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Ligation of CD27 on B Cells In Vivo during Primary Immunization Enhances Commitment to Memory B Cell Responses

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Ligation of CD27 on B cells has been shown to inhibit terminal differentiation of activated murine B cells into plasma cells. We show in this study that this inhibition is accompanied by an enhanced movement of activated B cells toward differentiation into memory cells. Treatment of mice with anti-CD27 during immunization leads to the generation of greater numbers of Ag-binding B cells in draining lymph nodes that persist for longer periods of time, and they contain a greater proportion of cells of a postgerminal center phenotype. Limiting dilution analyses reveal that they contain a higher frequency of cells that can be stimulated to secrete specific IgG, and adoptive transfer experiments confirm that they can generate higher secondary responses in carrier-primed recipients. Remarkably, significant secondary responses are also seen following primary immunization with a T-independent Ag in the presence of anti-CD27, confirming that ligation of CD27 on B cells during priming induces differentiation into the memory lineage. Treatment with anti-CD27 during priming also increases the average affinity of the secondary response, suggesting that high affinity clones generated early in a primary response may normally differentiate preferentially into plasma cells and are rescued from this fate by CD27 ligation. Anti-CD40 treatment shows similar effects in vivo. However, unlike CD27, CD40 coligation also enhances proliferation, survival, and isotype switching of LPS-stimulated B cells, suggesting that the two receptors may enhance commitment to B cell memory by different mechanisms, or that a common mechanism is used through both receptors that does not involve cell cycle control or survival. The Journal of Immunology, 2003, 171: 5876–5881.

Contact with specific Ag and cognate T cell help induces naive B cells in peripheral lymphoid organs to proliferate, undergo isotype switching and somatic mutation, and differentiate into plasma cells and memory cells. Many of these events occur in germinal centers (GCs), where clonal expansion, positive selection of high affinity clones, negative selection of potentially self-reactive clones, and commitment to the plasma or memory cell lineages occur (1–5). Various members of the TNFR family have been implicated in these processes directly or indirectly. Thus, they are required for the correct spatial arrangement of B and T cells within lymphoid organs and for the formation of GCs, and they can also directly influence cell proliferation and cell death (6–10). CD27, a lymphoid cell-specific TNFR, is expressed on a substantial proportion of human PBLs and appears to be a marker for human memory B cells (11–13). However, it is expressed on only a small proportion of murine B cells, and it is found predominantly in GCs and at sites of chronic B cell stimulation such as tonsils and Peyer’s patches (Refs. 14 and 15; our unpublished observations), suggesting that CD27 may identify a recently activated population of B cells in murine lymphoid organs. Ligation of CD27 on murine B cells stimulated in T-independent or T-dependent cultures has been shown to inhibit terminal differentiation of activated B cells to plasma cells (16, 17), and, as shown earlier with CD40 coligation, the effect seems to be mediated by an inhibition of Blimp-1 transcription (16, 18). Because terminal differentiation and memory cell generation are mutually exclusive events for an activated B cell, this raises the possibility that CD27 signaling may help commit B cells into the memory lineage. We have examined this possibility, and we report that CD27 ligation during primary immunization does increase the pool of Ag-specific secondary B cells in vivo by a B cell-specific mechanism that does not involve alteration of cell cycle or survival properties. We also report that cells rescued from early differentiation into plasma cells can enhance the average affinity of a secondary response.

Materials and Methods

Reagents

The following reagents were used: PE (Cyanotech, Kailua-Kona, Hawaii); 4-hydroxy-3-nitrophenylacetyl (NP) chicken γ-globulin (NP-CGG), NP-OVA, NP-BSA, and NP-Ficoll (Biosearch Technologies, Novato, CA); OVA (Sigma-Aldrich, St. Louis, MO); CFA (Difco Laboratories, Detroit, MI); alum (Superfos Biosector a/s, Kvistgaard, Denmark); LPS (Sigma-Aldrich or Difco); fluorescein/biotin/Cychrome anti-B220, fluorescein/biotin anti-CD38, PE anti-IgM, fluorescein anti-IgG1, biotin anti-CD138, PE CD44, anti-IgD, biotin anti-Thy-1.2, CyChrome streptavidin, azide-free anti-CD27, anti-CD40 (clone HM40-3), and anti-CD48 (BD PharMingen, San Diego, CA); fluorescein peanut agglutinin (PNA) (Vector Laboratories, Burlingame, CA); goat anti-mouse Ig, and biotin anti-mouse IgM, IgD, IgG1, IgG2a, and IgG2b (Southern Biotechnology, Birmingham, AL); PE streptavidin and fluorescein streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA); and streptavidin HRP (Genzyme, Cambridge, MA).

Abbreviations used in this paper: GC, germinal center; CGG, chicken γ-globulin; LDA, limiting dilution analysis; LN, lymph node; NF, 4-hydroxy-3-nitrophenylacetyl, PNA, peanut agglutinin.
Mice and immunizations

Six- to ten-week-old BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were used and maintained in the Small Animal Facility of the National Institute of Immunology, were used. Mice were immunized with 5 μg of PE or NP-CGG in CFA in the hind footpad or with 100 μg of NP-Ficoll i.p. for primary responses and with 100 μg of NP-OVA on alunum for secondary responses. Where indicated, groups of mice were treated i.p. with saline or with 100 μg of azide-free anti-CD27, anti-CD40, or anti-CD48 on the day of immunization. Approval from the Institutional Animal Ethics Committee was obtained for all experimental procedures involving animals.

B cells

Single cell suspensions of cells from peripheral lymph nodes (LN)s or spleen were obtained by mechanical disruption, and erythrocytes in the splenocyte population were lysed by treatment of the cell pellet with Geyer’s solution. B cells were enriched by treatment of the cell suspension with biotinylated anti-B220 or anti-IgD, followed by streptavidin or anti-rat IgG magnetic beads (Miltenyi Biotec, Auburn, CA), respectively, and separation on MACS columns (Miltenyi Biotec), according to the manufacturer’s instructions. Purified cells were routinely ≥90% pure by flow cytometric analysis.

Limiting dilution analysis (LDA)

Unfractionated lymphocytes or purified B cells from draining LN were titrated in 96-well flat-bottom plates (Falcon, Franklin Lakes, NJ) from 10^3/well to 100/well (1 plate/cell input) in Click’s medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM 2-ME, and antibiotics (Life Technologies, Rockville, MD). A total of 10^6 thymocytes from normal mice was added as a source of flier cells to all wells. The cultures were stimulated with 10 μg/ml of LPS for 7 days (12 wells in each plate were unstimulated controls), and culture supernatants were assayed for specific Ab by ELISA. Wells that showed an absorbance >3 times that of unstimulated controls in each plate were considered positive for Ab. Estimates of total Ig served as a normalizing control for LDA sensitivity.

Adoptive transfer

For adoptive transfer of primed B cells, draining LN were collected from mice immunized 2 wk earlier with NP-CGG or 6 days earlier with NP-Ficoll, and 10^7 cells were transferred i.p. into recipients that had been immunized with 100 μg of OVA in CFA 2–3 mo earlier and irradiated (600 rad from a Co^60 source) 18 h earlier. For adoptive transfer of T cells, T cells were purified from the draining LNs of mice immunized with OVA in CFA by MACS separation using biotin anti-Thy-1.2 and streptavidin-coupled beads, and transferred i.p. into mice primed with NP-Ficoll. Recipients were challenged with 100 μg of NP-OVA on alunum i.p.

ELISA

Ninety-six-well flexiplates (Falcon) were coated with PE (10 μg/ml), NP-BSA (1 μg/ml), or goat anti-mouse Ig (2 μg/ml). The plates were blocked with PBS containing 1% defatted milk and loaded with culture supernatant or diluted sera, and bound Ig was detected with biotinylated secondary Abs, streptavidin peroxidase, H_2O_2 (Merck, West Point, PA), and o-phenylenediamine (Sigma-Aldrich). Absorbances were read at 490 nm. Inhibition ELISAs to calculate average Ab affinity were done, as described earlier (19). Briefly, serial dilutions of soluble inhibitor (NP, NP-BSA) were added to plates coated with 1 μg/ml of NP-BSA, and selected dilutions of serum samples, determined previously to contain equivalent amounts of anti-NP Ab, were added. The percent inhibition at each inhibitor concentration was calculated, and the inhibitor concentration required for 50% inhibition was expressed as the IC_50.

Western blots

Cell pellets were lysed in cold buffer containing 1% Nonidet P-40, 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM MgCl_2 (Sigma-Aldrich), 10 μg/ml leupeptin, 10 μg/ml apropin, and 1 mM sodium orthovanadate (Boehringer Mannheim, Indianapolis, IN). An equal volume of 2X SDS loading buffer was added, and the lysates were sonicated for 5 s to decrease nuclear viscosity. Extracts from 0.5–1.0 × 10^6 cells were resolved by SDS-PAGE and blotted with rabbit anti-mouse Bcl-x (R&D Systems, Minneapolis, MN; a kind gift of A. Sarin (National Center for Biological Sciences, Bangalore, India)), followed by anti-rabbit HRP (Santa Cruz Biotechnolgy, Santa Cruz, CA). Bands were detected with ECL (Amersham Pharmacia Biotech, Little Chalfont, U.K.). Blots were stripped and reprobed with mouse anti-actin, followed by anti-mouse HRP (OncoGene Research Products, Damstadt, Germany).

Flow cytometry

Cells were incubated with appropriate staining reagents in buffer containing 0.1% sodium azide (Sigma-Aldrich) and 1% FBS for 45 min on ice. Samples were run on an Elite ESP (Beckman Coulter, Fullerton, CA) or a BD-LSR (BD Biosciences, San Jose, CA) flow cytometer. For identifying PE-specific B cells, between 0.3 and 0.5 million events were acquired for each sample. For determination of cell cycle progression, cells in cold PBS were permeabilized with ice-cold 70% ethanol, and treated with 5 μg/ml propidium iodide (Sigma-Aldrich) in PBS. Data were analyzed with FlowJo software (Treestar, San Carlos, CA).

Results and Discussion

Immunization of mice in the presence of anti-CD27 leads to the generation and persistence of greater numbers of Ag-binding cells in vivo

We have shown earlier that ligation of CD27 during B cell priming inhibits primary Ab responses in vivo (16) raising the possibility that a greater proportion of activated cells may be directed to the memory lineage. To address this, we immunized mice with PE in the presence or absence of anti-CD27 and tracked Ag-specific B cells over time. Mice immunized with PE mount a vigorous T-dependent B cell response and, as reported earlier (20), Ag-specific B cells can be visualized in draining LN by flow cytometry, as shown in Fig. 1(A and B), allowing calculations of Ag-specific B cell proportions (B220+, PE+) over time, their entry into GCs (B220+, PE+, PNA<sup>high</sup>), and the generation of secondary B cells (PE+, CD38<sup>high</sup>, IgM<sup>low</sup>, or IgG<sup>+</sup>) (21, 22). We found that the proportions as well as numbers of PE-binding B cells were similar in mice immunized in the presence or absence of anti-CD27 1 wk after immunization, and that similar proportions were of a GC phenotype (Fig. 1, C and D). By 2 wk, however, there were greater proportions of PE-specific cells in the anti-CD27–treated group, and this difference persisted for up to 6 wk (Fig. 1D). This group also had greater proportions of PE<sup>+</sup>IgM<sup>low</sup>CD38<sup>high</sup> cells (Fig. 1, E–H), a phenotype that identifies post-GC prememory B cells (21). This population constituted 29.3% of Ag-binding B cells in control mice and 41.3% in mice immunized under cover of anti-CD27 on day 7 (Fig. 1G), and 33 and 72%, respectively, on day 21 (Fig. 1H).

Immunization of mice in the presence of anti-CD27 enhances the proportion of restimulable Ag-specific B cells

We next addressed the issue of whether the increased numbers of Ag-binding B cells seen in mice immunized under cover of anti-CD27 can generate a higher secondary response upon subsequent encounter with Ag: in LDA s in vitro and in adoptive transfer experiments in vivo. To this end, mice were primed with NP-CGG in the presence or absence of anti-CD27, and 2 wk later the proportion of Ag-specific B cells in draining LNs was scored in the two assays. First, cells from each group were stimulated in LDA using the approach of polyclonal stimulation, followed by an Ag-specific ELISA (23, 24). As seen in Fig. 2A, the frequency of cells that can be stimulated by LPS to secrete anti-NP IgG is higher in mice that were immunized in the presence of anti-CD27 (frequency of 1/2 × 10<sup>4</sup> in the anti-CD27–treated group and 1/1.8 × 10<sup>4</sup> in controls). As expected, the frequency of NP-specific cells capable of secreting IgG in naive mice was very low (<1/10<sup>10</sup>).

Cells from the two groups, and from naive controls, were next adoptively transferred into irradiated, OVA–primed mice. The recipients were immunized with NP-OVA, and sera collected 6 days later were tested for anti-NP Ab. As shown in Fig. 2B, a higher secondary response is seen in recipients if the donor mice were immunized under cover of anti-CD27. No response was seen in recipients of naive cells at this time. Adoptive transfer of primed
cells from C57BL/6 mice into OVA-primed syngeneic B cell-deficient (μMT) mice or into naive C57BL/6 mice along with OVA-specific T cells from OT.II TCR transgenic mice showed similar results (data not shown), suggesting that the phenomenon is representative of murine in vivo responses to Ag. Interestingly, when we measured the average affinity of the secondary anti-NP Ab response by inhibition ELISA, we found that the IC_{50} values were much lower if primary immunization had been done under cover of anti-CD27 (Fig. 2, C and D), indicating that rescue of B cells from terminal differentiation very early in the primary response with anti-CD27 increases the average affinity of the secondary response in adoptive hosts. These data suggest the possibility that high-affinity clones generated early in the immune response may tend to undergo terminal differentiation to plasma cells rather than differentiating to the memory lineage, and are rescued from this fate by CD27 ligation. This is in keeping with other hypotheses suggesting that strength of signal may determine B cell differentiation into the memory lineage (27), it remained possible that administration of anti-CD27

CD27 ligation enhances secondary B cell responses in vivo by a direct effect on B cells

We have previously shown that anti-CD27 prevents plasma cell differentiation in T-independent cultures in vitro (16). However, because CD27 is also expressed on activated T cells, and its absence has been shown to affect T cell expansion and memory generation (27), it remained possible that administration of anti-CD27
in vivo may affect T cell priming, so that its effect on enhancement of secondary B cell responses may be mediated by enhanced or altered T cell responses rather than by its direct effect on B cell differentiation. To test this possibility, we examined the effect of treating mice with anti-CD27 during immunization with a T-independent Ag on the response to a subsequent T-dependent challenge. For this, mice were immunized with NP-Ficoll in the presence or absence of either anti-CD27 or a negative control Ab, anti-CD48. Treatment with anti-CD27, but not with anti-CD48, inhibited the primary response to NP-Ficoll (Fig. 3A). This confirms and extends our earlier observation that CD27 ligation inhibits terminal differentiation by a direct effect on B cells in vitro (16). Six days after immunization, spleen cells from these mice, and from naive control mice, were adoptively transferred into irradiated OVA-primed mice, and the recipients were immunized with NP-OVA on alum, as described above. As expected, negligible amounts of anti-NP IgM were detected in recipients of naive cells, and only marginal increments were contributed by transferring spleen cells from mice immunized with NP-Ficoll alone or with anti-CD48 treatment. However, treatment with anti-CD27 during primary immunization led to a substantial increase in the anti-NP IgM response in recipients by day 13, indicating that B cells rescued from terminal differentiation in the primary response can indeed mount a sustained secondary response. These data indicate that immunization with NP-Ficoll generates a good primary Ab response, but a poor restimulable B cell population, and that CD27 ligation in vivo induces this population even during such a T-independent response.

IgG responses were barely detectable in the recipients at this time. Since this could be because the number of Ag-specific cells successfully transferred was very small, we modified the adoptive transfer approach to assess IgG memory generation. Instead of transferring B cells from NP-Ficoll-primed mice into carrier-primed recipients, we adoptively transferred OVA-primed T cells into naive mice or variously treated NP-Ficoll-primed mice, and followed the transfer by immunization with NP-OVA. We found (Fig. 3, C and D) that naive mice mounted a good T-dependent Ab response, with IgG dominating the response by day 13 after immunization. However, mice that had been previously immunized with NP-Ficoll did not respond very well to the secondary challenge. IgM from the primary immunization could be detected in these mice at the time of secondary immunization, but the levels did not increase further, and 2 wk after immunization IgG levels were far lower in the previously immunized mice than in previously naive controls. This is likely to be due to the creation of a hole in the NP-specific B cell repertoire by depletion of such cells during the T-independent primary response. In striking contrast, mice that had been immunized with NP-Ficoll in the presence of anti-CD27 mounted a robust secondary response to NP-OVA immunization, and substantial levels of IgG were detectable even by day 6. Our data indicate that CD27 ligation during priming inhibits primary Ab responses and may direct activated cells into a pre-memory pool early in the immune response. CD27 is not expressed.
on dendritic cells or macrophages, and its expression on T cells is tightly controlled with up-regulation seen only when the cells are stimulated through the TCR. It is therefore unlikely that T cells are activated by the NP-Ficoll immunization and that the effect of anti-CD27 treatment is B cell specific. However, we cannot completely rule out the possibility that cells other than B cells may be affected by anti-CD27 in vivo and may contribute to the effect.

**Anti-CD27 and anti-CD40 have similar effects on B cell differentiation to memory in vivo**

CD40 is another member of the TNFR family, and it shares at least two features with CD27: both lack a death domain in their cytoplasmic tails, and signals through both inhibit terminal differentiation of activated B cells (16, 18). Because signals through CD40 are thought to be crucial for memory B cell responses, we compared the effect of ligation of the two molecules following immunization with PE. We found that mice immunized under cover of either anti-CD27 or anti-CD40 had almost twice as many PE-binding cells in their draining LN as control mice did (Fig. 4, A–C) and that the frequency of B cells that could be restimulated in vitro was higher in both treated groups (1/4.5 × 10^4 in controls, 1/1.8 × 10^4 in the anti-CD27-treated group, 1/1.1 × 10^4 in the anti-CD40-treated group; Fig. 4, D–F). These data indicate that signal transduction through CD27 and CD40 has similar effects on commitment to the memory lineage.

**CD27 and CD40 have differential effects on B cell activation**

The in vivo data indicate that ligation of either CD27 or CD40 during primary immunization can enhance memory B cell generation. Terminal differentiation of B cells into plasma cells is associated with an exit of activated cells from cell cycle, and this appears to be mediated by repression of c-myc gene expression by Blimp-1 (28). Signaling through both CD27 and CD40 is known to inhibit Blimp-1 transcription and plasma cell generation, suggesting that the enhanced commitment to memory may be linked to regulation of cell cycle exit or survival. We therefore examined the effects of CD27 and CD40 ligation on these events over a 6-day period in polyclonally stimulated B cells. As shown in Fig. 5, B cells proliferated extensively at 48 h in the presence of anti-CD40 (C, F, and I) for 6 days, analyzed for the expression of IgG1 (A–C), CD44 (D–F), and CD138 (G–I), Western blots for Bcl-XL and actin and the ratio of the two densities in extracts of cells stimulated with LPS ± anti-CD27 or anti-CD40 for various times. Data are representative of two experiments.
both groups than in cultures stimulated in the presence of anti-CD40 (data not shown).

Furthermore, a significant proportion of cells stimulated in the presence of anti-CD40 expresses IgG1 (Fig. 6, A–C) and a greater proportion is CD44hi (Fig. 6, D–F) on day 6. Neither phenotype is seen in cells stimulated in the presence of anti-CD27. However, as expected from earlier findings (16, 18), signaling through either CD27 or CD40 inhibited the generation of plasma cells (Fig. 6, G–I). Almost 9% of cells stimulated with LPS alone expressed high levels of CD138, compared with 1.6 and 1.2% in cultures containing anti-CD27 and anti-CD40, respectively. However, cell yields on day 6 were greater in cultures containing anti-CD40, and the actual numbers of plasma cells generated were 9.8 × 10^5 (LPS alone), 1.9 × 10^5 (LPS + CD27), and 3 × 10^5 (LPS + CD40). Thus, although both CD27 and CD40 change the balance of the plasma cell to B cell ratio, CD40 ligation during B cell stimulation is likely to inhibit primary Ab responses less than CD27 ligation. We also looked at the up-regulation and persistence of various pro- and antiapoptotic proteins over time in the stimulated cultures. Although anti-CD40 caused a dramatic up-regulation of Bel-2, anti-CD27 did not (Fig. 6J). Anti-CD27 did not affect levels of cytoplasmic inhibitor of apoptosis protein-1, cytoplasmic inhibitor of apoptosis protein-2, Bax, or Bim either (data not shown).

Together, our data suggest that signaling through CD27 or CD40 during B cell priming inhibits primary Ab responses and enhances the generation of secondary B cells. Such cells are demonstrable in vivo relatively soon (within 2 wk) after immunization and can be scored as cells capable of secreting secondary Ab responses that memory lineage B cells, with evidence of somatic mutations and antigen maturation, can be detected in the spleen within 7–8 days after primary immunization (29). The enhanced secondary responses are seen in the face of a depressed primary response, suggesting that anti-CD27 treatment does not simply boost B cell responses in vivo. Although CD40 has multiple effects on B cells, including effects on B cell activation, proliferation, and survival, CD27 seems to have a restricted and unique effect that directly skews B cells away from terminal differentiation and into a restimulable and persisting pool. Being more specific, it will hopefully serve as a handle for dissecting the molecular events a restimulable and persisting pool. Being more specific, it will hopefully serve as a handle for dissecting the molecular events.

**References**