, sB-1a IgM Ag affinity is sufficiently high to mediate complement activation for generation of C5a that, together with vasoactive mediators such as TNF-α released by FLC-sensitized mast cells, activate local endothelium for extravascular recruitment of effector T cells. We conclude by discussing the possibility of functional sB-1 cells in humans.

Keywords: activation-induced deaminase; AID; free antibody light chains; FLC; contact sensitivity; CS; delayed-type hypersensitivity; DTH; pneumococcal pneumonia; B cell; interleukin 4

Introduction

B-1 cells are often called innate B cells because they respond rapidly to immunization with a variety of antigens before acquired immunity. Uniquely among B cells, B-1 cells originate in the spleen and then, for the most part, migrate to the peritoneal cavity (PerC). Then, upon immunization, B-1 cells are stimulated by antigens (Ags) to migrate to the spleen to produce IgM antibodies (Abs).[1] B-1a cells are divided into two populations based on cell surface expression of CD5: $CD5^+$ B-1a and $CD5^-$ B-1b cells.[2] CD5 expression, an activation marker that distinguishes the two subsets, is usually expressed on T cells, not B cells; a further distinction is CD11b expression on PerC, but not splenic, B-1 cells.[3]

We have characterized a special B-1a B cell subset ("sB-1a" cells) that mediates linked processes very early after immunization that initiate cutaneous contact sensitivity (CS),[4] delayed-type hypersensitivity (DTH),[5] and immune resistance to pneumococcal pneumonia.[6] In CS and DTH, these initiation

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processes are elicited early after skin testing in immunized animals *required* for subsequent elicitation of the delayed onset and late-occurring classical T cell–mediated responses. For CS immunization by contact skin painting on the torso, a high dose of low molecular weight reactive hapten (such as that found in clinical contact dermatitis mediated by poison ivy and reactive metals) becomes a T and B cell–activating neoantigen by linking to a carrier self-protein. Subsequent contact skin painting challenge on the ears with a dilute solution of the reactive hapten again conjugates to self-proteins to now elicit a strong local immune inflammatory hypersensitivity response, classically with delayed onset and peaking at 24–48 hours. This is due to local diapedesis of hapten–self-specific $CD4^+$ or $CD8^+$ effector T cells from the circulation for local activation by hapten–self–peptides in a complex with major histocompatibility complex (MHC)–self molecules on the surface of local cutaneous antigen presenting cells (APCs) to produce T_H1 cytokines, such as IFN-γ, that then orchestrate the skin inflammation.[4,7–9]

The sB-1a cell–dependent early elicited processes precede and are required to initiate the classical delayed T cell aspects of CS and DTH, and for early IgM Ab-mediated resistance to pneumococcal pneumonia. The initiation processes consist of induced early *initiation* (Fig. 1A) and late *elicitation* (Fig. 1B) components. Together, they form an increasing cascade of early component Ag-specific steps dependent on sB-1a cell–derived IgM Ab of higher affinity for Ag than conventional B-1a cell (cB-1a)–derived natural IgM Ab (NAb). The higher affinity is due to immunoglobulin (Ig)-variable (V)-region mutations in the sB-1a cells mediated by activation-induced cytidine deaminase (AID).[4,7] Further, production and secretion of this Ab from sB-1a cells requires IL-4 from iNKT cells for activation and development of the sB-1a cells.[8,10,11] Initiation of CS to several different reactive haptens (TNP,[9] DNFB,[12] and oxazolone[10]) and metals (e.g., nickel sulfate[13]) all similarly depend on Ag-specific sB-1a cell–produced IgM Ab.

Surface phenotype and quantitation of sB-1a cells that initiate CS

The surface phenotype of sB-1a cells initially was defined by the depletion of CS-initiating activity with specific monoclonal antibodies (mAbs) plus complement (C′), for example, mAb to $CD5^+$ and $CD90^+$ (Thy-1), both markers usually associated with T cells. Subsequent multicolor flow cytometry analysis of specific hapten–phycoerythrin-binding sB-1a cells appears to confirm that these $CD5^+$ $CD90^+$ cells are a relatively rare activated subset of the B1-a cells in the spleen of immunized mice; these sB-1a cells begin to initiate CS on day 2 following immunization.[6] Further, the sB-1a cells appear to be IgM^{hi} and IgD^{hi} and express the conventional B cell markers CD19, CD20, CD21, CD23, B220, and Mac-1.[7] In CS, specific DNP hapten–Ag-binding immune-activated sB-1a cells are only about 0.05% of total splenocytes, or perhaps as few as 5–7000 spleen cells per immunized mouse.[7] Significantly, intravenous transfer of as few as 1500 hapten–Ag-binding sB-1a cells transfer CS initiation.[7]

In an analogous study of pneumococcal pneumonia in mice, we employed phosphoryl choline (PC), the dominant hapten of pneumococcal polysaccharide. As in the use of the relevant hapten in CS, we conjugated PC to phycoerythrin to form a hapten–fluorescent complex that we used to enrich (by cell sorting) the PC hapten–Ag-binding sB-1a cells. These cells contained the pneumonia-protective sB1a cells as a minor splenocyte subset, that is, about 0.6% of the PC hapten–binding total B-1a cells ($CD19^+$ $CD5^+$). This small number of Ag-binding sB-1a cells were active in pneumonia resistance, similar to the very small numbers that we found in effective CS; perhaps as few as about 6800 splenocytes harvested from individual mice at day 2 of infection were sufficient.[6] Also present among the PC hapten–binding total B-1a cells ($CD19^+$ $CD5^+$) were the major Ag-binding, but inactive cB-1a, splenocytes that do not mediate resistance to pneumonia.

Developmental, molecular, and functional phenotypes of sB-1a cells

As noted, among the total B-1 cells, there are B-1a cells that we hypothesize can be divided into two subsets. First, there are the established and numerically dominant cB-1a cells that have germline DNA sequences in their Ig V regions and that are independent of T helper cell cytokines in their development and function; these cells produce NAb present at immunization with little Ag specificity, but instead with poly-Ag specificity.[14] Second, there is the minor subset of sB-1a cells that we have found

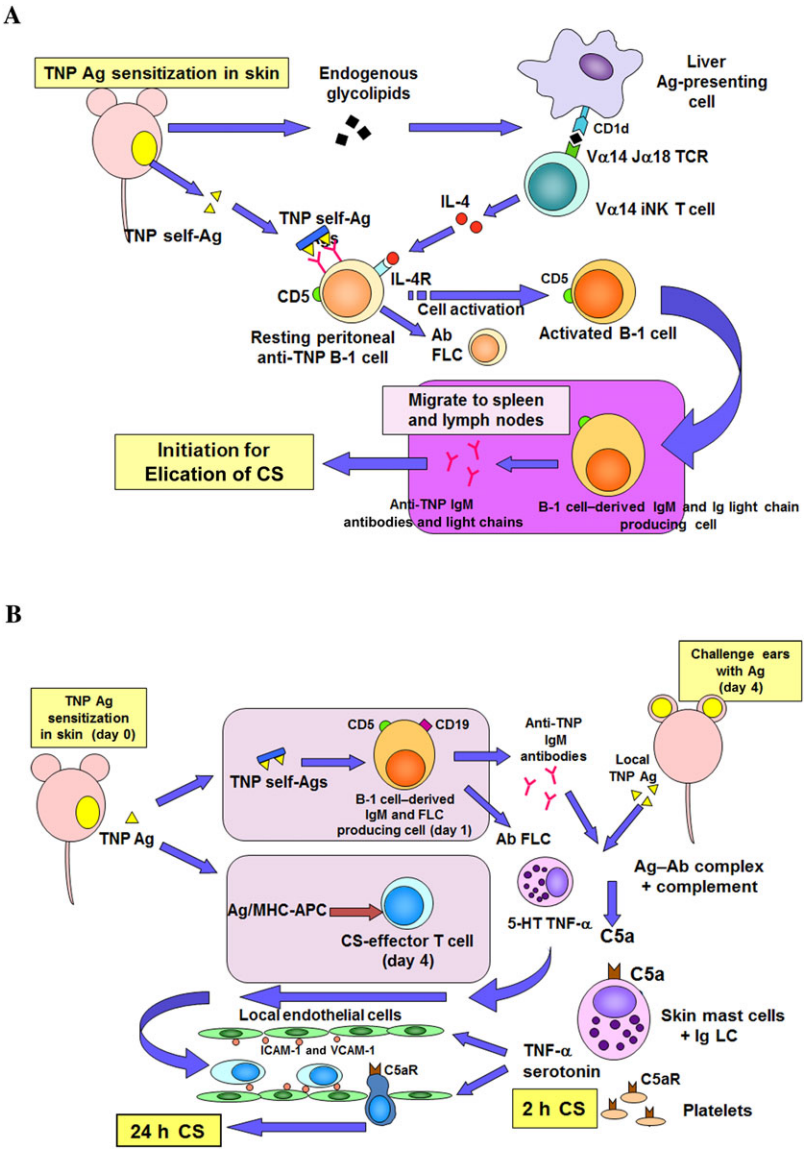

Figure 1. (A) Induction of the initiation of CS that leads to the late elicitation phase of local tissue recruitment of effector T cells. At priming with a high dose of the contact sensitizer (5.0%), there is induction of cutaneous sensitization for CS by skin painting with concentrated contact sensitizer TNP–Cl reactive hapten. The priming Ag then goes in two pathways. In one pathway, the reactive hapten covalently binds to local self-skin proteins. These hapten–Ag–self complexes are systemically released and bind to Ag-specific IgM-like surface receptors on sB-1a cells in the PerC. The sB-1a cells are simultaneously costimulated by IL-4 by liver iNKT cells stimulated by glycolipid antigens, allowing rapid production of anti-TNP IgM Abs and Ag-specific Ab free light chains (FLCs). In the second pathway, the TNP–self complexes are taken up by local skin antigen presenting cells that migrate to draining lymph nodes to activate recirculating CS effector T cells. (B) Elicitation of initiation of CS that leads to the local tissue recruitment of effector T cells. The late elicitation phase of CS is induced by secondary skin challenge, with dilute hapten Ag (0.4%) on the ears and generally on day 4. Compared to immunization with concentrated 5% hapten, the dilute hapten causes little local reactivity in naive nonimmune controls. Here, the challenging TNP–Cl hapten again forms local TNP hapten–Ag–self complexes. These are bound by sB-1a cell–derived IgM of high affinity to enable activation of complement and subsequent generation of the C5a fragment to stimulate local mast cells, platelets, and other cells. The activated circulating Ag-specific T cells bind endothelial adhesion molecules on the local postcapillary venules. Ag-specific FLCs that also are released by stimulated sB-1a cells bind the mast cell surface to sensitize them for Ag-induced release of their vasoactive serotonin and TNF-α. Together, these initiating processes of the late elicitation phase enables development of circulating, recently immunized anti-TNP–self-specific CS effector T cells that pass into local tissues.

to have AID-dependent Ig V-region mutations.[6,7] sB-1a cells and cB-1 cells differ in their molecular phenotypes that results in their having different functional properties in CS, DTH, and pneumonia resistance.[15]

The differing molecular phenotypes include, first, that the sB-1a cells have susceptibility to the action of AID on their Ig V region, resulting in mutations that influence Ag specificity and affinity of the encoded Ab.[6] Second, sB-1a cells have active and essential T_H2-like IL-4,[10,11] IL-5,[16] or IL-13[6] signaling pathways. In CS, sB-1a cells are dependent on IL-4 from iNKT cells, with an IL-4 signaling pathway as a molecular phenotype consisting of IL-4 binding to IL-4 receptors and downstream STAT6 signaling;[11] this is similar to B2 cells that are dependent on T cell–derived helper cytokines provided by $\alpha\beta TCR^+$ conventional T cells. These two differences in molecular phenotype (of requirement for AID and IL-4) seem to be acquired during the phylogenetic development of the sB-1a cells *before* migration from precursors in the neonatal spleen for eventual location in the PerC. B-1a cells at this stage in development are in the PerC when activated by Ag immunization to induce CS, DTH, or by the onset of pneumonia infection in the 5- to 7-week-old young adult mice.[15] It is not known what processes mediate the induction of an active helper cytokine signaling pathway and susceptibility to AID, with resulting AID-dependent Ig V-region mutations. As noted, these abilities appear to be acquired during development and thus are present when mice are immunized. Importantly, they do not appear to be due to the microbiome, as they occur in germ-free animals.[15] However, they could be the result of sB-1a stimulation by endogenous self or altered self-Ag, or by endogenous Toll-like receptor ligands. Overall, these two distinctive subtypes of B-1a cells (sB-1a and cB-1a) appear to have different developmental, molecular, and functional phenotypes.

Resemblance of CS-initiating sB-1a cells to B2 cells

sB-1a cells are reminiscent of B2 memory cells that also express Ab with high Ag affinity owing to AID-dependent somatic hypermutation, primed before the time of secondary immunization, and are dependent on helper T cell cytokines. We demonstrated the high affinity of the Ag-specific sB-1a subset *in vitro* by hapten-binding competition assay[6] and, separately, *in vivo* by their sensitivity to low doses of immunizing Ag (Fig. 2D).[7] Although there seem to be low numbers of V-region mutations in hapten-binding sB-1a cells in CS and pneumonia, they compare to none in AID-deficient mice.[6,7] The low mutation rate probably is likely an underestimate because the cB-1a cell subset that is in great excess compared to the sB-1a cells can also bind hapten-conjugated phycoerythrin used to isolate hapten–Ag-specific sB-1a cells, and thus some cB-1a cells are present among the cells sequenced. However, since cB-1a cells are not acted on by AID, and thus have only germ-line sequences, they are able to bind Ag only weakly as a manifestation of their weak Ag polyspecificity.[14] Thus, a clearer picture of the sB-1a cell V-region mutations should emerge from single-cell sequencing.

There are two other similarities between sB-1a cells and B2 memory cells. First, in CS, DTH, and pneumonia, like B2 cells, sB-1a cells produce high-affinity Ag-specific IgM Ab rapidly after initial Ag exposure. Second, B2 cells are well known to undergo clonal selection induced by repeated boosting exposures to Ag; this results in AID-induced progressive V-region mutations generating different combining site conformations for most optimal Ag specificity by selection of a best fit optimal affinity among various 3D conformations. This also may be true for sB-1a cells, perhaps according to a clonal selection process that however is not yet known for sB-1a cells, which are mutated, with an active IL-4 signaling pathway, and thus ready for rapid and Ag-specific activation in the PerC at the time of immunization.[6,17] Both sB-1a cells and B2 memory cells are dependent on T helper cytokines, such as IL-4 for CS-initiating sB-1a cells[8,10,11] and IL-13 for Ab initiators of pneumonia resistance,[6] compared to cB-1a cells that, by definition, are independent of T cell help.[1]

A role for sB-1a cell–derived Ab free light chains in murine CS

We first established that sB-1a cell–derived IgM Ab were responsible for CS and DTH initiation.[4,8,10,11] The IgM Ab forms immune complexes with hapten–Ag–self resulting from the eliciting hapten–Ag challenge in the skin to then be able to activate complement to generate C5a,[18–20] that activates release of vasoactive mediators to permit local recruitment of

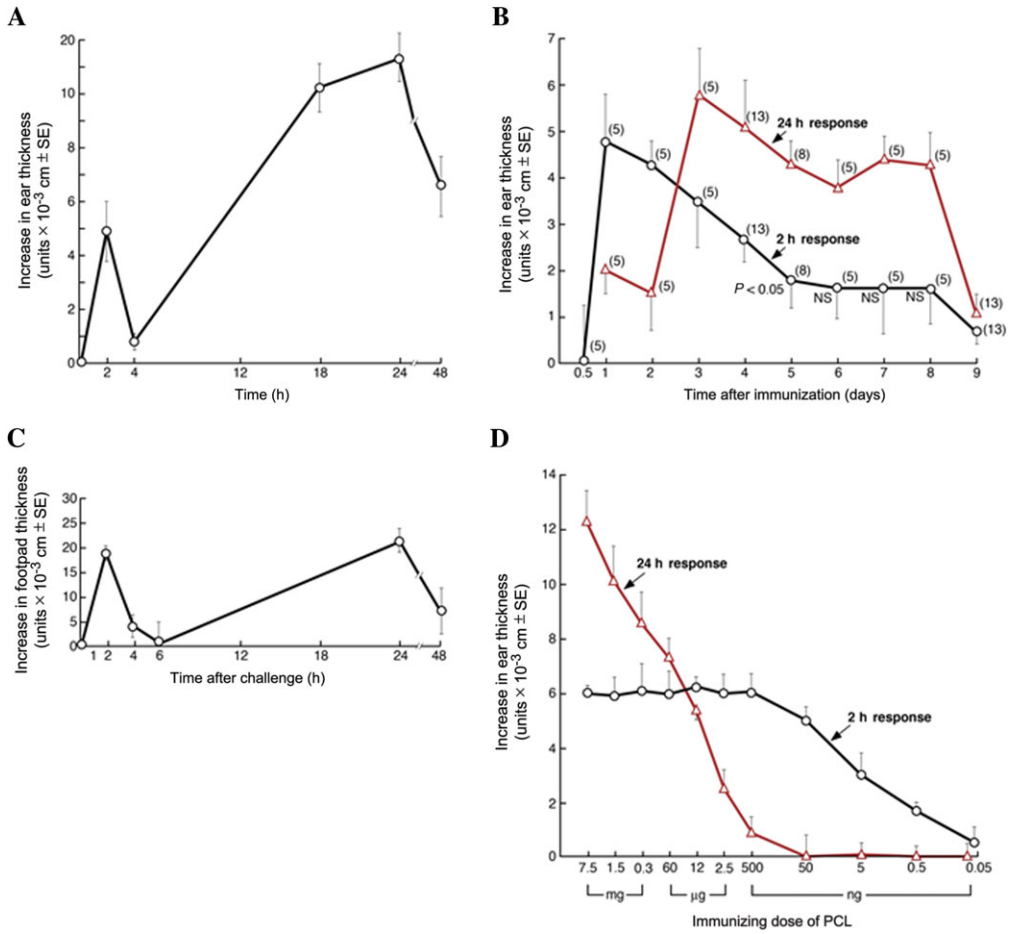

Figure 2. Two-hour peaking component required to elicit late classical CS and DTH responses and sensitivity to Ag. (A) Early phase 2 h maximal skin swelling in contact-sensitized mice challenged with dilute Ag on the ears on day 4; ear swelling rapidly diminishes to nearly nil at 4 h (when an IgG-complement-mediated Arthus-type response, if present, would be maximal) and then rises to a 24 h classic delayed peak, prolonged here to 48 h due to local release of inflammatory cytokines by CS effector T cells locally recruited. (B) Elicitation of CS on different days after immunization. sB-1a cell–mediated early phase is maximally elicited only 1–2 days after immunization and falls away thereafter, but remains at day 4 when the classical 24 h delayed response, evident at day 3, is elicited. (C) sB-1a cell–mediated early component and the effector T cell late component patterns apply to a DTH reaction elicited in the footpads of mice originally injected with syngeneic tumor cells and subsequently challenged in the feet with the tumor cells. (D) Immunization of different groups of mice with serial downward dilutions of the contact sensitizing reactive hapten TNP–Cl, starting with 7.5 mg. The first group shows the typical response of strong early and late components; thereafter, with downward dosing of sensitization, the 24 h late T cell response to the usual ear challenge with dilute nonirritating 0.4% TNP–Cl (0.6 mg) falls away rapidly and is gone by 2.5 µg (a 300-fold dose decrease), suggesting that these low doses are not sufficient to induce T cell immunity as complexes of hapten–self–MHC. In contrast, the sB-1a cell–mediated early phase is sensitive to immunization with low concentrations of Ag. Adapted from data originally published in Ref. 9.

the effector T cells. Further study led to discovery of an alternate pathway that also caused vascular activation for local T cell recruitment. It was due to an initiating Ag-specific IgE-like factor,[21] later shown to be sB-1a cell–derived free Ab light chains (FLCs).[22,23] Subsequently, in a series of publications over the last dozen years, Ab FLCs have repeatedly been shown to have initiating biologic activities and disease associations.[24–26]

FLC-initiating activity emerged from our finding that the early phase of CS is expressed clinically as a 2-h peaking skin swelling that is predominantly edema produced by Ab FLCs that mediate an IgE-like mast cell–sensitizing and

Ag-induced activation process for mast cell release of vasoactive mediators.[21–23] In CS and DTH, the newly uncovered early 2-h Ag-specific peak is due to sB-1a–derived IgM Ab and/or to Ab FLCs that are evident macroscopically by 2 hours after Ag challenge.[4,7,9] Thus, after this second Ag challenge to test skin responsiveness induced by primary immunization, the early response is elicited and which can be quantitated by measurement of ear swelling and by Evans blue vascular permeability;[27] continued measurement reveals the 24–48 h late component of ear skin swelling due to local inflammation.[27] The early 2-h peak has become an invariable marker of early initiating sB-1a cells that are required for the subsequent late classical 24–48 h T cell–mediated inflammatory ear swelling.[4–13] Besides ear thickness and permeability to dye, we additionally determine ear content of myeloperoxidase from neutrophils, the principal inflammatory cells,[28,29] and IFN-γ content.[4] IFN-γ is the principal proinflammatory cytokine secreted by the locally recruited CS/DTH-mediating effector T cells that are induced by primary immunization, and that are recruited locally by the sB-1a initiating mechanisms and are evident in the ears by 24–48 h after testing and, generally, occurring by 3–4 days after immunization (Fig. 2A–C). In a subsequent study of resistance to pneumonia, we described similar PC hapten–Ag-specific early and late cutaneous hypersensitivity components. In this early immunity to pneumonia infection, the early phase began to be elicited in the ears beginning at only 12 h after the onset of infection.[6]

Studies of sB-1a effects in murine CS

CS in mice first was described in the 1960s. It allowed in-depth studies of induction, elicitation, time course, and regulation because of easy cell transfers, fractionation, phenotyping, genetic requirements, and study of many groups at once. This led to our discoveries that negated previous rules excluding B cell participation in T cell–mediated CS[4,8] and DTH.[5] Crucially, we identified the unanticipated early sB-1a cell initiating ear swelling phase required to elicit subsequent classical T cell responses. Simply, although T cells were induced by cutaneous immunization, they could not mediate the classical late phase of CS without the prior action of the initiator sB-1a subset. An important finding, relevant to B1 cells in general, is that the Ag-specific sB-1a cell subset seems to be rapidly and specifically activated in the PerC in all CS and DTH Ag systems evaluated. The cells arrive in the PerC before the time of immunization in 5–7-week-old animals, likely by transit from their earlier development in the spleen.[15] At immunization, the Ag-sensitive sB-1a cells require IL-4 to be activated within only 1 h of Ag stimulation, and then for migration to the spleen that is completed by 12–18 hours.[17] In the spleen, they rapidly differentiate into plasma cells that secrete Ag-specific IgM and Ab FLCs as soon as 24 h after sensitization.[17]

Early sB-1a cell initiation involves a cascade of mast cells, complement, and vasoactive mediators

In the increasing multistep CS-initiating cascade process, sB-1a cells play a crucial role in the *initiation* of the early edematous component (Fig. 1A) that is required for the *elicitation* of the late inflammatory component that is dependent on local recruitment of the effector T cells (Fig. 1B). In the early phase, sB-1a cells produce IgM and Ab FLCs that induce two vasoactivating pathways that facilitate recruitment of the effector T cells.[22] Both pathways involve mast cells, newly recognized in these studies to play an essential role in CS and DTH by being required in the early component.[30–33] In the canonical initiating pathway, secondary skin challenge to elicit CS causes the higher affinity IgM Ab to form immune complexes with eliciting Ag,[19,20] leading to complement activation[18–20,22] (also shown here for the first time in DTH and CS). This generates C5a fragment[18–20] that stimulates C5a receptors on mast cells,[34] platelets,[35,36] endothelium,[37] and even T cells[38] to cause mast cell and platelet release of vasoactivating serotonin (5-HT)[39] and TNF-α.[28] As part of the middle of the sB-1a cell–dependent initiating cascade, these mediators activate early vascular permeability[31] and subsequent expression of adhesion molecules on endothelium.[40] This enables binding and then diapedesis of the newly immunized and circulating CS–effector T cells, which are then activated by local hapten–self-peptides in MHC molecules on APCs at the skin site of the secondary ear skin challenge. A few of the effector T cells recruited into the extravascular tissues have hapten–Ag/self–MHC-specific αβTCRs, and had been induced by the primary contact sensitization. After local extracellular recruitment, these

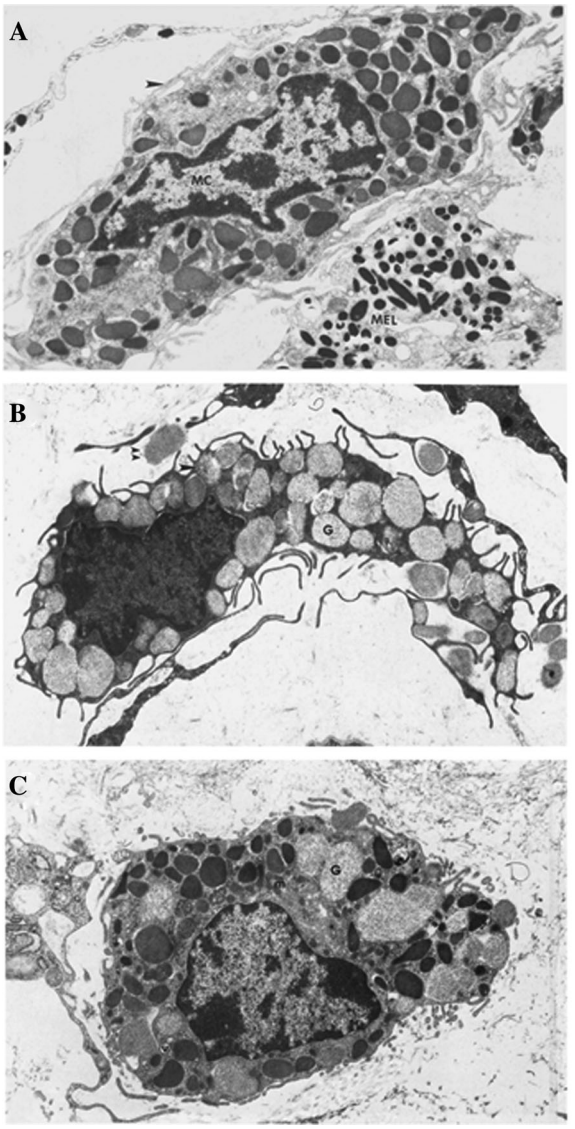

Figure 3. **Partial, piece-meal mast cell degranulation in CS. Degranulation, shown by electron microscopy, is mediated by sB-1a cell–derived IgM Ab, with sufficient binding affinity to activate C5a and binding to mast cell C5a receptor, and by mast cell–sensitizing sB-1a cell–derived Ab FLC. (A) Nonactivated nonreleasing mast cells. (B) Mast cells conventionally activated for compound sequential exocytosis via classical IgE mechanisms. (C) C5a- and sB-1a cell–derived Ab FLC–activated mast cells.[42] Originally published in Ref. 32.**

T cells then are activated by skin Ag challenge–induced Ag-peptide/MHC expressed on local extravascular APCs to stimulate release of cytokines that mediate inflammation by stimulating local stromal tissue cells to release chemokines.

In the alternate CS-initiating pathway,[22] the Ag-specific sB-1a cell–derived Ab FLCs bind the mast cell surface,[23,33] perhaps due to a particular combinations of lipids,[41] thereby sensitizing the mast cells[23] for Ag-induced mediator release[8] via piece-meal degranulation[39] (Fig. 3C) for differential release[42] of vasoactive 5-HT[39,43] and TNF-α production.[28,40] This is analogous to IgE Ab–dependent mechanisms.[21,32,43] In fact, we first characterized the Ab FLCs as a mast cell–sensitizing and –releasing serum factor like IgE,[21,44] but one

that did not bind IgE Fc receptors nor other Fc receptors,[23] as anticipated because FLCs have no Fc portion.

Hepatic iNKT cell–derived helper cytokines are required to activate sB-1a cells

The characterization of sB1-a–mediated CS initiation led to the finding of a required role for iNKT cell activation for the production of IL-4,[10,11,45] needed to act on IL-4 receptors,[11] to trigger STAT6 signaling,[11] for stimulation of sB-1a cell production of IgM and Ab FLCs. The finding of T helper cell cytokine dependence of sB-1a cells was novel (cB-1a cells are defined as strictly T cell independent), as was the finding of the helper B cell function of iNKT cells. This was similar to the dependence of memory B2 cell on helper cytokines from conventional αβT cells. Therefore, at this level, the sB-1a cells in CS, DTH, and in pneumonia protection again are similar to B2 cells and other mature B cells in their dependence on T helper cells.

An unusual element in the sB-1a cell–dependent initiating cascade is that IL-4 release in CS initiation is from iNKT cells, preferentially those in the liver (Fig. 4) rather than spleen or lymph nodes,[10] and not IFN-γ often also released by iNKT cells.[10] Hepatic iNKT cell IL-4 production is detected only 18 min after skin immunization, with a peak doubling as early as 2 h (Fig. 4).[10] This rapid IL-4– and liver-specific activation of iNKT cells may be via glycolipid ligands for their iTCRs that are released during skin sensitization and which specifically target the liver. Because glycolipids are the Ag for the unusual CD1d-dependent iTCR on iNKT cells, we phenol–chloroform extracted the liver after immunization and found iNKT cell–stimulating lipids increased at 30 minutes.[46] It is thought that these putative glycolipids are released by skin irritation caused by the high-dose reactive haptens releasing endogenous glycolipids.

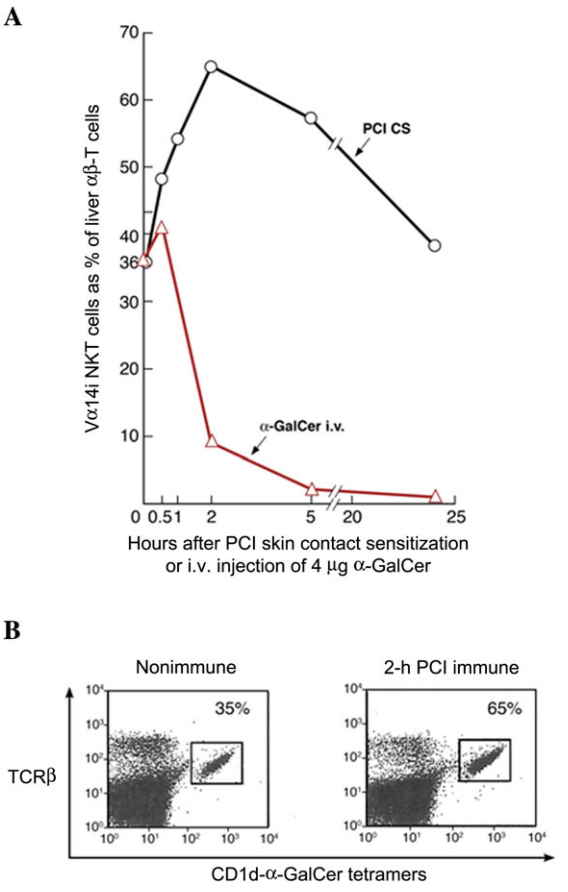

Figure 4. Induction of CS initiation: contact sensitization rapidly activates hepatic iNKT cells. (A) Contact skin sensitization with TNP hapten likely releases endogenous glycolipids that migrate to the liver; the glycolipids bind to hydrophobic CD1d on hepatic antigen presenting cells to induce rapid activation of iNKT cells by 1 h, peaking by 2 h, for expression and release of IL-4 by only 18 minutes. (B) The liver iNKT cell doubling, measured as TCRβ^+ cells binding tetramers of CD1d–α-GalCer, reaches a peak at 2 h after skin sensitization and remains elevated for nearly 24 hours. Systemic injection of α-GalCer produces a profound decrease. Originally published in Ref. 10.

sB-1a cells in early immune protection from pneumococcal pneumonia

sB-1a cells also mediate very early resistance to pneumococcal pneumonia in mice.[6] These sB-1a cells, like the initiator sB-1a cells in CS, also are acted on by AID for induction of Ag specificity and higher affinity compared with the cB-1a cell–derived IgM NAb with germ-line V-region sequences.[6] Again, the rapidity of sB-1a cell actions suggests that the AID mutating activity was prior to infection. In this frequently studied system, cB-1a cells, particularly those with the T15 idiotype in Balb/c mice, are thought to mediate early resistance to pneumococci.[48] However, our data show in contrast that the sB-1a cells produce the T15$^+$ IgM,[6] and that these cells are responsible for the rapid bacterial clearance (by only 2–3 days) after onset of infection.[6] Further, sB-1a cell IgM also mediates a CS/DTH-like

2-h ear swelling responsiveness to the pneumococcal hapten PC conjugated to albumin in infected mice that is elicitable within only a half-day following the onset of infection.[6] Finally, there is a significant correlation of IgM anti-PC Ab responses with onset of maximal ear swelling response at only a half-day after beginning the infection.[5] The sB-1a cell–derived high-affinity V-region-mutated anti-pneumococcal IgM Ab enables rapid activation of complement[6] to mediate Ag-specific bacterial lysis and generation of chemotactic activity likely due to C5a. The essential role of complement activation was shown by loss of the sB-1a cell–mediated early pneumonia resistance in mice rendered complement depleted by treatment with cobra venom factor.[6] Further, the higher affinity IgM Ab likely produces significantly greater opsonization for more efficient local phagocytosis of growing organisms and rapid infiltration of the lungs with leukocytes, particularly neutrophils, that together quickly eliminates the pathogen and clears the pneumonia.

Finally, AID-deficient mice had no resistance but an adequate $T15^+$ IgM response to infection.[6] Resistance to pneumococcal pneumonia was restored by intraperitoneal transfer of naive wild-type $T15^+$ sB-1a cells given prior to infection of the AID-deficient mice.[6] Therefore, the T15 idiotype is expressed on both cB-1a cell NAb and on V-region-mutated IgM of sB-1a cells, the latter being AID dependent and, as we showed, responsible for mediating pneumonia resistance. Thus, the PC-binding T15 idiotype is not a marker of protective Ab but of IgM in Balb/c mice, encoded in both sB-1a cell V region of IgM that mediates resistance and in the unmutated germ-line DNA–encoding IgM NAb that do not mediate resistance, likely because of lower Ag affinity of the latter.[6] Therefore, we theorize that the T15 idiotype is not in the combining site encoded by the V region or in its germ-line portions. Finally, a further similarity of the sB-1a cells in pneumonia resistance to those in CS and generally to B2 cells is that there is a requirement for IL-13, a Th2-type B cell helper cytokine, again unusually derived from very early activated iNKT cells.[6]

Discussion

Biological relevance to other disease models

Our studies have defined crucial roles for sB-1a cells in eliciting late T cell responses associated with CS, DTH, and pneumonia resistance. It is not known whether this initiating process occurs in other T cell–mediated models and diseases. Certainly, elicitation of an early skin response at about 2 h or less after testing, such as in CS, DTH, and in pneumonia infected mice, suggests a wider incidence of initiation. We are alerted to this by finding such an early phase response in other systems we and colleagues have studied, including immunity to syngenetic lymphoma,[49] tumor suppression of immunity with decreased late T cell DTH but presence of early sB-1a cell–like DTH component,[50] and in responses to helminthic parasites.[51]

Further, the early sB-1a cell aspect may also pertain to infections other than pneumococcal pneumonia, since B cell–deficient mice[53] have defects in immune protection from several infectious diseases that previously were assumed to be solely T cell mediated. We theorize that the B cell dependence of T cell protection could be due to a loss of sB-1a cell–mediated initiation for T cell participation in B cell–deficient animals, or the sB-1a cell response itself mediates protection, as in the murine model of pneumococcal pneumonia. We previously reviewed the dependence on B cells of such T cell-dependent infection responses, and in some instances of elements of sB-1a cell initiation in many of these, such as those caused by mycobacteria, cryptococci, *Toxoplasma*, tularemia, *Salmonella*, *Chlamydia*, *Candida*, *Leishmania*, malaria, filarial, and viral infections.[8] In the further investigation of these models, we recommend testing the primary responses for early skin reactivity to specific Ag and the infection resistance itself in for possible involvement of the sB-1a cell subset in AID-deficient versus wild-type mice. The same considerations apply to autoimmune diseases thought to be dominantly controlled by T cells. We also previously reviewed the presence of elements of sB-1a initiation in many of these: collagen and rheumatoid arthritis, autoimmune encephalomyelitis and multiple sclerosis, diabetes mellitus, systemic lupus, inflammatory bowel disease, hepatitis, and thyroiditis.[8]

The sB-1a cell component of CS need not be detectable macroscopically and may play a role in IgE-mediated atopic allergic diseases

To determine quantitative aspects of sB-1a cell initiation and if other Ab isotypes could participate, we used IgE monoclonal anti-hapten Ab to

imitate IgM and FLCs of CS. As a mast cell activator, IgE might recapitulate the early essential sB-1a cell vasoactive steps of CS initiation.[52] Further, by employing this anaphylactic Ab, we might determine whether atopic allergic Th2 T cell–mediated diseases also might require an initiating phase, and thus if IgE, famous for immediate hypersensitivity, could in this instance also be important in effector T cell aspects via initiating mechanisms similar to sB-1a cell–dependent recruitment of effector T cells into the tissues.

Naive wild-type mice received two different intravenous transfers that separately modeled the early and late phases of CS. For CS-effector T cells, they received intravenous transfer of 4-day TNP–Cl immune cells that had been treated *in vitro* with anti-B220 MAb plus complement;[52] this removed sB-1a cells so that the T cells were present without early initiation and thus no CS was elicited (Fig. 5, group A). In this case, to elicit the early phase, they received 10-fold decreasing intravenous doses of monoclonal anti-TNP IgE. Then, TNP–Cl ear challenge elicited an IgE mast cell–dependent early phase that allowed the expression of the T cell late phase in mice that optimally received as little as 100 ng of IgE systemically, compared to those given the IgE that had not received the isolated T cells (Fig. 5; groups F vs. E). Remarkably, an IgE dose of as little as 10 ng, and even somewhat at 1 ng systemically, had negligible or no early macroscopic phase, yet had a significant late phase, compared to mice that received no IgE (Fig. 5; groups C and D vs. A). These findings indicate that even microscopic initiation provided by very small doses of IgE is sufficient to allow significant recruitment of effector T cells. Interestingly, the very low systemic IgE doses that initiated T cell recruitment for elicitation of a macroscopic late-phase T cell response elicited no macroscopically measurable early component, but still facilitated elicitation of the late CS component due to the competent effector late-acting T cells present in these CS-immunized JH-deficient mice.[52]

Thus, the early CS-initiating component could be provided by IgE-sensitized mast cells mediating vasoactive responses that imitated the sB-1a cell–dependent processes. Importantly, this experiment showed that the required early component need not be manifest as a macroscopic response. Therefore, release of low amounts of mast cell–derived vasoactive mediators like 5-HT and TNF-α likely caused only microscopic vasculature activation, which was sufficient to facilitate local extra vascular recruitment of the few[54] needed effector T cells to mediate a macroscopic, that is, a clinical delayed T cell response.[52] This could be relevant to human allergic diseases, in which the initiating component might not be detected macroscopically (i.e., clinically), but still would be sufficient to elicit the manifestations of the late T cell–mediated component of the disease, such as in so-called nonatopic asthma, where pathology identical to atopic/allergic IgE–mediated asthma is present without high levels of IgE nor associated with clinical atopic allergy.

Clinical correlates of pneumonia protection by the Ag-specific sB-1a cells

Many immunodeficient and geriatric patients with impaired immune systems are particularly susceptible to severe pneumonia, particularly due to pneumococci. An important finding of our pneumonia studies shows that AID-deficient mice lack effective acquired sB-1a cell IgM responses and consequently have a severe defect in the early clearance of bacteria from the lung, which can be reconstituted by transfer of the sB-1a subset.[6] Interestingly, these animals have significant serum elevation of unmutated anti-pneumococcal IgM from cB-1a cells that are of low Ag affinity and that mediate no resistance. Similarly, some AID-deficient patients with hyper-IgM syndrome, who solely have-IgM NAb from cB-1a cells without mutations, are not protected from respiratory tract infections.[55,56] Therefore, large amounts of unmutated, polyreactive IgM Ab are able to bind pneumococcal Ag via their germ-line-encoded Ig V regions, but this binding is weak and of lower affinity, and thus is insufficient to provide complement fixation, bacterial neutralization, and thus pneumonia protection.

Early CS-initiating cascade of sB-1a cells, iNKT cells, mast cells, and complement

There seems to be a set cascade of interacting innate and acquired components that we have uncovered in CS, DTH, and pneumonia protection. These components center on the very early activation of a rare sB-1a cell subpopulation that seems to already have acquired AID-dependent Ig V-region mutations by unknown means (e.g., by previous Ag or Ag-like exposure). Prior immunization with self-Ag, perhaps abnormally presented or as altered

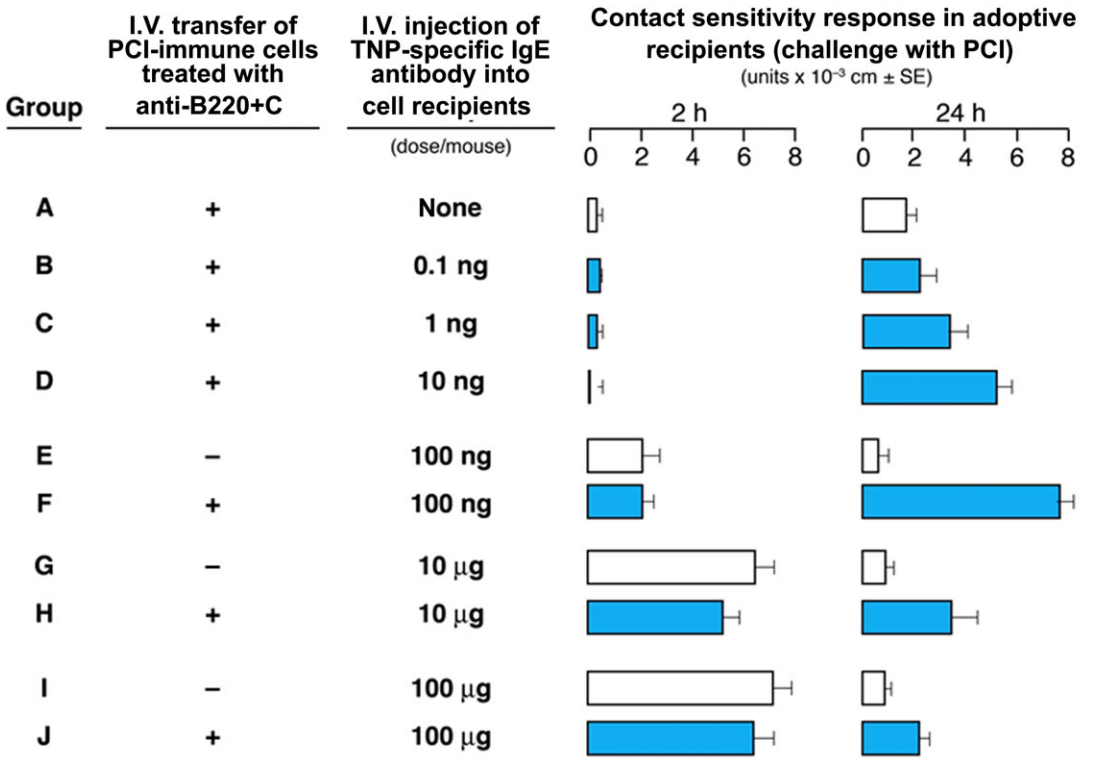

Figure 5. Low systemic intravenous doses of monoclonal anti-TNP IgE Abs initiates CS microscopically.[52] Naive wild-type mice received two different intravenous transfers that modeled the early and late phases of CS. For CS effector T cells, they received 4-day TNP–Cl immune cells that were treated *in vitro* with anti-B220 mAb plus complement to remove sB-1a cells; thus, T cells were present without early initiation and CS was not elicited (Group A). To elicit the early phase, mice received 10-fold decreasing doses of monoclonal anti-TNP IgE, followed by TNP–Cl ear challenge that elicited an IgE mast cell–dependent early phase that allowed expression of the T cell late phase in mice that optimally received as little as 100 ng of IgE, systemically, versus those receiving the same dose of IgE without the T cells (Group F vs. E). Even as little as 10 ng (or 1 ng), which produced negligible or no early macroscopic phase, produced a significant late phase, compared to mice that received no IgE (Group C and D vs. A). Thus, mere microscopic initiation provided by very small doses of IgE is sufficient to allow significant recruitment of effect T cells. A further point concerns the progressive decline of the late phase at the higher 10 and 100 μg doses of IgE (Groups H and J) that we showed was due to vigorous IgE-mediated mast cell histamine release, stimulating inhibiting H2 receptors that were blocked by treatment with a H2 receptor antagonist to restore the strong late-phase responses.[64] Originally published in Ref. 52.

self, or release of endogenous TLR ligands[47] may have activated the sB-1a cells for AID-mediated mutations. Early activated sB-1a cells require helper cytokines, likely from hepatic iNKT cells, seemingly stimulated by glycolipids released from the skin at immunization.[46] These iNKT cells express a primitive iTCR consisting of the first rearranged and monoclonal TCRα chain (Vα18),[57] and limited Vβ chains for iTCR recognition of glycolipid Ag bound in CD1d, which is a minor histocompatibility Ag that is the first to appear in the thymus,[57] rather than peptide Ag in MHC like in recognition by αβT cells. We postulate that in other responses thought to be purely T cell mediated, that sB-1a cells, as an initiator of innate-like immune effectors, may center a group of similarly required ancient innate immune mechanisms, namely, iNKT cells, mast cells, and complement, to act in a set cascade sequence. We theorize that in some instances, this may lead to an essential final endothelial activation step for eventual binding and then extravasation of effector recognition cells (here αβT cells), enabling their recruitment into the tissue site of Ag challenge, and in others may constitute just an early needed set of protective components as we found in pneumococcal pneumonia.

Overall, there seem to be similar interactions of seemingly related common components that we hypothesize are possibility linked in an ancient phylogenetic effector pattern we demonstrated as essential in CS, DTH, and pneumonia protection. Therefore, we hypothesize that the involved components also may participate in a group pattern in other systems, including (1) rapid activation; (2) innate-like cell activation, that is, sB-1a cells induced to express some surface phenotypic of markers usually found on T cells (CD5 and CD90) and on myeloid cells (CD123/IL-3R);[7] (3) other innate-like elements such as iNKT cells;[58] (4) reacting with CD1;[59] (5) reacting with mast cells;[60] (6) reacting with complement;[61,62] (7) sB-1a cell production of Abs within just 1 day;[6,17] (8) Ab produced is IgM[63] and derived FLCs;[22,23] (9) early elicited cutaneous response peaking macroscopically at 0.5–2 h in animals skin tested at 1–2 days after immunization;[6,9] (10) Ag specificity of the early response and the IgM Abs due to AID acting previously on Ig Ab V region of the sB-1a–like cells;[6,7] (11) dependence on iNKT cell–derived B cell helper cytokines;[6,10,11] (12) released glycolipids from the host or an infecting organism[64] that complex with CD1d[63] (the first histocompatibility Ag expressed in the thymus[57]) to activate primitive iNKT cells via their iTCR;[57,58] and (13) dual activation of the sB-1a subset by Ag acting on their BCRs, together with a helper cytokine, such as rapidly produced IL-4 in CS[10,11] and IL-13 in pneumonia.[6]

The linkage of these characteristics in CS, DTH, and pneumonia protection, theorized to occur in many other responses, suggests that together this specific cascade of cells and molecules may be an evolutionarily conserved innate immune response pattern with constituents that may have evolved together or in sequence by Darwinian-like natural selection, and thus may exist in phylogenetically earlier vertebrates that indeed have dominant IgM responses.[63] Evolution of such an innate tissue reactive pathway required for extravascular recruitment of effector T cells into the tissues—the highest level of acquired immunity—seems to have evolutionary advantages in responses against microbes and parasites and thus in protective immunity. However, in modern western countries, where parasites now are relatively absent and bacteria are eliminated by antibiotics, these responses seem to have increased participation in tissue pathology and destruction triggered by self-antigens, or by innocuous environmental antigens like allergens, respectively in autoimmunity and allergic diseases.

Speculations arising from identification of the B2 cell-like sB-1a cells in CS

We have found that sB-1a cells can have important properties beyond CS and DTH, such as early protection from pneumonia, whereas cB-1a cells that are widely thought responsible are not involved. Crucially, AID-deficient mice have increased IgM NAb produced by cB-1a cells, but have no CS[7] or resistance to pneumococcal pneumonia,[6] compared to wild-type mice that have AID-mediated Ig V-region mutations encoding higher affinity, and thus more reactive and protective, IgM Ab. In contrast, past studies of infection responses with remarkably early protection have ascribed this effect to cB-1a cell–derived IgM NAb. Instead, we postulate that such early protection likely is due to the minority population of sB-1a cells because of their high Ag-affinity IgM Ab.

Finally, it is conceivable that clinical anti-B cell therapy, which is surprisingly effective in a broad number of immunological, autoimmune, and inflammatory diseases, may interfere with early sB-1a cell responses. This may include clinical pathogenic properties of Ab FLCs that are present and potentially acting in a variety of autoimmune, allergic, and immunologic diseases.[24–26] Therefore, some diseases that seem to solely depend on pathogenic T cells or B2 cell Abs may have an unappreciated requirement for sB-1a cells.

Summary

Our published work has characterized a unique sB-1a cell subset that resembles memory B2 cells and is stimulated within the first hour after immunization. This subset is required to mediate an early initiation process required for eliciting the classical late-phase T cell–mediated component of CS and DTH, and also acts very rapidly after the onset of pneumonia to mediate protection. The sB-1a cells produce Ag-specific IgM Abs and Ab FLCs of high affinity for Ag due to AID-dependent mutations in their Ig V region. This enables more avid Ag binding of IgM Ab to activate complement for generation of C5a, and for the FLCs that bind to mast cell surfaces and thereby sensitize them to Ag-dependent

release of vasoactive mediators. Together, these are needed to recruit effector T cells into the tissues via their activation of the local microendothelium that then allows binding and subsequent extravasation of effector T cells to enter tissues to elicit the delayed components of CS and DTH, as well as bacterial clearance in pneumonia.

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Conflicts of interest

The authors declare no conflicts of interest.

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