Blood Dendritic Cells Interact with Splenic Marginal Zone B Cells to Initiate T-Independent Immune Responses

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Summary

Marginal zone (MZ) and B1 B lymphocytes participate jointly in the early immune response against T-independent (TI) particulate antigens. Here we show that blood-derived neutrophil granulocytes and CD11c^{lo} immature dendritic cells (DC) are the primary cells that efficiently capture and transport particulate bacteria to the spleen. In a systemic infection, CD11c^{lo} DC, but not neutrophils, provide critical survival signals, which can be inhibited by TACI-Fc, to antigen-specific MZ B cells and promote their differentiation into IgM-secreting plasmablasts. In a local TI response, peritoneal cavity macrophages provide similar support to B1 B-derived Ag-specific blasts. In the absence of soluble TACI ligands, Ag-activated MZ- and B1-derived blasts lack survival signals and undergo apoptosis, resulting in severely impaired antibody responses.

Introduction

When bacterial pathogens enter a vertebrate organism, orchestrated activities of a variety of effector cells and molecules attempt their elimination. Immediately, innate components, including neutrophil granulocytes, macrophages, dendritic cells, and complement and circulating natural antibodies act in concert to effectively eliminate most of the circulating antigen (Ag). Subsequent to this early phase, repetitive antigenic epitopes exposed on the surface of pathogens activate Ag-specific B lymphocytes, which in the absence of T cells initiate a rapid T-independent (TI) immune response.

In contrast to the large body of work detailing the cellular and molecular events in T-dependent (TD) responses, little is known about the cellular and molecular requirements of a TI immune response. In addition, most of the experimental systems used conventional soluble "laboratory" Ag to describe the precursor B cell types, cellular kinetics, and topographical location of plasma cells participating in TI as well as TD responses (Liu et al., 1991; Jacob and Kelsoe, 1992; Toellner et al., 1996). These types of molecules do not entirely mimic the reality of antigen, normally exposed on bacterial surfaces. In order to understand the mechanisms that make the spleen critical for effective protection against blood-borne pathogens, we analyzed the early immune response to intact bacteria as a particulate Ag. We showed that the ensuing response to surface phosphorylcholine (PC) epitopes involved the activation of Ag-specific B cell clones of MZ and B1 B cell origin (Martin et al., 2001). These studies, together with recent reports of targeted mutations that affect MZ and B1 B cell development and function, implicate these B cell compartments as a major source of precursors for TI immune responses (reviewed in Martin and Kearney, 2001).

In contrast to TD responses, where T-DC cell interactions are necessary for efficient B cell effector responses, no such accessory "helper" cells have been described in TI immune reactions. In vitro generated DC have been shown to influence in vitro B cell proliferation, isotype switching, and plasma cell differentiation (Fayette et al., 1998). However, a need for DC in TI responses was first suggested by the topographical association and bidirectional expansion of spleen "myeloid" DC and plasmablasts in response to a soluble TI Ag (Garcia de Vinuesa et al., 1999). Additionally, it was shown recently that in vitro generated, bone marrow-derived DC (BMDC) support TI antibody secretion (Colino et al., 2002). Collectively, these results, showing that TI responses develop rapidly and generate large amounts of IgM from a massive plasmablast pool, suggest a role for dendritic cells in the process leading to plasma cell differentiation. However, the identity of the cell type(s) involved, and the cellular and molecular interactions in vivo that influence Ag-specific plasma cell generation, are not known.

Here, we describe the capture and transport of Ag to the spleen by blood-derived, CD11c^{lo} DC, which are singularly responsible for initiating and supporting the TI immune response. We also provide insight to the in vivo molecular mechanisms involved in the DC-mediated B cell response. Recently described members of the TNF superfamily, BLyS (*B* lymphocyte *S*timulator, also called BAFF) and APRIL (*A PR*oliferation-*I*nducing *L*igand) are derived from monocyte/macrophages, DC, and subsets of T cells. Their receptors (TACI [*T*ransmembrane *A*ctivator and *C*alcium modulator cyclophilin ligand *I*nteractor], BCMA [*B C*ell *M*aturation *A*ntigen], and BAFF-R, which is specific for BLyS/BAFF) have been suggested to provide survival signals for the B cell lineage during development and activation (reviewed in MacKay and Browning, 2002). We show that these DC-derived soluble ligands (BLyS and APRIL) bind to their B cell receptor(s) and provide a crucial cosignal required for plasma cell generation in TI immune responses.

Results

Ag-Specific MZ B Cells Interact with CD11c^{+} Dendritic Cells at the Site of Plasma Cell Differentiation

In order to trace Ag-specific B cell clones after TI B cell activation, we utilized the M167, heavy chain transgenic (Tg) mouse (V_{H1} heavy chain derived from the anti-PC hybridoma M167). In this model the dominant B cell clone, V_{H1}-$V_{\kappa 24}$, reactive with PC, is enriched in the MZ B cell subset (CD21^{hi}IgM^{hi}CD23^{lo}IgD^{lo}) and located predominantly in the MZ. 24 hr after i.v. administration of

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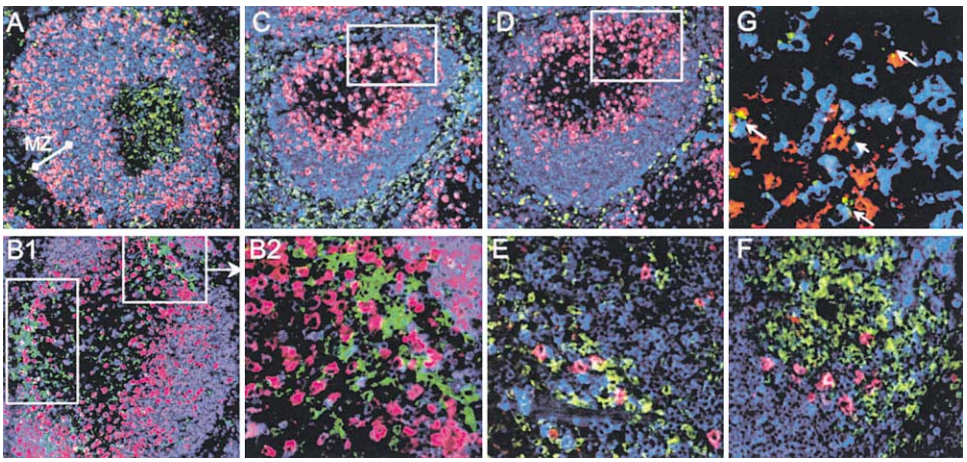

Figure 1. TI Ag-Specific B Cells Associate with Ag-Loaded Dendritic Cells at Sites of Plasmablast Differentiation

(A) Histology of the spleen from an unimmunized M167 mouse, stained with IgM (blue), M167 Id (red), and CD11c (green). Purple (red and blue) Id+ B cells are enriched in the MZ.

(B) M167 mice, immunized with *S. pneumoniae*, were sacrificed at day 1 after i.v. immunization. Ag-specific B cells (purple; M167-Id red and IgM blue) have migrated from the MZ to the T-B border and bridging channels (B1) that connect the white and red pulp, where they are blasting in physical association with DC (CD11c, green; B1 and B2). Ag-specific Id cells enlarge and become brighter for intracellular immunoglobulin (plasmablasts).

(C and D) In the same M167 mice at day 1 after immunization, spleen macrophages (F4/80, green, C) and neutrophil granulocytes (Gr-1, green, D) do not participate in these primary B cell clusters stained with M167-Id (red) and IgM (blue).

(E and F) C57BL/6 (E) or T cell$^{-/-}$ (F) mice adoptively transferred with M167 B cells and immunized with bacteria i.v. were sacrificed and stained with IgM (blue), M167-Id (red), and CD11c (green) at day 1. Ag-driven migration and association with DC occurred regardless of the presence (M167→C57BL/6; E) or absence of T cells (M167→T cell$^{-/-}$; F).

(G) A functional triad composed of MZ derived B blasts (M167-Id, blue) and green bacteria taken up in vivo by DC (CD11c, red) is formed in the bridging channels.

PC-bearing, heat-killed *Streptococcus pneumoniae* (henceforth referred to as "bacteria"), Ag-specific B cells had moved from the MZ to the T-B border and bridging channels that connect between the white and red pulp (Figure 1A), and were blasting in physical association with CD11c-expressing DC in the bridging channels (Figures 1B1 and 1B2). No other myeloid cells (Gr-1$^+$ granulocytes, F4/80$^+$ red pulp macrophages, or the MOMA-1$^+$ and ER-TR-9$^+$ splenic specialized macrophages) were found in the primary clusters (Figures 1C–1D and data not shown). The plasmablast production in the bridging channels continues until day 3–4, generating large numbers of plasmablasts. When very large numbers of Ag-specific B cells were transferred in transfer experiments or in transgenic animals, cells from the T-B border form T-less germinal centers as previously described (data not shown; de Vinuesa et al., 2000; Martin and Kearney, 2000a). To determine whether these events, including the interactions with DC, occurred in the absence of T cells, we adoptively transferred B220$^+$ B cells from M167 mice to T cell-deficient (TcR$\beta^{-/-}$TcR$\delta^{-/-}$, Figure 1F) or C57BL/6 (Figure 1E) recipients 20 hr prior to i.v. immunization with bacteria. As in the intact mice, MZ B-derived blasts were located at the bridging channels in contact with DC, 24 hr after Ag challenge, regardless of the presence or absence of T cells (Figures 1E–1F). Fluorescent bacteria were used to confirm that the injected particulate Ag also colocalized at these sites of apparent DC-B cell interaction (Figure 1G).

These findings suggested that the formation of a potentially interactive triad composed of Ag, DC, and B cells initiated the observed Ag-specific plasma cell generation at this site.

Myeloid Subsets Stimulate Ag-Specific Plasmablast Differentiation In Vitro

To identify the cellular requirements for the in vitro survival and differentiation of Ag-specific MZ B cells, we cocultured M167 B220$^+$ B cells for 3 days with various unprimed or in vitro bacteria-primed myeloid subsets. Coculture of Ag-specific B cells with in vitro bacteria-primed bone marrow-derived dendritic cells (BMDC) resulted in a large-scale generation of Ag-specific plasmablasts (Syn-1^{hi}IgM^{hi}Id^{hi}, Figure 2A). Although the low viability ($<$2%), seen with B cells alone or B cells plus free bacteria, improved to $\sim$30% by the inclusion of "empty" BMDC, Ag-specific plasmablasts differentiated only in the presence of primed BMDC (Figure 2A). Despite the fact that these cytokine-dependent, in vitro generated BMDC were highly effective in vitro, their in vivo counterparts have not yet been identified. We also found that primed, but not empty, total peritoneal cells or FACS-sorted peritoneal macrophages (PEC Mϕ) showed similar potent support in this in vitro system (Figure 2B).

The use of this experimental model was prompted by the generation of the dominant T15 anti-PC plasmablast clone (Claflin et al., 1974). We cultured total peritoneal cavity cells, containing PEC Mϕ and B1 B cells, from normal BALB/c mice together with *S. pneumoniae* and found effective support of PEC Mϕ for B1 B cell differentiation (data not shown).

Since the systemic immune response to blood-borne bacteria, including plasma cell differentiation, takes place largely in the spleen, we determined whether freshly isolated spleen DC could provide signals needed for in vitro plasmablast generation. We sorted splenic

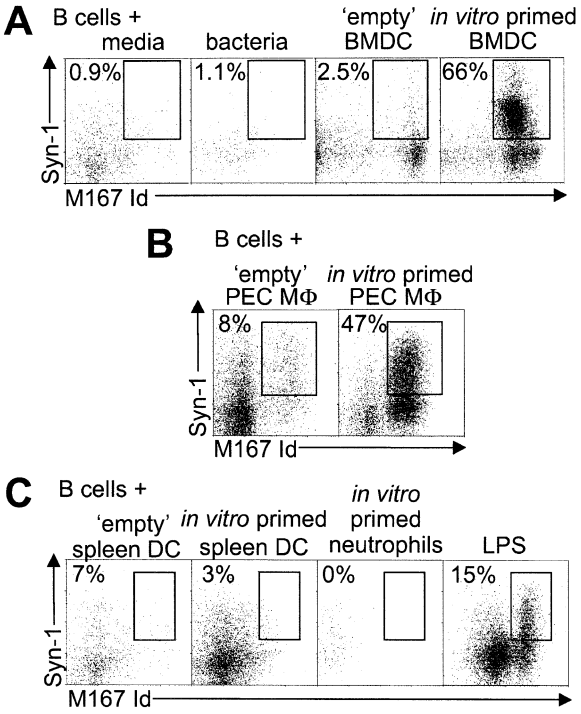

Figure 2. Myeloid Cells Support Ag-Specific Plasmablast Differentiation In Vitro

B220+ B cells from M167 mice were cultured for three days in media, alone, or with various combinations of bacteria and bone marrow-derived DC (BMDC) (A), PEC macrophages (PEC Mϕ; B), spleen Mac-1^{hi}CD11c^{hi} DC, blood neutrophil granulocytes, or LPS (20 μg/ml) (C). Harvested cells were stained with IgM^a-FITC, Syndecan-1-PE (Syn-1), and M167-Id, developed with SA-APC. Plots show Syn-1 and M167-Id profiles of B cells, previously gated on IgM^{a+} populations. Numbers indicate percentages of Ag-specific Syn-1 expressing plasmablasts of total IgM^{a+} cells. FACS profiles show one representative experiment of 3–15 for each condition.

CD11c^{hi}Mac-1^{neg} and CD11c^{hi}Mac-1^{hi} DC (commonly termed "lymphoid" and "myeloid" DC, respectively), primed them with bacteria, and cultured them with M167 B cells. Neither unprimed nor primed splenic CD11c^{hi}-Mac-1^{neg} DC supported the differentiation of Ag-specific MZ B cells in 3-day cultures (data not shown). Although MZ B cells survived better in the case of primed spleen CD11c^{hi}Mac-1^{hi} DC (25%) than unprimed (10%), plasmablasts were not generated (Figure 2C). Increased B cell viability was very likely due to nonspecific stimulatory signals derived from bacteria-activated DC (Kadowaki et al., 2001). Similarly, coculture with primed spleen Mϕ (Mac-1^{hi} excluding DC and granulocytes) enhanced the B cell survival without supporting strong plasmablast differentiation (not shown). In addition, FACS-sorted, bacteria-primed neutrophil granulocytes (Gr-1^{hi}Mac-1^{hi} CD11c$^-$) did not promote survival of Ag-specific B cells (Figure 2C). Polyclonal B cell stimulation with LPS for 3 days generated only a minor population of Id$^+$ plasmablasts, proportional to the initial representation of Id$^+$ MZ B cells in the culture (Figure 2C).

These results showed that selected populations of macrophages (PEC Mϕ) and in vitro-generated BMDC participate as supporting cells in TI responses. However, these cells do not have a known in vivo counterpart in the spleen and blood stream during a systemic TI response to blood-borne bacteria. In addition, the classic CD11c^{hi} DC subpopulations, isolated from the spleen, that are perfectly capable of stimulating T cells (Maldonado-Lopez et al., 1999) failed to provide efficient B cell cosignals, suggesting that a different cell subset was involved in this process.

CD11c^{lo} Blood DC Capture and Transport Ag, and Activate B cells

Since a variety of cells with defined costimulatory activity isolated from the spleen, where TI immune responses take place, failed to efficiently support in vitro plasmablastogenesis, we revisited the in vivo time course of cellular events following i.v. bacterial immunization. The first cells with potential to scavenge and transport i.v. injected bacteria are located in the blood, so we searched for subpopulations of cells with these capabilities. Analysis of myeloid cells at time points within the first day after i.v. bacteria revealed an 8–10 fold influx of neutrophil granulocytes (Gr-1^{hi}Mac-1^{hi}, granular cells) into the spleen in the first 15–60 min (Figure 3A). These granulocytes had captured bacteria, and accumulated mostly in the MZ and bridging channels (Figure 3A, histology). In addition to granulocytes, the entire blood compartment of CD11c^{lo} DC migrated into the spleen by 1 hr after i.v. immunization (Figure 3B). After $\sim$4 hr, the depleted CD11c^{lo} population began to recover (not shown), and by day 1, complete recovery of blood and spleen myeloid subsets had occurred (Figure 3B). To directly visualize and enumerate the cell types with the ability to capture intact bacteria, i.v. immunizations were performed with fluorescent bacteria. We also found that most bacteria were taken up by blood neutrophils, which migrated into the spleen within the first few hours after Ag introduction (Figures 3A, 3C, and 3D). In addition to the neutrophils, blood CD11c^{lo} DC also captured antigen and migrated from the blood into the spleen (Figures 3B–3D). Neither of the splenic resident DC subsets (CD11c^{hi}Mac-1^{hi} or CD11c^{hi}Mac-1^{neg}) appeared to have access to the bacteria in vivo at these time points (Figure 3D). To further dissect the antigen-handling capability of these myeloid subsets, an in vitro phagocytosis assay was performed using fluorescent bacteria and magnetically enriched Mac-1$^+$ splenocytes. The three subsets examined showed similar uptake of bacteria in vitro (Figure 3E, black lines). However, trypan blue treatment, which quenches fluorescence of surface, but not intracellular particles, pinpointed that CD11c^{lo} as well as Mac-1^{hi} cells (including neutrophils and macrophages), but not CD11c^{hi} DC, successfully internalized the bacteria (Figure 3E, colored filled histograms).

The cells expressing low levels of CD11c were Mac-1^{hi} and were negative for CD4, CD8α and B220. They also expressed high levels of B7.1 and CD43, low levels of Gr-1, B7.2 and CD40 and very low levels of MHC class II (Figure 3F and not shown). In addition to capturing bacteria and migrating from blood into the spleen, these blood DC displayed upregulated activation markers $\sim$6 hr after bacterial uptake (Figure 3F). Treatment of FACS sorted blood-derived CD11c^{lo} DC with GM-CSF for 24 hr induced upregulation of CD11c, class II, B7.1 and

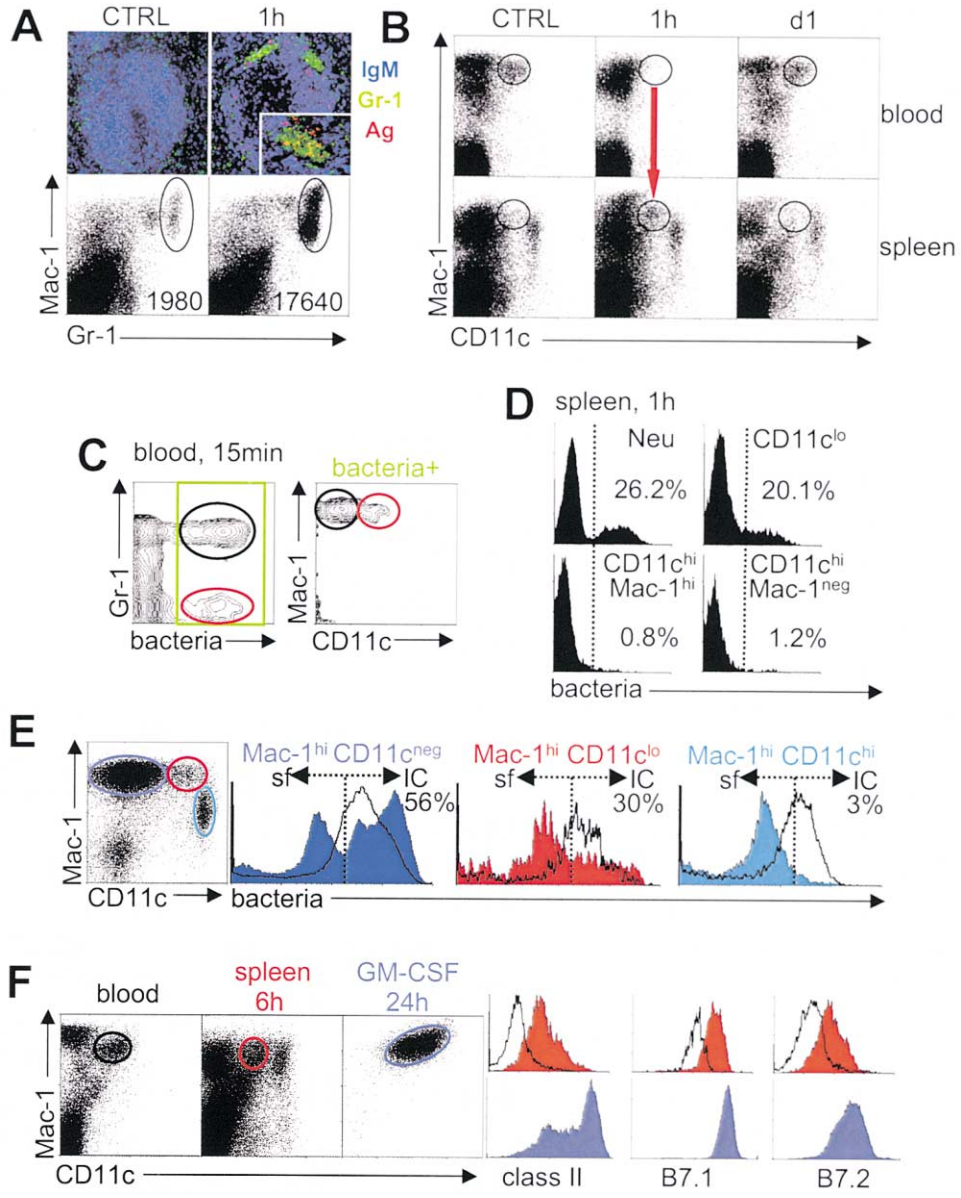

Figure 3. Blood-Derived Neutrophils and CD11c^{lo} DC Capture and Transport Ag to the Spleen

(A and B) Neutrophil granulocytes (A) and CD11c^{lo} DC (B) rapidly migrate from the blood into the spleen following i.v. bacterial immunization. C57BL/6 mice were injected with *S. pneumoniae* and sacrificed, and splenocytes were stained with Mac-1, CD11c, and Gr-1 at different time points. Numbers represent total cells out of 250,000 live splenocytes falling into the appropriate gates. Spleen sections were stained with antibodies against IgM (blue) and Gr-1 (green), while the fluorescent bacteria are red.

(C) Neutrophil granulocytes and CD11c^{lo} DC are the major subsets that capture intact bacteria in the blood. Peripheral blood from C57BL/6 mice 15 min after i.v. immunization with green fluorescent bacteria was stained with CD11c, Mac-1, and Gr-1 mAbs, and analyzed by FACS. The green rectangular gate shows all cells with captured bacteria, and black and red gates represent bacteria-bearing neutrophils and CD11c^{lo} DC, respectively.

(D) Neutrophil granulocytes and blood-derived CD11c^{lo} DC transport intact bacteria into the spleen, while resident splenic CD11c^{hi} DC do not have access to bacteria in vivo at any time. C57BL/6 mice, injected with green fluorescent bacteria, were sacrificed 1 hour after i.v. immunization and splenocytes stained with CD11c, Mac-1, and Gr-1 mAbs and analyzed by FACS. Numbers show percentages of bacteria-bearing cells from each subset.

(E) Spleen Mac-1^{hi}CD11c^{neg} (granulocytes and macrophages [56 $\pm$ 5%]) and CD11c^{lo} (30$\pm$3%), but not Mac-1^{hi}CD11c^{hi} (3 $\pm$ 1%), DC subsets phagocytose intact bacteria in vitro. Splenocytes were enriched for Mac-1 and exposed in vitro to fluorescent bacteria. Black histograms show green bacteria on the particular subsets; filled colored histograms represent surface (sf) versus intracellular (IC) bacteria after trypan blue treatment.

(F) Blood-derived CD11c^{lo} cells represent the major DC population in the blood. They have low levels of class II and B7.2, and upregulate activation markers upon bacterial uptake. Blood mononuclear cells were stained with CD11c, Mac-1, and different DC subset-specific and activation markers. Bacteria-bearing CD11c^{lo} DC were phenotyped after 6 hours of i.v. immunization from the spleens of neutrophil granulocyte-depleted C57BL/6 mice, immunized with green fluorescent bacteria. FACS-sorted blood-derived CD11c^{lo} DC were cultured with GM-CSF for 24 hr and phenotyped. Histograms show the resting phenotype of blood DC (empty) overlaid with bacteria-activated CD11c^{lo} DC (red filled) and DC cultured with GM-CSF (blue filled).

All results are representative of 3 experiments with 3–5 mice in each group.

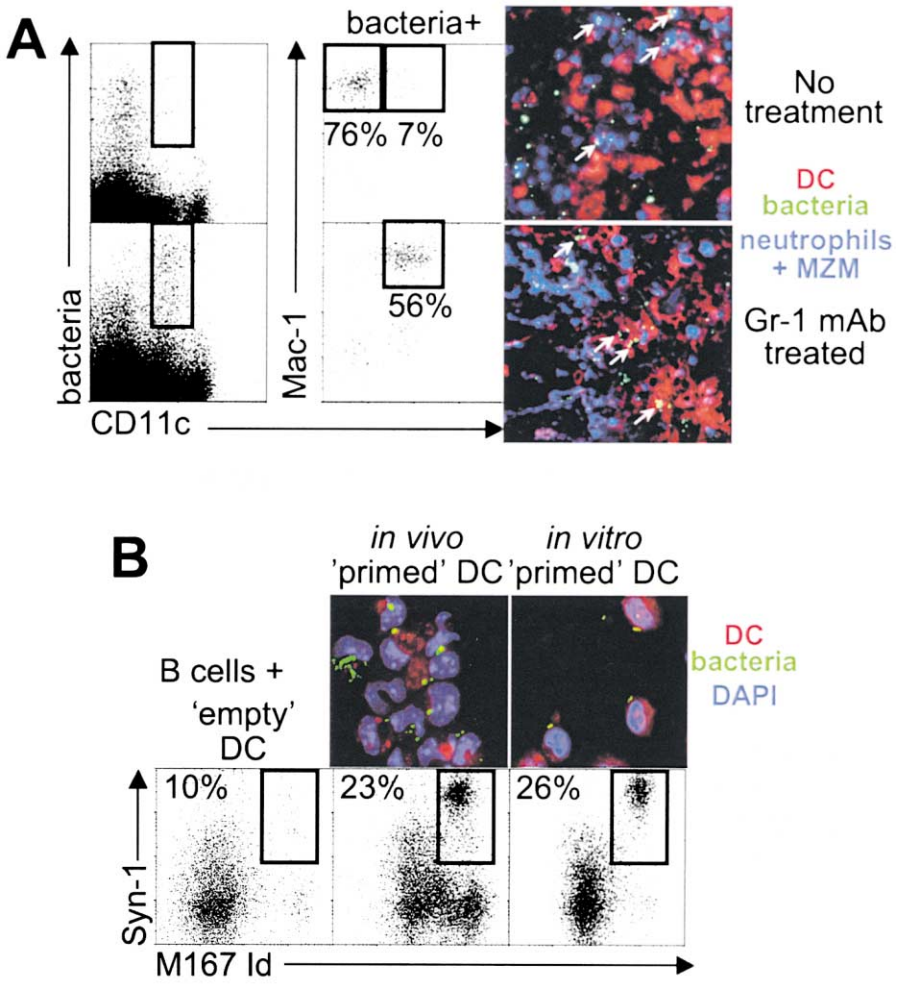

Figure 4. Bacteria-Primed Blood-Derived CD11c^{lo} DC Support Naïve MZ B Cells to Differentiate into Plasmablasts

(A) In vivo depletion of neutrophil granulocytes results in enrichment of bacteria-capturing CD11c^{lo} DC. Untreated or anti-Gr-1 mAb treated C57BL/6 mice were immunized with green fluorescent bacteria and analyzed at 4 hours after bacterial challenge. Sections were stained with antibodies against CD11c (red) and Gr-1 and ER-TR-9 recognizing MZM (Marginal Zone Macrophages) (both subsets are blue).

(B) CD11c^{lo} DC from spleens of neutrophil-depleted mice, 4 hours after in vivo bacteria-priming or blood-derived in vitro bacteria-primed CD11c^{lo} DC were cultured with B cells from M167 mice for 3 days and stained for IgM^{a}, Syn-1, and M167-Id. Profiles show IgM^{a+} gated cells and the numbers represent the percentage of cells with plasmablast (M167^{+}Syn-1^{+}) phenotype. Cytospins of primed DC were stained with CD11c (red), followed by nuclear DAPI counterstaining (blue), while the bacteria are green. Profiles are representative of 3 experiments.

B7.2 molecules, but not of B220 and Gr-1 (Figure 3F and not shown). Fresh sorted (as well as 2 hr LPS activated) CD11c^{lo} blood derived cells were also potent as antigen presenting cells in a mixed lymphocyte reaction (not shown). All these data suggest that CD11c^{lo}Mac-1^{hi} cells belong to the dendritic cell lineage and are exceptionally proficient in blood-borne particulate antigen capture.

Although blood neutrophils were a major population migrating into the spleen and were clearly efficient in the phagocytosis and transport of bacteria in this model, they failed to support survival and differentiation of plasmablasts in the previously described in vitro culture system (Figure 2C).

Temporary removal of neutrophils by in vivo anti-Gr-1 mAb treatment was used to enrich and facilitate the analysis of the small numbers of CD11c^{lo} DC cells in the blood, prior to immunization with fluorescent bacteria. In addition to neutrophils, the Gr-1 treatment also depletes the IFN-secreting plasmacytoid DC subset (Asselin-Paturel et al., 2001); however, it did not affect the magnitude or kinetics of the antibacterial immune response (not shown). The absence of neutrophils enabled better access of CD11c^{lo} DC to Ag in vivo (Figure 4A, left panels), and the number of bacteria-bearing CD11c^{lo} DC was increased (Figure 4A, middle and right panels). To determine the functional capabilities of these cells, we cocultured M167 B220^{+} B cells with FACS-sorted, in vivo-primed, or empty CD11c^{lo} DC from these neutrophil granulocyte-depleted mice. In the same in vitro system, we tested the supporting ability of FACS-sorted blood-derived CD11c^{lo} DC after in vitro bacterial priming. Only in vivo- or in vitro-primed, but not empty, CD11c^{lo} DC promoted the generation of Syndecan-1^{+} Ag-specific plasmablasts from naïve MZ B cells in vitro (Figure 4B).

Taken together, these data showed that blood-derived CD11c^{lo} DC play a necessary and sufficient role in the

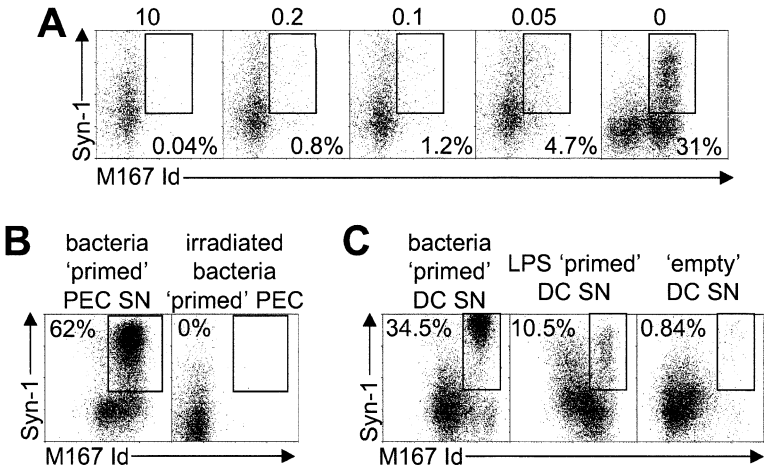

Figure 5. TI Ag-Specific Plasmablast Differentiation Requires DC Derived Soluble Factors and Is Inhibited by TACI-Fc In Vitro

(A) In vitro bacteria-primed PEC cells were pretreated with TACI-Fc fusion protein in decreasing concentrations for 30' at 37°C; B220+ B cells from M167 mice were added and cultured for 3 days. Profiles are representative of 3 independent experiments.

(B) Soluble factors from in vitro-primed PEC, but not membrane molecules, provide the signals for Ag-specific plasmablast differentiation. PEC cells from C57BL/6 mice were cultured for 3 days with intact *S. pneumoniae*. SNs were harvested and filtered and cells were irradiated with 2500 cG. SNs and the irradiated cells were cultured with M167 B220+ B cells for 3 days and analyzed by FACS, as described above. Profiles are representative of 3 independent experiments.

(C) SN from bacteria-primed blood-derived CD11c^{lo} DC support Ag-specific plasmablast generation. CD11c^{lo} DC, FACS-sorted from blood, were cultured with bacteria, LPS, or media for 3 days. SNs were harvested and cocultured with M167 B220+ B cells for a further 3 days, stained, and analyzed by FACS as describe above. Profiles show one representative of 3 independent experiments.

initiation of a TI immune response against blood-borne *S. pneumoniae*.

TI Ag-Specific Plasmablast Differentiation Is Inhibited by TACI-Fc Treatment In Vitro

Having identified the functionally relevant accessory cells involved in TI plasma cell differentiation, we next wished to determine the molecular mechanisms of this DC-B cell interaction. We analyzed the effect of TACI-Fc, which effectively blocks BLyS and APRIL binding to their respective receptors on B cells. In cultures of M167 B220$^+$ B cells and PEC-supportive cells, TACI-Fc, but not a control LFA-3-Fc, inhibited the differentiation of naïve MZ B cells into Ag-specific plasmablasts in a dose-dependent manner (Figure 5A). To analyze whether soluble ligands or membrane contact between supporting cells and B lymphocytes would induce the B cell differentiation, conditioned supernatant (SN) from PEC Mϕ cultured with bacteria for 3 days, or bacteria-loaded, irradiated PEC Mϕ, were added to M167 B220$^+$ B cells. As seen in Figure 5B, Ag-specific plasmablasts were generated in the presence of conditioned SN, but not with irradiated, bacteria-loaded PEC Mϕ. The supportive effect of the SN was blocked entirely by TACI-Fc added to the culture media (not shown). Similarly, successful plasmablast generation occurred when we used day 3 SNs from FACS-sorted, blood-derived bacteria-primed CD11c^{lo} DC (Figure 5C). As a control, SN from LPS-stimulated CD11c^{lo} DC supported polyclonal expansion; however, in this case, the Id+ subset did not dominate the culture.

These data showed that TACI-ligands secreted by supporting cells provided a necessary and sufficient cosignal to Ag-specific MZ B cells to differentiate into plasmablasts in vitro.

TI Ag-Specific Plasmablast Differentiation Is Reduced after In Vivo TACI-Fc-Adenovirus Treatment

To block in vivo the interaction between BLyS and APRIL and their respective ligands, adenovirus-expressed TACI-Fc (TACI-Ad) was injected 2 days before immunizing with bacteria. Using this protocol, high levels (50–100 μg/ml) of circulating TACI-Fc were obtained as early as 2 days after viral administration (not shown).

High and low doses, 2×10^9 and 2×10^8 viral particles/mouse, of TACI-Ad inhibited the differentiation of splenic Ag-specific plasmablasts at 3 days after immunization by ~75% and ~50%, respectively. Control virus (LacZ-Ad) treatment did not affect the anti-PC immune response (Figure 6A). This short-term treatment of TACI-Ad selectively affected only the Ag-activated Id$^+$ MZ B cells, leaving non Ag-specific, bystander follicular and Id$^-$ MZ B cells unaltered (Figure 6A, graph).

To determine whether the observed reduction in plasma cell numbers by day 3 reflected a different kinetics of differentiation, we analyzed the time course of the response of adoptively transferred M167 B cells in C57BL/6 recipients. At each time point, Ag-specific B cell numbers in TACI-Ad treated mice were increasingly reduced as the immune response progressed compared to the response in control virus groups (Figure 6B).

In normal, nontransgenic mice, the antibody response to PC is the net result of a major B1 and a minor MZ B cell derived component. To determine whether soluble TACI-ligands were also crucial for the differentiation of plasma cells from B1 B precursors, we immunized normal BALB/c mice i.v. and i.p. following TACI-Ad and control virus injection. In BALB/c mice, B1 B cells are normally responsible for 85% of the anti-PC response (Martin et al., 2001). The inhibition of plasma cell generation in the spleen after TACI-Ad treatment was similar to that observed when the response was entirely derived from transgenic MZ B cells, and occurred with both i.v. and i.p. immunizations (Figure 6C).

These results confirmed in vivo that the major effect of TACI-Fc was to block DC-B cell interactions in TI responses, and also highlighted the selectivity of short-term TACI-Ad treatment for Ag-activated, but not bystander, B cells.

Inhibition by TACI-Ad Treatment Is Due to Preferential Apoptosis of Ag-Activated MZ-Derived Blasts

The TACI-Ad blockade of plasma cell generation may have been due to an effect on Ag-specific MZ B cell

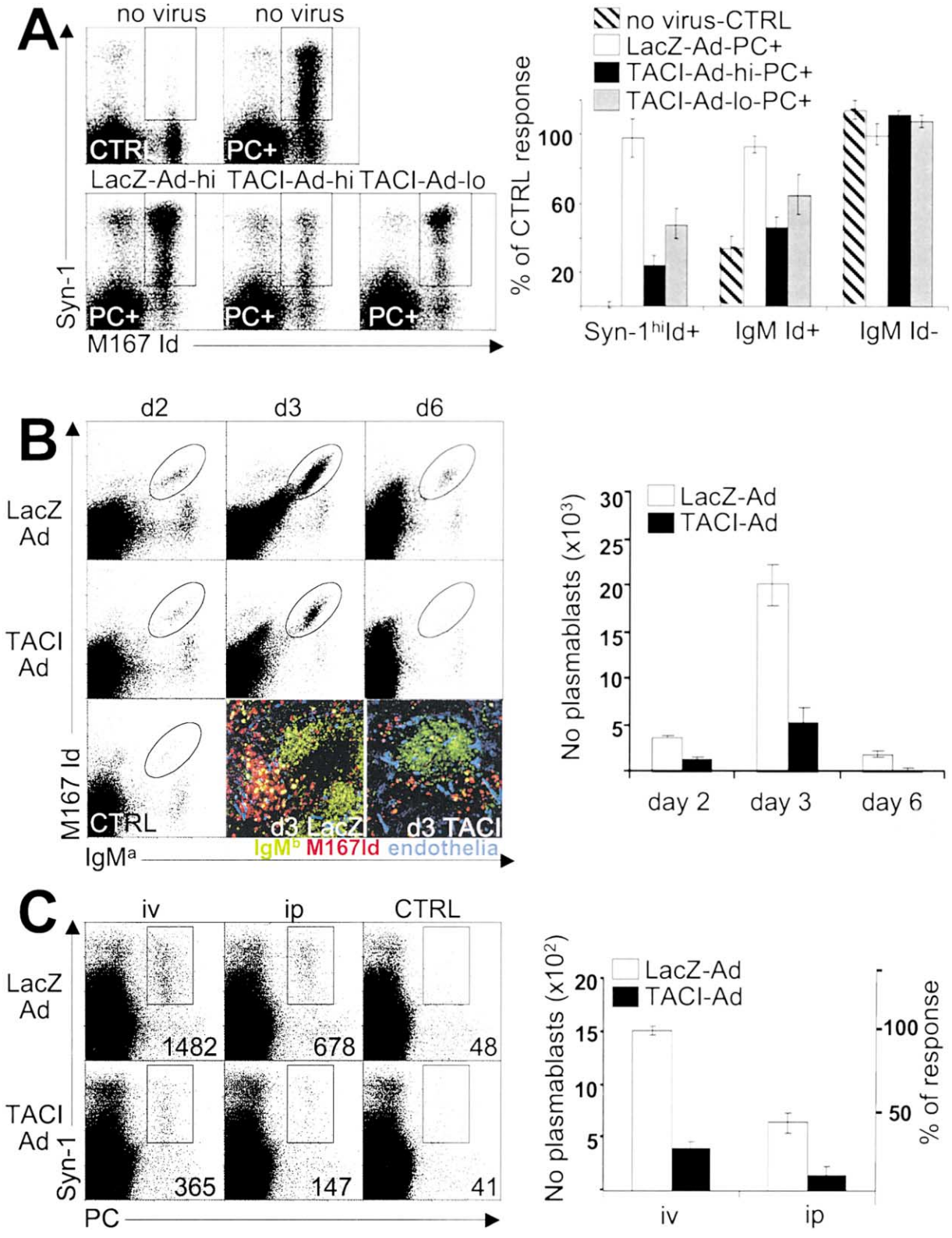

Figure 6. TI Ag-Specific Plasmablast Differentiation Is Diminished after TACI-Ad Treatment In Vivo

(A) M167 mice injected with TACI-Ad or control virus (LacZ-Ad) were immunized with *S. pneumoniae*, sacrificed at day 3 after bacterial challenge, and stained with antibodies against IgM^a, M167-Id, and Syn-1 for FACS analysis. Profiles show splenocytes gated on IgM^{a+} population. Graph displays changes in Ag-specific plasma cell ($Syn\text{-}1^{hi}Id^{hi}$), total Ag-specific B cell (IgM^+Id^+), and non-Ag-specific B cell (IgM^+Id^-) compartments after Ad treatments compared to the regular immune response (no virus treatment).

(B) 2×10^6 MACS-purified M167 B220+ B cells were injected into TACI-Ad and control virus-treated C57BL/6 recipients, which were immunized with bacteria 24 hours later. Mice were sacrificed at days 2, 3, and 6. Transferred Ag-specific MZ B cells were followed by staining for IgM^a and M167-Id. Sections from the same mice were stained with IgM^b (green), M167-Id (red), and IBL-7/1 (blue). Graph shows absolute numbers of Ag-specific plasma cells (IgM^+M167-$Id^{hi}Syn\text{-}1^{hi}$) through the first 6 days of the response.

(C) BALB/c mice injected with TACI-Ad and control virus were sacrificed at 3 days after i.v. or i.p. bacterial immunization. Spleen cells were stained with PC and mAbs against Syn-1 and T15 idiotype (AB1.2).

Profiles are representative of two experiments with 4–5 mice in each group.

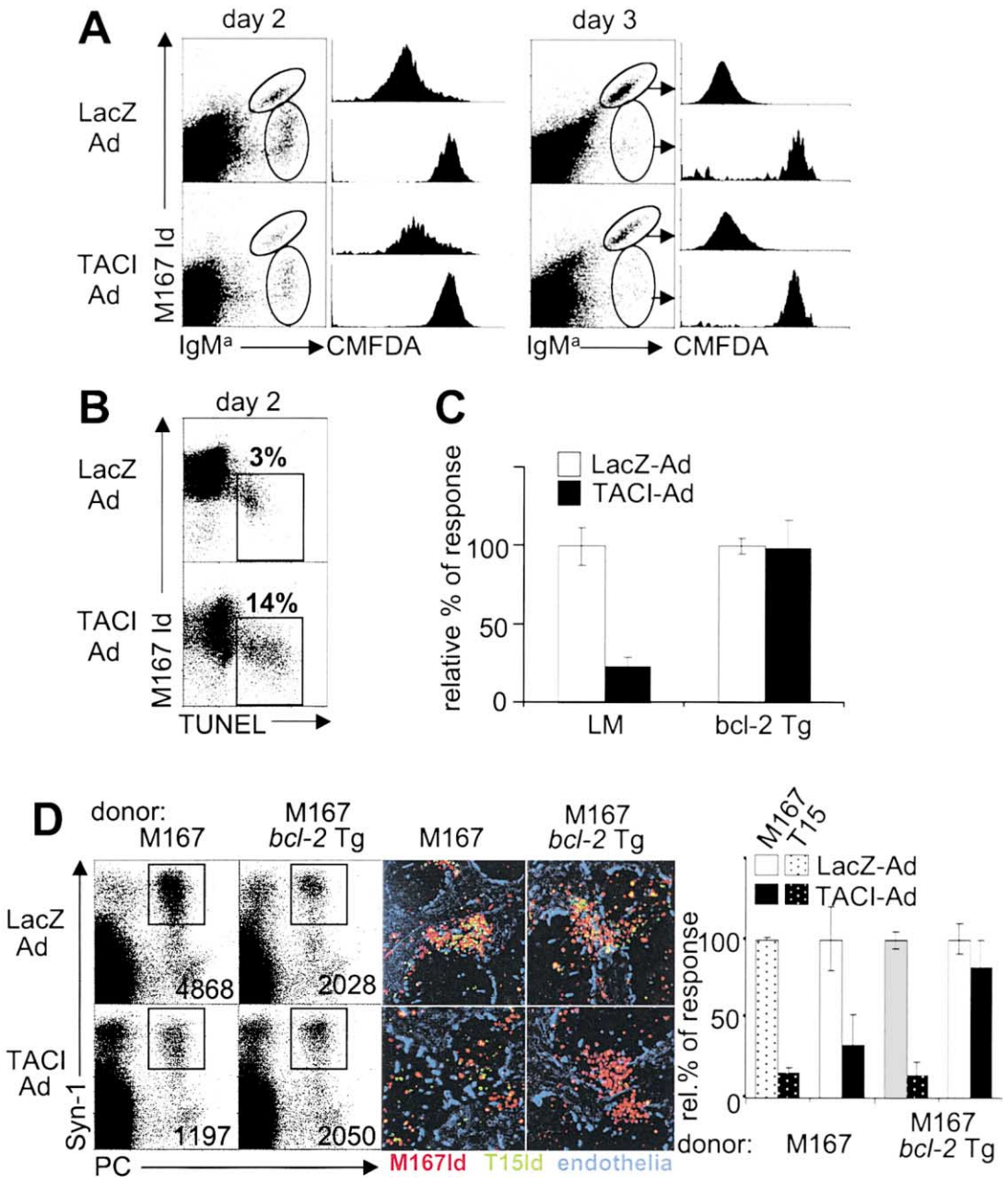

Figure 7. Ag-Specific MZ-Derived Blasts Undergoing Preferential Apoptosis in the Presence of Circulating TACI-Fc Can Be Rescued by Constitutive Expression of *bcl-2* in B Cells

(A) 2×10^6 CMFDA-labeled splenocytes were injected into TACI-Ad and control virus-treated C57BL/6 recipients, which were immunized with *S. pneumoniae* after 24 hours. Recipients were sacrificed at days 2 and 3 and spleen cells were stained with antibodies against IgM^a and M167-Id.

(B) TACI-Ad and control virus-treated M167 mice were sacrificed at day 2 after bacterial immunization, and spleen cells were stained for Syn-1, TUNEL, cytoplasmic IgM, and M167-Id. Plots show TUNEL levels on total plasma cells (Syn-1^{hi} and cytoplasmic IgM^{hi} gated). Profiles are representative of three mice in each group.

(C) B1 B cell-derived anti-PC plasma numbers were rescued from the blocking effects of TACI-Fc by forced expression of *bcl-2* in B cells. TACI-Ad and control virus-treated *bcl-2* Tg C57BL/6 and non-Tg littermate mice were immunized with bacteria and sacrificed at day 3. Graph shows percentage of anti-PC immune response in TACI-Ad-treated groups compared to the control virus ($n = 4$).

(D) The MZ B cell-derived anti-PC response was rescued by *bcl-2* transgenic expression. 2×10^6 M167 *bcl-2* double Tg and M167 littermate splenocytes (containing 5×10^3 M167 Id^+ MZ B cells) and 10^7 filler cells were transferred into TACI-Ad and control virus-treated C57BL/6 recipients, which were immunized with bacteria. Recipients were sacrificed at 3 days after bacterial challenge and spleen cells were stained with PC and mAbs against Syn-1, M167, and T15 idiotypes. Sections from the same mice were stained with antibodies against T15 Id (green), M167-Id (red), and IBL-7/1 (blue). Graph displays percentages of anti-PC immune responses, constituted by two dominant B cell clones (T15 and M167), in TACI-Ad compared to the control virus-treated groups. Profiles are representative of 4 mice in each group.

proliferation or alternatively on B cell survival. To determine whether the inhibition of plasma cell differentiation resulted from arrested proliferation, CMFDA-labeled M167 B220$^+$ B cells were transferred into C57BL/6 recipients previously injected with TACI-Ad or control virus. No striking differences in the CMFDA levels in Ag-specific B cells were found at day 2 or 3 after immunization (Figure 7A), indicating that the reduction in plasmablast numbers was not correlated with a major decrease in proliferation. At all time points, Id$^-$ B cells remained nonactivated, while Ag-specific MZ-derived blasts diluted the CMFDA equally in both control and TACI-Ad treated mice.

We next determined, by TUNEL staining of spleen cells from TACI-Ad injected M167 Tg mice 2 days after immunization, if the decreased plasmablast numbers were due to increased cell death. We found that there was a 4- to 5-fold increase in apoptosis, which occurred preferentially in the Ag-specific plasmablast compartment, after TACI-Ad treatment compared to the control virus-treated groups (Figure 7B).

The upregulation of survival-associated genes is common during lymphocyte activation, permitting the favored clone to expand or differentiate further and form effector cell types (Carey et al., 2000). We next asked whether constitutive overexpression of the antiapoptotic gene, *bcl-2*, would counteract the effect of TACI-Ad on plasmablast differentiationm and we immunized *bcl-2* Tg mice with bacteria after TACI-Ad pretreatment. As a readout for the effects of *bcl-2* on TACI-Fc inhibition, B1-derived (T15 clone) plasmablasts were analyzed at day 3.5 after immunization. Enforced *bcl-2* expression fully rescued the anti-PC plasmablast compartment, suggesting that an antiapoptotic signal was a major component of the differentiation process blocked by TACI-Fc (Figure 7C). To determine if TACI-Ad treatment inhibited MZ-derived plasma cell differentiation by a similar mechanism to that shown for B1 cells, limited numbers of M167/*bcl-2* double Tg B cells were transferred into TACI-Ad or control virus-injected C57BL/6 recipients. Plasmablasts were assayed at day 3 after immunization. In this transfer experiment, the anti-PC plasmablast pool in the recipient is composed of donor-derived MZ B cells, expressing the *bcl-2* Tg and recipient-derived wild-type B1 B cells, without the *bcl-2* Tg. As seen in Figure 7D, donor MZ (M167/*bcl-2* Tg), but not endogenous B1 B-derived (T15), plasmablasts were rescued in the same TACI-Ad treated hosts. The immune response in all *bcl-2* Tg mice was slightly reduced compared to non-Tg littermates, most likely due to the partial inhibition of proliferation by the constitutive hyperexpression of *bcl-2* in B cells (O'Reilly et al., 1997).

These results showed a requirement for a survival signal received from TACI-ligands for both MZ and B1 B cell compartments involved in TI responses.

Discussion

We have identified the cellular participants and the molecular interactions required for the Ag-specific plasmablast differentiation that occur early in the TI immune response against blood-borne particulate Ag. DC and B cells, but not other myeloid cells, were found in the primary clusters of MZ B cell-derived blasts in the bridging channels of the spleen (Figure 1). These blood-derived DC appeared to be the only DC subset in this system with a significant capacity to capture and transport intact bacteria to the spleen in the first few hours after i.v. immunization (Figure 3). This support, both in vitro and in vivo, depended on critical interactions of TACI-ligands with their receptors on MZ and B1 B cells, respectively (Figures 5 and 6). It was previously shown that BLyS signaled the upregulation of survival genes (*bcl-2*, *bcl-X$_L$*) (Do et al., 2000), and here we provide direct in vivo evidence that constitutive overexpression of *bcl-2* rescues plasmablasts from the blocking effects of TACI-Ad treatment.

The seemingly unique functional ability of blood-derived DC to initiate the TI immune response is likely due to their increased ability to phagocytose and migrate (Figure 2E). Early work has clearly shown myeloid/dendritic cells migrating from the bone marrow to the site of inflammatory injury (Oghiso et al., 1992). CD11c$^+$Mac-1$^+$Ly6C$^+$ preimmunocytes were detected in bone marrow and blood, and gave rise to all mature DC subsets and macrophages (Bruno et al., 2001). Also, recent reports have identified immediate precursors for mouse mature DC subsets, characterized by CD11c$^+$ and various levels of B220, Gr-1, Mac-1, and CD4 (del Hoyo et al., 2002; Asselin-Paturel et al., 2001; Shortman and Liu, 2002). Splenic CD11c^{lo}Mac-1^{hi} DC have been described (Daro et al., 2000), but their functional potential in immune responses was not evaluated. The outstanding attribute of these DC seems to be their initial access to blood-borne Ag, due to their presence in the blood stream. These studies raise questions regarding the function of these blood DC. What are the molecular mechanisms permitting this efficient antigen capture, and what is the basis for their superiority over other DC subsets in providing B cell help? Since these DC have greater accessibility than organ-associated DC to blood-borne antigens, it is very likely that they are part of the first line of innate defense mechanisms involved in the capture and presentation of antigen to MZ and B1 B cells.

The molecular basis of this DC-B cell interaction appears to be complex, since it involves at least three receptors (BCMA, TACI, BAFF-R) and 2 ligands (BLyS, APRIL). In several other reports, similar to our data, a partial reduction of antibody levels occurred in both TI (NP-Ficoll, Pneumovax) and TD (NP-CGG, etc) responses (Yan et al., 2001; von Bulow et al., 2001). Although previous studies in TACI$^{-/-}$ mice suggested that this molecule was involved in TI responses, these results (von Bulow et al., 2001) are puzzling, since there were marked alterations in B cell numbers and the appearance of hyperresponsive B cells (Yan et al., 2001). A constant higher level of responsiveness may have resulted in the exhaustion of precursor subsets responsible for TI responses. We have readdressed this problem, using a model in which Ag-specific B cells are normal, the blocking is done in real time, and the Ag-reactive B cell clones were traced throughout the activation period. A possible explanation for the partial inhibition with TACI-Fc treatment is the existence of an as yet undefined ligand/receptor pair, which was not blocked in

these experiments. Future use of receptor-specific antagonists will distinguish between the individual roles of each ligand/receptor pair in TI activation.

Long-term (>2 weeks) absence of circulating BLyS leads to the loss of almost the entire mature B cell compartment (Schiemann et al., 2001; Schneider et al., 2001). Likewise, long-term TACI-Fc treatment leads to the loss of B cells, although it is crucial to note that short-term (within the first six days) administration of TACI-Ad affected mostly Ag-activated B cells (Figure 6A). That B cells in various stages of activation exhibit differential sensitivity to blocking effects on this pathway is crucial, and might provide possible therapeutic manipulation of immune responses and autoimmunity. It is likely that blockade of TACI will inhibit at least the TI component in certain pathological immune responses. In addition, the resolution of the role that APRIL and its receptors play in this reaction is of interest for alternative therapeutic manipulations.

DC have been previously suggested to facilitate B cell functions; however, most of these studies did not directly survey Ag-specific B cell activation. DC were suggested to transport and transfer antigen to naïve B lymphocytes in initiating and modifying TD responses (Wykes et al., 1998; Fayette et al., 1998). In other mouse models, the use of in vitro generated BMDC also suggested a need for DC in B cell activation (Colino et al., 2002), or the in situ association of DC with TI plasmablasts (Garcia de Vinuesa et al., 1999). However, our work defines a DC subset, derived contemporaneously with the in vivo introduction of Ag, that promotes Ag-specific plasma cell differentiation in vitro and in vivo. These findings raise further questions related to T dependent immune responses. Is the DC-B cell interaction we have described a normal component of the primary TD immune response, in which DC-B cell communications may occur in the extrafollicular reaction, in addition to the T-DC and T-B interactions? Additionally, do Mac-1^+CD11c^+ pre-immunocytes in the bone marrow (Bruno et al., 2001) play a supportive role in the survival of long-lived plasma cells associated with TD immune responses?

Infiltrating cells exhibiting a MZ B cell phenotype have been recently described in two distinct, organ-specific autoimmune diseases, involving the thyroid gland in Graves' disease and the salivary gland in Sjogren's syndrome (Segundo et al., 2001; Groom et al., 2002). Higher levels of circulating BLyS were also found in patients with SLE and Sjogren's syndrome (Zhang et al., 2001; Groom et al., 2002). It is likely that DC, by providing constant high levels of soluble TACI ligands, sustain pathological B cell activation and survival, characteristic of these diseases. Our observation that their short-term in vivo blockade preferentially targets the TI Ag-activated B cell compartment also suggests rational therapeutic approaches to autoimmune diseases.

Finally, the versatile, Ag-capturing capabilities of the blood DC described here make them ideal candidates as targets to deliver particulate packaged therapeutic compounds.

Experimental procedures

Animals

8- to 12-week-old C57BL/6, BALB/c, μT-C57BL/6, T $cell^{-/-}$ ($TcR\beta^{-/-}TcR\delta^{-/-}$) C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). μT-C57BL/6 and *bcl-2* Tg *(C57BL/6-TgN (BCL2) 22Wehi)* mice (gift of M.D. Cooper, UAB, Birmingham, AL) were bred with M167-C57BL/6 (gift of Dr. J.J. Kenny, NIH, Washington, DC).

Adoptive Transfer and CMFDA Labeling

2×10^6/injection of M167 splenocytes (in some cases labeled with CMFDA as described earlier [Martin and Kearney, 2000b]) were adoptively transferred into C57BL/6 recipients, followed by i.v. immunization. Mice were sacrificed and analyzed at day 2 and 3. For earlier time points, 20–40 $\times$ 10^6 magnetically sorted M167 B220+ cells were transferred into T $cell^{-/-}$ or C57BL/6 mice and analyzed at day 1.

Immunizations

8- to 12-week-old mice were immunized i.v. or i.p. with 10^8 heat-killed, pepsin-treated *S. pneumoniae* (strain R36A) (Briles et al., 1981) per injection. To track Ag uptake in vivo, *S. pneumoniae* were conjugated with Alexa-488 (Molecular Probes, Eugene, OR) according to the manufacturer's protocol. Ag-specific B cells were identified and traced using a monoclonal anti-idiotypic Ab (M167 Id) or with labeled Ag (PC). Mice were analyzed at various time points (1, 4, and 8 hr; days 1, 2, 3, and 6).

Flow Cytometry and Cell Sorting

FITC-mAbs specific for mouse CD11c, PE-mAbs recognizing mouse markers (CD11c, Syndecan-1, CD11b, CD43), biotinylated mAbs specific for mouse antigens (CD11c, CD4, CD8, CD80, CD86, CD40, CD1, CD44, CD62L, Gr-1, B220), and Streptavidin-APC were purchased from PharMingen (San Diego, CA). Goat anti-mouse IgM-Cy5 was purchased from Jackson Immunoresearch Inc. (West Grove, PA). PC-Dex-FITC and anti-M167-PE and biotin were a gift from Dr. Jim Kenny (NIH, Washington, DC). AB1.2-Alexa-488 (anti-T15 Id), anti-Igh6a (RS3.1-Alexa-488), and anti-Igh6b (MB.86-Alexa-488) were described (Martin et al., 2001). Three- and four-color surface staining and analysis was performed as previously described (Martin et al., 2001). TUNEL was performed using a Fluorescein-labeled In Situ Cell Death Detection Kit (Roche Diagnostics Corporation, Indianapolis, IN).

To sort Mac-$1^{hi}CD11c^{lo}$, Mac-$1^{hi}CD11c^{hi}$, Mac-$1^{hi}CD11^{neg}$, and Mac-$1^{hi}Gr\text{-}1^{hi}$ cells, splenocytes (from 20–40 mice) or blood mononuclear fraction (from 70–120 mice) were magnetically enriched for Mac-1^+ and stained with CD11c-FITC, Mac-1-PE, and Gr-1-biotin mAbs. $CD11c^{hi}$Mac-1^{neg} cells were sorted from spleens magnetically enriched for $CD11c^+$, stained with CD11c-FITC and Mac-1-PE mAbs. In vivo-primed $CD11c^{lo}$ DC were obtained from 20–25 spleens from mice treated with anti-Gr-1 mAb 24 hr prior to immunization with Alexa-488-*S. pneumoniae*. Cells were sorted with a FACStar Plus (Becton Dickinson, Mountain View, CA) or a MoFlo (Cytomation, Ft. Collins, CO).

Immunofluorescence on Tissue Sections

Tissue sections were processed and analyzed as described (Martin et al., 2001). Frozen sections were stained with a cocktail of AMCA-goat-anti-IgM (Vector Laboratories, Burlingame, CA) and PE-M167 and either anti-CD11c or Gr-1 or F4/80 (Caltag, Sac Francisco, CA) conjugated with biotin. After washing, biotinylated Abs were developed with Streptavidin-Alexa 488 (Molecular Probes, Eugene, OR). When Alexa-488 labeled bacteria were traced, we stained the sections with ER-TR-9 (generous gift from Dr. Georg Kraal, specific for mouse splenic MZ macrophages) and anti-Gr-1 mAbs, both developed with goat anti-rat Ig-AMCA (Jackson Immunoresearch Inc, West Grove, PA), blocked with normal rat serum (Peel-Freeze, Rogers, AR), washed, and stained with PE-CD11c. When staining for plasma cells, we stained the sections with a cocktail of biotinylated IBL-7/1 (recognizes marginal sinus and red pulp endothelium, Balazs et al., 1999), PE-M167, and either AB1.2-Alexa-488 or FITC-anti-Igh6b. Biotinylated Ab was developed with Streptavidin-AMCA (Vector Laboratories, Burlingame, CA).

Magnetic Cell Sorting

To enrich for spleen DC cells, typically 5–10 C57BL/6 spleens were incubated with anti-CD11c magnetic beads (Miltenyi Biotec, Auburn, CA), followed by double positive selection on an AutoMACS (Miltenyi

Biotec, Auburn, CA). When isolating CD11c^{lo} cells, enrichment for Mac-1^{+} cells with anti-CD11b magnetic beads and double positive selection were performed. Mac-1^{hi}CD11c^{lo} and CD11^{hi} cells were then FACS sorted as described above. B220^{+} cells from M167 transgenic mice were obtained with greater than 96% purity using anti-B220 beads and single or double positive selection.

In Vitro Cultures and Phagocytosis

2.5×10^5 M167 B220+ cells were plated in 0.2 ml of complete RPMI 1640 media in U-bottom tissue culture plates and cultured alone or with bacteria (10^6/ml) or with LPS (20 μg/ml) or with various myeloid cells (in 1:5 ratio to B cells), unprimed or previously primed (10^7 bacteria/10^6 cells, for 2 hr at 37°C). In blocking experiments, primed PEC cells were preincubated with 0.002–20 μg/ml anti-human TACI-Fc (a gift from Human Genome Sciences) or 20 μg/ml anti-human-LFA-3-Fc (gift of Paul Rennert, Biogen, Cambridge, MA) for 30 min. 3-day supernatants were used from bacteria or LPS-primed FACS-sorted blood CD11c^{lo} DC cells or PEC in cocultures with M167 B220+ cells. After 72 hr, the cells were harvested, washed, and used for FACS. Bone marrow-derived DC from C57BL/6 mice were generated as described (Inaba et al., 1992). FACS-sorted blood CD11c^{lo} cells were cultured with 100 U/ml GM-CSF (Immunex, Seattle, WA) for 24 hr and cells were used for FACS. Phagocytosis assays with and without trypan-blue were performed using Mac-1^{+} magnetically enriched splenocytes and gating on each subset (Ramet et al., 2002).

Construction of the Recombinant Adenoviral Vector Encoding TACI-Fc

A fusion gene construct was obtained from Human Genome Sciences, in which the extracellular domain of TACI was fused with the Fc portion of human IgG1. The cDNA was cloned into an adenoviral shuttle vector from Quantum Biotechnologies, Inc. (Vancouver, Canada). The recombinant adenoviral vector was made by cotransfection of 293 cells with the linearized shuttle vector containing the TACI-Fc with a large fragment of the adenovirus DNA. Recombinant adenoviral vectors were screened by in situ immunofluorescent staining of the plaques with anti-human IgG1 mAb (PharMingen, San Diego, CA).

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