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Contrasting the Roles of Costimulation and the Natural Adjuvant Lipopolysaccharide During the Induction of T Cell Immunity

Joseph R. Maxwell,* Carl Ruby,† Nancy I. Kerkvliet,† and Anthony T. Vella²*

The requirements for circumventing tolerance induction in favor of memory T cell development are poorly understood. Although two signals (Ag and costimulation) are necessary to drive effective T cell clonal expansion, few memory T cells remain after the response wanes. The adjuvant LPS can increase numbers of long-lived Ag-specific T cells, but its mechanism of action is not understood. In this report, it is shown that LPS, when combined with two-signal stimulation, profoundly enhances T cell survival in vivo. This survival does not appear to be dependent on the cytokines TNF-α, IL-1β, IL-6, and IFN-γ, nor is it dependent on the transcription factor NF-κB. However, in vivo proliferation of NF-κB-deficient T cells was comparable to that of wild-type T cells, yet their early accumulation in the lymph nodes was severely reduced unless the mice were treated with LPS and an agonistic CD40 mAb. Most importantly, we found that activation of two different costimulatory signals, CD40 and OX40, could not substitute for LPS in rescuing T cells from peripheral deletion. Perhaps surprisingly, these data show that LPS delivers a qualitatively different signal than multiple costimulatory signals. *The Journal of Immunology, 2002, 168: 4372–4381.

A major concept in the study of T cell activation is the two-signal hypothesis first proposed by Bretsch and Cohn (1). After modifications by others, it is generally believed that specific recognition of Ags/MHC complexes by the TCR is not enough to drive a productive and long-lasting T cell response (2, 3). Only concurrent delivery of a costimulatory signal to the T cell will facilitate rapid clonal expansion of the Ag-specific T cell population and differentiation of those cells into effectors (4–6). Although CD28 is the most studied costimulatory molecule, ligation of many other cell surface molecules such as CD40, OX40, and 4-1BB has been described as promoting enhanced T cell clonal expansion in vivo (7–9). After the clonal expansion phase, the majority of the responding T cells undergo apoptosis, leaving behind a small cohort of memory T cells that can respond faster and more potently upon Ag re-exposure (10, 11).

Although the two-signal hypothesis effectively describes T cell activation, it does not fully address how long-lived memory T cells develop and stay alive. It is certain that memory T cell responses can develop after two-signal stimulation (8, 12), but it is not yet clear whether these responses are optimal or what factors direct T cells into a surviving state. A variety of poorly understood compounds known as adjuvants can enhance an immune response and may be central to uncovering how T cells survive.

LPS, a natural adjuvant, is well known for having proinflammatory properties. LPS is a component of Gram-negative bacterial cell walls that induces potent inflammatory responses (13). Binding of LPS to CD14 or Toll-like receptors on cells triggers the secretion of reactive oxygen species and inflammatory cytokines such as TNF-α, IL-6, and IL-1β (14, 15). Additionally, LPS can activate the transcription factor NF-κB, a molecule that is well documented in its ability to trigger cellular proliferation and cytokine secretion among many other effects (16, 17).

LPS was previously shown to promote the long-term survival of T cells stimulated with the superantigen (SAg)³ staphylococcal enterotoxin A (SEA) or with nominal peptide Ags (8, 18). This survival effect could develop in the absence of CD28 signaling and was at least partially dependent or partially substituted by proinflammatory cytokines like TNF-α, IL-1β, and IFN-γ (19).

Subsequent work with LPS found that, although it promoted significant levels of T cell survival, its effects were synergistic when coupled with OX40 costimulation in the presence of either SEA or OVA as the Ag (8). Delivery of these three signals not only enhanced T cell expansion compared with delivery of two signals in the form of Ag and costimulation, but it significantly enhanced the number of T cells expressing a memory phenotype for at least 2 mo. Thus, delivery of a natural adjuvant like LPS in the context of the two-signal model can potentiate T cell clonal expansion, but even more importantly, it can drive the long-term survival of those responding cells.

In this paper, we sought to better understand the mechanism of how LPS was working in a three-signal model of T cell stimulation involving TCR ligation (signal 1), CD40 stimulation (signal 2), and LPS (signal 3). SAgS like SEA have proven very useful when studying clonal expansion and tolerance of T cells in vivo (20, 21). Their specificity for T cells possessing particular variable β-chains provides a reliable method for tracking T cells responding to the SAg in vivo (22). CD40 is a member of the TNFR family that is found primarily on APCs (23–25) and that upon stimulation can
enhance the activation and survival of APCs during an immune response (26–28). SEA and CD40 stimulation together enhance T cell clonal expansion in a CD28-dependent manner, but this is followed by profound deletion (7). We show that LPS stimulation in conjunction with SEA and CD40 stimulation markedly enhanced T cell survival in vivo. Interestingly, the survival effect was not a result of enhanced co-stimulation, because combined CD40 and OX40 costimulatory signals could not substitute for LPS. We found that LPS-dependent T cell survival did not rely on proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and IFN-γ. Moreover, LPS-independent short-term T cell survival was NF-kB dependent, but inclusion of LPS could rescue T cells in the short and long term in the absence of NF-kB. The results presented in this paper begin to define a third signal, which cannot be substituted for by two different costimulatory signals and is NF-kB independent.

Materials and Methods

Mice

B10.A mice were purchased from the National Cancer Institute (Frederick, MD). C57BL/6, TNFR1 knockout (KO), and B6, D2-TgN(LCK-NFKBIA)5Dwb mice (30) (hereafter referred to as inhibitory protein that dissociates from NF-kB α transgenic (IκB-α Tg) mice) were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in our animal facility at Oregon State University under specific pathogen-free conditions in accordance with federal guidelines. All mice were between 6 and 12 wk of age.

Reagents, mAbs, and flow cytometry

SEA, staphylococcal enterotoxin B (SEB), LPS, and rat IgG were purchased from Sigma-Aldrich (St. Louis, MO) and administered to mice as i.p. injections in balanced salt solution (BSS) or PBS. The recombinant human IL-1Ra was a kind gift from Angen (Boulder, CO).

The anti-CD40 mAb-producing hybridoma FGK45.5 was a kind gift from Dr. A. Rolink (Base Institute, Basel, Switzerland) (31). The anti-IFN-γ-producing hybridoma XMG1.2 (32), the anti-TNF-α-producing hybridoma MP6-XT22 (33), and the anti-IL-6-producing hybridoma MP5-20F3.11 (34) were all obtained from the American Type Culture Collection (Manassas, VA) with anti-IL-6 being obtained with permission from DNAX (Palo Alto, CA). Supernatants from each of the above hybridomas were purified over protein G agarose (Life Technologies, Grand Island, NY) and dialyzed against PBS for injection.

For flow cytometric staining, Abs purchased from BD Pharmingen (San Diego, CA) were used: anti-CD4 was conjugated to either PE or APC, anti-CD8 was conjugated to either PE or APC, and anti-TCR Vβ8 was conjugated to FITC. The anti-TCR Vβ3 mAb KJ22-607.7 (35) was purified from hybridoma supernatant over protein G agarose (Life Technologies) and conjugated to FITC as described previously (36). Briefly, purified Ab was dialyzed against 0.1 M NaHCO3, pH 9.4–9.6. Protein concentration was adjusted to 1 mg/ml and incubated with FITC-Celite (Sigma-Aldrich) for 30 min at room temperature. Free celeite was removed by centrifugation and the Ab was dialyzed against PBS for use.

Injection schedule

All injections were i.p. The injection of the SAgs SEA and SEB was considered day 0. Injection of anti-CD40 mAb was done 2 days before SAg injection. LPS was always injected 24 h after the SAgs (day +1). The anti-OX40 mAb was injected 24 and 36 h after SAg injection. For the cytokine blocking experiments in Fig. 5, b and c, we used the neutralizing mAbs anti-TNF-α, anti-IL-6, and anti-IFN-γ, as well as IL-1Ra. These reagents were injected 20 and 30 h after SEA.

Cell processing and flow cytometry

Spleens and peripheral lymph nodes (inguinal, axillary, and bronchial) were teased through nylon mesh sieves (Falcon, BD Pharmingen) and RBCs were lysed with ammonium chloride. After washing, cells were counted with a Z1 particle counter (Beckman Coulter, Miami, FL), and spleen cells were teased through nylon wool as described previously (37). Briefly, 3-ml syringes were filled with 0.12–0.15 g of washed and brushed nylon wool. The columns were prepared with warm BSS 5% FBS, after which the cells were loaded in a 0.5-ml volume and incubated for 30 min at 37°C. After draining 0.5 ml away, the columns were incubated for an additional 30 min, followed by elution with BSS 5% FBS.

For two- and three-color staining, cells were incubated on ice with the primary Abs in the presence of 5% normal mouse serum, culture supernatant from hybridoma cells producing an anti-mouse FcR mAb, 2.4.G2 (38), and 10 μg/ml human γglobulin (Sigma-Aldrich) to block nonspecific binding. After a 30-min incubation on ice in staining buffer (BSS, 3% FBS, 0.1% sodium azide) with primary Abs, the cells were washed twice and analyzed by flow cytometry, or if a secondary reagent was necessary, the incubation and wash procedures were repeated. Flow cytometry was conducted on a FACSCaliber flow cytometer (BD Biosciences, Mountain View, CA), and the data were analyzed using CellQuest software (BD Pharmingen).

Bromodeoxyuridine (BrdU) staining

Mice were injected with 60 μg of SEB, 0.20 mg of anti-CD40 mAb, and 10 μg of LPS at the times described above. Additionally, the mice were injected with 1 mg of BrdU (Sigma-Aldrich) dissolved in PBS on days 0, 1, and 2. On day 3, T cells from the peripheral lymph nodes (LNs) and spleens from the treated mice were isolated and stained with biotin-conjugated anti-TCR Vβ3 mAb and then with PE-conjugated streptavidin (BD Pharmingen). The cells were then stained with a modified BrdU staining protocol (39). Briefly, the cells were dehydrated and fixed in ice-cold 95% ethanol and then fixed in BSS containing 1% paraformaldehyde and 0.01% Tween 20. Next, cellular DNA was lightly digested with 50 Kunitz U of DNase I (Sigma-Aldrich), and the cells were stained with anti-BrdU-FITC (BD Pharmingen).

In vitro proliferation

Mice were injected with 0.30 μg of SEA, 0.25 mg of anti-CD40 mAb, and 10 μg of LPS as described above. Ten days after SEA injection, spleen cells were isolated for culture. Cells from each in vivo treatment group were plated in triplicate at 5.0 × 106, 2.5 × 107, 1.25 × 107, and 0.63 × 107 cells per well in complete tumor medium (CTM). CTM consists of minimal essential medium with FBS, amino acids, salts, and antibiotics. SEA was added to the wells at a concentration of 1 μg/ml. The cultures were left for 72 h, with 1 μCi of [3H]thymidine (ICN Pharmaceuticals, Costa Mesa, CA) being added for the last 8 h. Incorporation of [3H]thymidine was measured on a 1450 Microbeta Trilux Scintillation Counter (Wallac, Turku, Finland).

RT-PCR

B10.A mice were injected with 0.25 mg of anti-CD40, 0.30 μg of SEA, and 10 μg of LPS as described above. At the time points corresponding to 0.5, 1.5, and 6 h after LPS injection (or 24.5, 25.5, and 30 h after SEA injection), peripheral LNs and spleens were removed, and total RNA was recovered from the cell suspensions using RNAzol according to the manufacturer’s suggested protocol (Ambion, Austin, TX). Synthesis of cDNA was performed with 1 μg of RNA using the Reverse Transcriptase System (Promega, Madison, WI) and accompanying protocol. Aliquots of cDNA were amplified with the GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, CT). Amplification conditions consisted of a 4-min denaturation step at 94°C, followed by 30 cycles of amplification (95°C for 1 min, 53°C for 1 min, 72°C for 1 min) and a final extension at 72°C for 7 min. PCR products were electrophoretically separated on a 2% agarose gel containing ethidium bromide. The polyclonotizer plasmid PQRS (40) was a kind gift from Dr. Reiner (University of Chicago). This plasmid contains cDNA sequences for each cytokine amplified and was used as a positive control. The sequences of the primers are as follows: hypoxanthine phosphoribosyltransferase, 5'-GGT GAA TAC CCA GAC TTT GTT G 3'- and 5'-CAG CTG ATG GTA GCT TTA GAC TTT GGG GTA G-3'; TNF-α, 5'-GAA GCA TTA TCC AGC CAG TTA GCG CTG TTG CCC AGA AGC TCA-CA-3' and 5'-TAC CAG GGT TAG ATC ACC TCA GC-3'; IL-1β, 5'-AGG CCT TCC ACC ACC TCA GTT AGC AG-3' and 5'-CTC AAA CCT CAC TCT GGT CCT GTT CA-3'; IL-2, 5'-TGG ACA GAC CAT AAC ACA GAC TCA ATG GAC-3' and 5'-GAG TCA AAT CCA GAT CAT GCC-3'; IL-6, 5'-GCT CTG GTC TGT TCG AGT ACC AT-3' and 5'-GGC ATA ACG CAC TAG TGT TGC CG-3'; IL-15, 5'-GAT CAC GAT GCC TTT GCA GGG AGC CAC-3' and 5'-ATG ACC CAG AGG CAC CCT TTA TCA-3'; IFN-γ, 5'-CAT TGA AAG CCT AGA TAC GAG TCT G-3' and 5'-CTC ATG AAT AGA TCC TTT TTC G-3'. All primers were made by the Central Services Laboratory at Oregon State University (Corvallis, OR).

Subcellular fractionation

Combined LN and spleen cells from C57BL/6 and IκB-α Tg mice were cultured overnight at a concentration of 10 × 106 cells/ml in CTM in the

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presence of 2 μg/ml Con A. T cells were purified out nylon wool to between 50 and 90% purity.

Nuclear and cytoplasmic extracts were prepared as described (41). Briefly, cell pellets were resuspended in sucrose buffer (0.32 M sucrose, 5 mM CaCl₂, 0.1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 2 mM Mg acetate, 1 mM DTT, 0.5 mM PMSF) with 0.5% (v/v) IGEPAL by gentle pipetting and were centrifuged. To the cytoplasmic fraction, 0.22 volumes of 5% (v/v) IGEPAL was added. The supernatant was transferred to a fresh tube containing 25% (v/v) glycerol and stored at −80°C. The nuclei were then resuspended in low salt buffer (20 mM HEPES, 25% glycerol, 1.5 mM MgCl₂, 0.02 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF), and then 1 volume of high salt buffer (20 mM HEPES, 25% glycerol, 1.5 mM KCl, 0.2 mM EDTA, 1% IGEPAL, 0.5 mM DTT, 0.5 mM PMSF) was carefully added in 1/4 increments. Nuclei were incubated on ice for 30 min, diluted 1/2.5 with diluent (25 mM HEPES, 25% glycerol, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF), and centrifuged at 15,000 rpm in a microcentrifuge, and the supernatant was transferred to a fresh tube containing 25% (v/v) glycerol and stored at −80°C.

DNA binding assay

EMSA were used to assess sequence-specific binding of DC2.4 nuclear NF-κB/Rel to DNA (41). Briefly, a synthetic 20-bp consensus κB element probe (under strand, 5′-GAT CGG CAG GGG CAT CCT CC-3′ and lower strand, 5′-GAT CGG CAG GGG CAT CCT CC-3′) was labeled with [α-32P]dATP using Klenow fragment (Invitrogen, Carlsbad, CA) and used for DNA binding assays. Nuclear extracts were prepared as described above. Samples (5 μg) were incubated with binding buffer (12 mM HEPES, pH 7.3; 4 mM Tris-HCl, pH 7.5; 100 mM KCl; 1 mM EDTA; 20 mM DTT; 1 μg/μl BSA, 4 μg of poly(dI-dC) (Amersham Pharmacia Biotech, Piscataway, NJ), and 100,000 cpm of 32P-labeled κB response element for 20 min at room temperature. Anti-RelA, anti-RelB, anti-c-rel, anti-p50, and anti-p52 were added to the reaction mixture according to manufacturer’s instructions (Santa Cruz Biotechnology, Santa Cruz, CA) and incubated for 10 min at room temperature. Samples were analyzed on a 5% polyacrylamide gel in 0.5% Tris-buffered EDTA (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA) and visualized by autoradiography.

Immunoblotting

Cytoplasmic extracts were subjected to SDS-PAGE as described (42). Proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) in 25 mM Tris (pH 8.3), 192 mM glycine, 20% methanol using a Genie Electrolotter (Idea Scientific, Minneapolis, MN), Membranes were blocked overnight at 4°C in TBS (25 mM Tris, pH 7.4, 150 mM NaCl) containing 5% nonfat dry milk and were incubated with primary Abs for at least 1.5 h at room temperature. Anti-IκB-α IgG (Santa Cruz Biotechnology) and HRP-conjugated secondary Abs, donkey anti-rabbit IgG (Amersham Pharmacia Biotech) were used according to the manufacturer’s instructions. After each Ab treatment, blots were washed in TBS containing 0.05% Tween 20. Abs complexes were visualized with chemiluminescent reagents (Pierce, Rockford, IL).

Results

LPS and CD40 stimulation synergize to enhance Ag-specific T cell survival

Our previous work with CD40 stimulation found that agonistic anti-CD40 mAb treatment could enhance SEA-mediated T cell expansion in vivo; however, it could not keep the responding T cells alive for very long (7). After 2–3 wk, Ag-specific T cell numbers declined to levels observed in mice treated with SEA alone. This death process was not prevented, only delayed. A very similar trend was observed during treatment with an anti-OX40 agonistic mAb (8). OX40 stimulation enhanced Ag-specific T cell expansion and, much like CD40 stimulation, could only yield weak long-term survival. However, injection of LPS into mice treated with both Ag and anti-OX40 mAb strongly enhanced T cell survival beyond 2 mo.

With these observations, we hypothesized that LPS injection would prevent the death of T cells from mice treated with SEA and anti-CD40 mAb. Thus, mice were injected with the anti-CD40 agonistic mAb, SEA, and LPS. At various time points after Ag treatment, T cell populations were examined in the lymphoid tissues. Fig. 1 shows a time course of the clonal expansion and survival of splenic CD4 Vβ3 (Fig. 1, a and c) and CD8 Vβ3 (Fig. 1, b and d) T cells stimulated by a combination of SEA, anti-CD40 mAb, and LPS.

In mice treated with SEA alone, CD4 Vβ3 T cell percentages (Fig. 1a) and numbers (Fig. 1c) declined on day 5 below their starting levels. The peak of clonal expansion that normally occurs on day 2–3 was not examined. Co-injection of either anti-CD40 mAb or LPS with SEA produced increased percentages and numbers of T cells on day 5, with LPS yielding more potent T cell expansion and short-term survival than CD40 stimulation. By day 14, however, T cells from these SEA/anti-CD40 or SEA/LPS-treated mice had slightly increased numbers of Vβ3 T cells compared with those mice treated with SEA alone (Fig. 1, c and d). Based on percentages of CD4 Vβ3 T cells on day 14, mice treated with SEA/LPS had 1.5-fold more splenic Ag-specific T cells (3.40 ± 0.49%), and SEA/anti-CD40-treated mice had 2-fold more splenic Ag-specific T cells (4.70 ± 0.25%) than SEA-treated mice (2.31 ± 0.12%). Similar differences were observed when examining T cell numbers (Fig. 1c). Thus, a low level of T cell survival is produced with SEA/anti-CD40 or SEA/LPS treatment.

What is most striking about the results is that a combination of the three signals (SEA, anti-CD40 mAb, and LPS) prevented many more T cells from deleting. This effect is observed by examining both percentages and numbers. Vβ3 T cells from mice stimulated by the three signals accumulated almost 10-fold from their starting populations by day 5 and had declined slightly in number after 2 wk. On day 14, CD4 Vβ3 percentages were increased by 16-fold over SEA alone to 38.46 ± 3.08% (Fig. 1a), whereas the absolute numbers were increased by nearly 13-fold to 40.27 × 10⁴ ± 2.69 (Fig. 1c).

![FIGURE 1](http://www.jimmunol.org/)
A very similar response to that described above was observed in the LN (data not shown) as well as in the Ag-specific CD8 T cell population (Fig. 1, b and d). In mice injected with SEA, anti-CD40 mAb, and LPS, splenic CD8 Vβ3 T cells accumulated to a much greater level than any other treatment on day 5 and then began to gradually decline in both percentage (Fig. 1b) and number (Fig. 1d). By day 14, there was a 26-fold increase in the percentage of CD8 Vβ3 T cells in three-signal-treated mice (29.90 ± 4.86%) vs SEA-treated mice (1.13 ± 0.02%). In contrast, SEA/LPS- and SEA/anti-CD40-treated mice only produced a 4-fold increase in Ag-specific T cell accumulation. Thus, both Ag-specific CD4 and CD8 T cell populations can be signaled to survive beyond the effector stage by the combined action of signal 1 (SAg), signal 2 (costimulation), and signal 3 (LPS).

We next tested whether these rescued T cells were functional by stimulating them in vitro with recall Ag using SEA. As shown in Fig. 2, mice treated with three signals in vivo for 10 days proliferated effectively when restimulated in vitro. This proliferation was less on a per cell basis than other weaker in vivo treatments yielded in vitro, which was likely due to the depleted resources and energetic costs of undergoing such a potent response. These data show that the T cells that are rescued from death by the combination of SAg, costimulation, and LPS are not anergic.

Multiple costimulatory signals cannot substitute for LPS

Stimulation of APCs through CD40 causes an increase in the expression of both B7 molecules as well as MHC class II (7, 43). In addition, LPS stimulation can also enhance B7 expression and prolong OX40 expression on activated T cells (8, 44). We hypothesized that because LPS can synergize with both CD40 and OX40 stimulation to promote Ag-specific T cell survival, its mechanism of action may involve combined signaling via CD28 and OX40 on the SAg-specific cells.

To test this idea, we injected mice with a triple combination of SEA, an agonistic anti-CD40 mAb, and an agonistic anti-OX40 mAb. The time course of this experiment is shown in Fig. 3, a and b, and the same scale as in Fig. 1 is used to allow a direct comparison between the two figures.

The percentages of both CD4 (Fig. 3a) and CD8 (Fig. 3b) T cells expressing Vβ3 behaved in a similar manner. Treatment of mice with SEA and anti-OX40 mAb enhanced the percentage of Ag-specific T cells in the spleen above that observed with SEA alone on day 3, but on subsequent days, the difference between the two treatments was minimal. SEA and anti-CD40 mAb treatment enhanced the CD4 Vβ3 and CD8 Vβ3 percentages in the spleen at the peak of expansion by 4- to 5-fold above that observed with SEA alone. By days 7 and 12, however, there was less than a 2-fold increase in the percentage of T cells remaining. When SEA, anti-CD40 mAb, and anti-OX40 mAb were injected in combination, the observed response was very similar to that from injecting SEA and anti-CD40 mAb. Ag-specific T cell expansion was enhanced 4- to 5-fold above starting levels on day 3, but after day 3, little survival was observed. There was a slightly larger percentage of Ag-specific T cells remaining on day 12 after the combined treatment (~4-fold above SEA alone), but this was a small fraction of that observed with LPS (Fig. 1). Thus, costimulation via CD40 and OX40 can yield small levels of T cell survival, but when combined with signals produced upon LPS injection, the survival effect is greatly potentiated, suggesting that LPS does not rescue Ag-specific T cells merely by stimulating through OX40 or CD40.

Because the survival response observed during three-signal stimulation does not appear to be a sum of multiple costimulatory signals being delivered, the next focus of study was whether the LPS response could be enhanced further. Because LPS can synergize with either CD40 or OX40 stimulation to improve Ag-specific T cell survival, it was necessary to examine whether combining all of these signals could enhance the amount of surviving T cells.
Mice were injected with a combination of SEA, LPS, anti-CD40, and anti-OX40. After 10 days, the T cell populations were examined. In mice injected with SEA, anti-CD40, and LPS, 18.96 ± 2.86% of the CD4 T cells (Fig. 3c) and 8.54 ± 1.49% of the CD8 T cells (Fig. 3d) were SEA specific. A slightly larger percentage was observed in mice injected with SEA, anti-OX40, and LPS: 19.50 ± 3.81% CD4 Vβ3 and 9.26 ± 2.23% CD8 Vβ3 T cells. In mice treated with both mAbs, SEA and LPS, little enhancement of T cell survival was observed, increasing to 24.29 ± 4.27% in the CD4 Vβ/H9251 and 11.56 ± 3.17% in the CD8 Vβ/H11006 population. In these experiments, we used 2.5 times less anti-CD40 mAb to conserve on reagent, and thus the magnitude of the response is lessened compared with Fig. 1. Increasing the LPS dose to 15 μg did not further enhance T cell survival, yielding slightly lower percentages and numbers of SEA-specific T cells than observed with 10 μg (data not shown). Again, the numbers of SEA-specific T cells as well as the LN cells both behaved in a similar fashion (data not shown). Thus, costimulation of T cells via either CD40 or OX40 stimulation in the presence of LPS can produce a substantial population of surviving Ag-specific T cells that cannot be significantly augmented by additional costimulation through these receptors. This again suggests that single or multiple costimulatory signals are not the major factor determining T cell survival beyond the effector phase in vivo.

Proinflammatory cytokines are not necessary for T cell survival in the presence of anti-CD40 stimulation and LPS

LPS stimulation of APCs can trigger the production of many proinflammatory cytokines. To begin to understand how LPS promoted survival in our model, mRNA expression of various cytokines was examined at early time points after LPS injection by RT-PCR (Fig. 4). The goal was to identify candidate cytokines that may be contributing to the survival response.

Expression of many of the important proinflammatory cytokines examined in Fig. 4 were enhanced upon LPS treatment in the LN and spleen. All reactions were normalized to hypoxanthine phosphoribosyltransferase, which allowed for semiquantitative comparisons to be made. TNF-α, IL-1β, IL-6, IL-15, and IFN-γ mRNA were all up-regulated to varying degrees during the first 6 h after LPS treatment compared with mice that received no LPS but were examined at the exact same time. IL-2 was also examined, but it was not observed in any treated or untreated mouse at the times tested (data not shown).

Previous work with mice injected with SEA and LPS showed that TNF-α can substitute to a small degree for LPS in keeping Ag-specific T cells alive (19). The survival effect was not nearly as potent as that observed with LPS injection, but it was significant. This result, coupled with the fact that TNF-α message expression was increased by LPS injection in the three-signal model system, suggested that TNF-α might be at least partially responsible for long-term T cell survival in the model described in this report.

To investigate the importance of this cytokine in the three-signal model system, mice deficient in either or both TNFRs were used (29). Because the knockout mice are on a C57BL/6 background, we used SEB instead of SEA to avoid the effect of endogenous mouse mammary tumor viruses on Vβ3 T cells. Each mouse strain was injected with SEB and anti-CD40 mAb with or without LPS, and lymphoid tissues were examined on day 10 for the presence of SEB-specific T cells (TCR Vβ/H9253).Fig. 5a shows the percentage of CD4 Vβ/H9253 T cells in the spleens of the treated mice 10 days after SEB injection.

In C57BL/6 mice treated with SEB and anti-CD40 stimulation, CD4 Vβ/H9253 T cells declined to an average percentage of 18.00 ± 0.92% after 10 days. When those mice were given LPS, the percentage of SEB-specific T cells rose to 32.69 ± 2.85%; an 85% increase. Mice deficient in TNFR1 had 22.30 ± 1.18% splenic CD4 Vβ/H9253 T cells in the absence of LPS treatment, but a 63% increase to 36.34 ± 2.13% was observed when LPS was co-injected. T cells from TNFRII knockout mice increased by 69% upon LPS treatment, rising from 18.35 ± 0.79% CD4 Vβ/H9253 T cells without LPS to 31.13 ± 3.30% with LPS.

In the absence of one TNFR, there remained the possibility that the other receptor was still delivering a survival signal due to redundancy of function between the two receptors. Thus, mice deficient in both TNFRs were treated with three signals as in the other strains. Mice receiving SEB and anti-CD40 mAb had a CD4 Vβ/H9253 T cell percentage on day 10 of 20.03 ± 1.00%, but when injected with LPS, the percentage rose 63% to 32.65 ± 1.79%, similar to that observed in the other mice. Overall, these data suggest that signaling through TNFRI and TNFRII is dispensable for long-term T cell survival when CD40 stimulation is used in combination with LPS.

The observation that T cell survival can still occur in the absence of TNFR signaling does not mean that such signaling cannot contribute to survival. Other proinflammatory cytokines may be contributing a survival signal and thus may be substituting for TNFR signaling. To get at the issue of whether other cytokines were delivering the signals necessary to keep activated T cells alive, a combination of cytokines was blocked in vivo during SEA, anti-CD40, and LPS stimulation.

B10.A mice were injected with SEA, anti-CD40, and LPS, but before and after LPS injection, IL-1Ra, anti-TNF-α mAb and anti-IL-6 mAb were all injected to try to neutralize as much activity of these cytokines as possible. Because the RT-PCR data showed that IFN-γ expression was also increased after LPS injection (Fig. 4), another treatment group was set up in which IFN-γ was blocked with a mAb to investigate what effect this cytokine had on survival. The percentages (Fig. 5b) and numbers (Fig. 5c) of CD4 Vβ/H9253 T cells 10 days after SEA injection are shown.

FIGURE 4. Examination of cytokine gene expression by RT-PCR within hours after LPS injection. Total RNA was isolated from LNs and spleens of B10.A mice and converted into cDNA for PCR analysis as described in Materials and Methods. The un.injected lane represents an un injected mouse. Numbers represent the hours after LPS injection, whether or not LPS was given. The plasmid lane represents the control plasmid pPQRS, which contains all of the amplified cytokine genes. The PCR product from this plasmid is slightly larger than the actual cytokine. αCD40, anti-CD40; HPRT, Hypoxanthine phosphoribosyltransferase. Results are from one representative experiment of three performed.
SEA treatment led to the expected decline in T cell percentages, decreasing from a 6% CD4 Vβ3 T cell population in uninjected mice to a little over 2% by day 10. SEA/anti-CD40 mAb and SEA/LPS treatments both produced small increases in T cell percentages in comparison to SEA alone, again showing that two signals can generate some T cell survival post effector stage. Mice treated with SEA/anti-CD40/LPS and the control IgG exhibited a large increase in percentage of SEA-specific T cells to 22.12 ± 4.32%. What was most interesting, however, was that when IL-1β, TNF-α, and IL-6 were all neutralized, the percentage of surviving T cells actually increased slightly to 27.42 ± 5.34%. Even blocking IFN-γ did not decrease survival, yielding 23.25 ± 6.56% CD4 Vβ3 T cells after 10 days in vivo.

A much better enhancement of T cell survival after cytokine neutralization was observed based on CD4 Vβ3 T cell numbers (Fig. 5c). Mice receiving three-signal stimulation and the control rat IgG had 23.28 × 10^3 ± 6.31 SEA-specific T cells remaining after 10 days. Blocking TNF-α, IL-1β, and IL-6 increased the numbers of surviving T cells to 42.40 × 10^5 ± 11.2, whereas IFN-γ inhibition increased the numbers to 35.83 × 10^3 ± 14.62.

CD8 Vβ3 T cells responded a little differently to the cytokine blocking studies than the CD4 Vβ3 T cells did (data not shown). Both percentages and numbers of CD8 T cells expressing Vβ3 in the spleen were increased poorly, if at all, by inhibition of proinflammatory cytokines. This could represent a difference in activation/survival requirements between CD4 and CD8 T cells. However, what is identical between both kinds of T cells is that blocking TNF-α, IL-1β, IL-6, and IFN-γ did not adversely affect survival and, perhaps surprisingly, enhanced T cell survival to varying degrees.

To verify that the cytokines were inhibited by the neutralizing mAbs, serum samples were taken from the injected mice 1 day after the LPS and Ab injections. ELISAs were performed using anti-rat IgG to detect the neutralizing Abs in the serum using a sandwich ELISA. In the control rat IgG group, quantities of rat IgG were detected in the serum that exceeded the maximum detectable amount (data not shown). In the anti-TNF-α-, anti-IL-6-, and IL-1Ra-treated groups there was slightly less Ab detected, but still large amounts remained in the serum (data not shown). The anti-IFN-γ-treated mice also had detectable levels of rat IgG in their serum (data not shown). The ELISA data show that there were large amounts of rat IgG Abs present in the serum over 24 h after LPS treatment. This suggests that the cytokines were neutralized soon after they were secreted, and thus this data is consistent with the data collected from the TNFR KO mice. Furthermore, attempts to substitute LPS with TNF-α, IL-1β, and IL-6 to induce SAg-specific T cell survival failed, even though large doses of the cytokines were used (data not shown). Collectively, these data suggest that CD40 stimulation circumvents a role for proinflammatory cytokines in long-term T cell survival.

Similar results were observed when IL-1β and IL-6 were inhibited in TNFR double-knockout mice (data not shown). No adverse effects on long-term T cell survival were observed in this model system, and some slight increases in surviving T cell numbers were observed. Thus, from these data it appears that the proinflammatory cytokines TNF-α, IL-1β, IL-6, and IFN-γ are not necessary for long-term T cell survival and in fact may actually be somewhat detrimental, possibly playing a role in attenuating an Ag-specific CD4 T cell response to Ag, strong costimulation, and LPS treatment. This is consistent with previous reports showing that TNF-α and IFN-γ readily exert cytotoxic effects on lymphocytes (40, 45, 46). IL-1β and IL-6, however, appear to be more protective (47, 48), although IL-β does play a role in the death of β-cells in diabetes (49).

A mixed role for NF-κB during T cell survival

The transcription factor NF-κB is very important for inflammatory responses as well as T cell proliferation and survival (16, 17, 50–52). Many inflammatory cytokines, as well as LPS, activate transcriptional activity of this molecule (53–56). To examine the role
of NF-κB in T cell survival in vivo, mice Tg for a mutant IκB-α molecule that cannot be degraded were used (30). IκB-α is a repressor of NF-κB that prevents the translocation of NF-κB into the nucleus (57). When IκB-α is phosphorylated, it is targeted for ubiquitination (58, 59). Subsequent degradation of IκB-α releases NF-κB and allows it to translocate to the nucleus and initiate transcription. Thus, these mutant IκB-α Tg mice have reduced NF-κB binding to DNA response elements. Additionally, this transgene is under the control of the Iκc promoter, thus limiting its expression, and ultimately NF-κB inhibition, to the T cell population, allowing an examination of how effective T cell survival is in the presence of reduced NF-κB activity.

As shown in Fig. 6a, the percentage of splenic CD4 Vβ8 T cells in C57BL/6 mice given SEB and anti-CD40 mAb without LPS was 20.91 ± 0.83% and rose to 33.26 ± 3.35% when LPS was injected. A very similar increase was observed in the IκB-α Tg whose T cell populations increased from 22.97 ± 1.12% without LPS to 33.90 ± 3.03% with LPS (Fig. 6b). These numbers correspond to a 59% difference between treatments in C57BL/6 mice and a 48% difference in the Tg mice. A similar trend was observed in the absolute numbers of CD4 Vβ8 T cells, which increased by 32% in the C57BL/6 mice and by 31% in the Tg mice that received LPS (data not shown). A few individual mice had much higher levels of CD4 Vβ8 T cells than observed in control strains. One Tg mouse in particular had over 70% of its CD4 T cells bearing Vβ8 in the spleen 10 days after SEB injection. This is in contrast to C57BL/6 mice, which never rose above 50%.

Expression of IκB-α by Western blot and EMSAs assessing NF-κB activity were performed using T cells from mice shown in Fig. 6b. The percentages shown in the lanes of the Western and EMSA blots represent the percentage of CD4 Vβ8 T cells in SEB/anti-CD40/LPS-treated mice used in Fig. 6b. Thus, the 39% lane represents cells from a mouse that had 39% CD4 Vβ8 T cells in Fig. 6b. The data show little variation in transgene expression regardless of the amount of rescue (Fig. 6c). Furthermore, data measuring NF-κB activity showed profound inhibition of NF-κB in the cells taken from the transgenic mice as expected (Fig. 6b). Therefore, these controls support our hypothesis that survival of Ag-activated T cells beyond the effector stage is not dependent on NF-κB.

LPS can circumvent proliferation defects in NF-κB-deficient T cells

Because LPS-induced long-term T cell survival can still occur in NF-κB-deficient T cells, it was important to investigate whether T cell responses were deficient and whether LPS could somehow overcome that deficiency. Thus, IκB-α Tg or C57BL/6 mice were injected with different combinations of SEB, anti-CD40 mAb, and

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**FIGURE 6.** The transcription factor NF-κB is not necessary for long-term T cell survival. a and b, C57BL/6 (a) or IκB-α Tg (b) mice were injected with 0.2 mg of anti-CD40, 60 μg of SEB, and 10 μg of LPS as described in Materials and Methods. Ten days after SEB injection, the CD4 Vβ8 T cell populations were analyzed by flow cytometry. Each graph represents the percentage of CD4 Vβ8 T cells in the spleen for each individual mouse used. The lines in the graphs signify the mean value from all the data points for each treatment. Graphs represent pooled data from six separate experiments. c and d, LN and spleen cells from control or IκB-α Tg mice were restimulated with Con A overnight. Cytoplasmic and nuclear extracts were prepared from purified T cell populations as described in Materials and Methods. A Western blot (c) was performed on the cytoplasmic extracts to detect IκB-α protein. Shown are results from naive C57BL/6 cells and from one C57BL/6 and four IκB-α Tg mice treated with three signals in vivo. Percentages correspond to the approximate percentage of CD4 T cells expressing Vβ8 shown in b for each transgenic mouse examined. Nuclear extracts from the above preparations were subjected to an EMSA (d) to detect NF-κB DNA-binding activity. Shown are data from six IκB-α Tg mice treated with three signals in vivo. The last lane is from naive wild-type (WT) T cells. Percentages correspond to the approximate percentage of CD4 T cells expressing Vβ8 shown in b for each transgenic mouse examined. Robust NF-κB activity was observed in rescued T cells from control mice receiving three-signal stimulation (data not shown). The blots were performed in two separate experiments and were similar. The Western blot data represent a different experiment than the EMSA blot.

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**FIGURE 7.** NF-κB is important for short-term T cell survival. C57BL/6 (a and b) and IκB-α Tg (c and d) mice were injected with SEB, anti-CD40 (αCD40), LPS, and BrdU as described in Materials and Methods. On day 3 after SEB injection, Ag-specific Vβ8 T cells were analyzed by flow cytometry. These data represent the mean percentage (a and c) and number (b and d) ± SEM of Vβ8 T cells incorporating BrdU in the LN. Bars represent a total of three to five mice combined from three separate experiments.
LPS and were given BrdU injections for 3 days. After this treatment, the percentages and numbers of Ag-specific T cells incorporating BrdU were evaluated (Fig. 7).

Both C57BL/6 and IκB-α Tg mice had similar percentages of Vβ8 T cells incorporating BrdU in the LN on day 3 (Fig. 7, a and c). Based on these percentages of BrdU incorporation, no major proliferation defect was apparent. SEB treatment mediated ~40% of the responding T cells to take up BrdU in both normal and IκB-α Tg mice. Three-signal treatment only slightly enhanced the incorporation of BrdU to ~50% of the Vβ8 population.

When the absolute numbers of Vβ8 T cells taking up BrdU was examined, however, a major defect was observed (Fig. 7, b and d). C57BL/6 mice had similar numbers of Vβ8 T cells incorporating BrdU regardless of whether SEB alone or three signals were delivered. The number of NF-κB-deficient T cells taking up BrdU was strongly reduced in SEB-treated mice, strongly supporting the notion that NF-κB activity promotes survival during clonal expansion. However, co-injection of anti-CD40 and LPS with SEB increased the numbers by 4.5-fold, suggesting that such stimulation enhances short-term survival, as measured by accumulation of Ag-specific T cells, in an NF-κB-independent manner.

Discussion

The focus of this paper was to better understand the relationship between costimulatory signals and signals induced by LPS that promote long-term T cell survival. Because LPS could synergize with CD40 stimulation to enhance T cell survival, it was initially reasoned that LPS might be merely a summation of various costimulatory signals. Thus, we delivered OX40 and CD40 costimulatory signals in place of LPS to see whether they could synergize to increase levels of surviving T cells. Fig. 3, a and b, shows that combined signaling via CD40 and OX40 cannot yield the same level of survival as observed in SEA/anti-CD40/LPS-treated mice. The percentages of SEA-specific T cells observed in Fig. 3, a and b, are increased 3–5-fold above SEA-treated mice when both costimulatory signals are delivered, a response similar to what is observed when only two signals are given (Fig. 1). Also, we found that simultaneous stimulation of both CD40 and OX40 did not significantly enhance the LPS response (Fig. 3, c and d), suggesting that under these circumstances, when analyzing T cell survival, the CD40 and OX40 signal is redundant or overlapping on a cellular level. It is likely that LPS induces other kinds of responses that OX40 and CD40 stimulation cannot. Thus, optimal T cell survival occurs when three qualitatively different signals are delivered to the T cell. We conclude that delivery of signals 1, 2, and 3 is not the same as the delivery of signal 1 and two signal 2s (Fig. 8).

To better understand the mechanism of how this survival response is produced, proinflammatory cytokines were examined. Cytokine analysis by RT-PCR confirmed the LPS-dependent increase in expression of many cytokines such as TNF-α, IL-1β, IL-6, and IFN-γ (Fig. 4). TNF-α was the first cytokine studied because previous work found that blocking TNF-α signaling in vivo with a mAb inhibited T cell survival in response to SEA/LPS injection (18). Subsequent work found that injection of TNF-α in place of LPS promoted some T cell survival after SEA injection (19). However, the percentage of SEA-specific T cells surviving after SEA/TNF-α treatment was only about one-third of that observed with injection of SEA and LPS. The fact that TNF-α or IL-1β could not completely substitute for LPS in enhancing survival implied that other cytokines might also contribute to the overall survival response. Thus, whereas some decrease in T cell survival might be expected in the absence of TNF signaling, it may not be completely abolished.

What is most apparent and perhaps surprising about the TNFR KO mouse data shown in Fig. 5a is that neither TNF-α is required for effective T cell survival resulting from three-signal stimulation. This is different from the results from SEA/LPS alone-treated mice. It has been shown that TNF-α does have survival-inducing capabilities during antigenic signaling (60) and is an important component of LPS-mediated T cell survival after SEA stimulation (18). Nevertheless, we show for the first time that a strong costimulatory signal via CD40 can bypass the requirement for TNF-α.

One possibility is that other cytokines may be substituting for TNF-α in the TNFR KO mice. Earlier work suggested that IL-1β and IFN-γ could also contribute to the survival response (18, 19). Thus, combinations of these proinflammatory cytokines were neutralized in the presence of three-signal stimulation to test whether signals from multiple cytokines delivered the survival stimulus. TNF-α, IL-1β, and IL-6 were all neutralized in the presence of SEA, anti-CD40, and LPS. Again, the inhibition of these three cytokines did not block T cell survival, but slightly enhanced it in the SEA-specific CD4 T cell population (Fig. 5, b and c). Furthermore, the survival response was also unaffected or slightly enhanced by a blockade of IFN-γ. Thus, there appears to be no significant role for these important inflammatory cytokines in T cell survival induced by three-signal stimulation.

Because the proinflammatory cytokines we tested were not involved in survival in this model, we reasoned that analysis of transcription factors may narrow our search for mediators of survival. NF-κB is an important molecule in T cell responses and is activated by signals from LPS and many proinflammatory cytokines (50, 53). Activation of NF-κB has been shown by many laboratories to inhibit apoptosis (54–56, 61). Additionally, NF-κB is responsible for transcribing many survival genes such as A1, A20, and Bcl-xL (62, 63). Thus, we tested the hypothesis that a deficiency in NF-κB signaling would alter three-signal-induced T cell survival by using Tg mice that express a T cell-specific transdominant form of the NF-κB repressor, IκB-α, that cannot be degraded (30). These mice and similar strains were shown to be deficient in T cell proliferation and IL-2 secretion in vitro (64–66). Additionally, the T cells were found to be more prone to apoptosis in vitro after activation. The majority of these studies were conducted in vitro, and very little is known about how these unusual peripheral T cells respond in vivo.

FIGURE 8. Schematic representation of possible differences between signals 2 and 3. The data in this report suggest that in T cells, signal 1 (TCR ligation by MHC) and signal 2 (mediated by CD40 ligation on APC) promote clonal expansion with little long-term survival in vivo. Addition of LPS-induced responses enhances ligation of a hypothetical third signal, which promotes survival. The identity of signal 3 remains obscure, but it is likely to be a result of inflammatory responses. αCD40, Anti-CD40. Arrow thickness indicates level of intensity.
Injecting the Tg mice with SEB, anti-CD40 mAb, and LPS continued to produce effective survival that was comparable to that observed in C57BL/6 mice (Fig. 6, a and b). It was shown in this report that upon exposure to three signals, the NF-κB-deficient T cells can survive for long periods of time in vivo even though activated NF-κB-deficient T cells are readily susceptible to apoptosis in vitro.

Although Western blots showed that IkBα was expressed in equivalent amounts in all Tg mice examined (Fig. 6c), it was possible that this mutation did not completely inhibit NF-κB activity. Thus, the possibility remained that some small level of transcription by NF-κB was still occurring in the Tg cells that transcribed the survival factor. To further test this idea, EMSAs were performed on some of the T cells taken from the mice in Fig. 6b. Although there were very low and somewhat variable levels of detectable NF-κB DNA-binding activity in cells from the Tg mice, no correlation was observed between NF-κB activity and the level of T cell survival in vivo. It is possible, however, that p50 homodimers are present in these cells at very low levels as previously reported (30). Thus, NF-κB does not appear to be important for long-term survival of Ag-specific T cells in this model.

The NF-κB data are further supported by the cytokine data. In the experiments where cytokines were neutralized, there presumably would be less NF-κB activation. Both TNF-α and IL-1β activate NF-κB, and in the absence of signals through the receptors of these cytokines, there should be less overall NF-κB transcriptional activity. In this situation, long-term T cell survival still occurred. Thus, the role of NF-κB in T cell survival appears to be dispensable.

A recent study from Mitchell et al. (67) suggested that adjuvants such as LPS may be causing T cell survival by inducing Bcl-3, which assists NF-κB transcription. Such transcription was suggested to produce important proteins responsible for survival. Our data suggest that NF-κB may not be necessary for long-term T cell survival, but we cannot rule out the possibility that NF-κB activity can produce a survival factor. Because Bcl-3 plays a role in NF-κB transcription and T cell responses, it may be that NF-κB is most significant in the early phases of a T cell response for short-term survival.

Proliferation, as measured by BrdU incorporation, was not affected significantly by NF-κB inhibition based on the percentage of T cells incorporating BrdU (Fig. 7). However, when the absolute numbers of T cells were examined, a dramatic decline in T cell numbers incorporating BrdU was observed after SEB injection (Fig. 7). Thus, the SEB-specific T cells divide normally, or at least attempt to divide normally, but they do not effectively accumulate in the lymphoid tissues without NF-κB signaling.

We tested whether CD40 and LPS stimulation could circumvent the defective accumulation of T cells in this response. Injection of these three signals enhanced the numbers of SEB-specific T cells incorporating BrdU on day 3 by ~4-fold. Numbers were not stored to levels observed in C57BL/6 mice, but they were enhanced, implying that three-signal stimulation can override some of the inhibition of short-term T cell survival that results from NF-κB deficiency. These data support the novel hypothesis that NF-κB is important for short-term survival of Ag-specific T cells as demonstrated by others (67) but that nevertheless can be circumvented by combined CD40 and LPS stimulation.

Although it is now clear that Ag is not necessary for maintenance of memory CD4 or CD8 T cells (68, 69), the question that remains is what external factors drive the decision of a cell to develop into a memory cell vs one that dies? We previously have proposed that three signals optimally induce the development of memory T cells, and in this report we show that LPS and CD40 stimulation do not obligatorily rely on proinflammatory cytokines as in other systems (18, 19, 70). Collectively, these data show that CD40 stimulation can bypass the need for LPS-induced proinflammatory cytokines, and they demonstrate that two different costimulatory signals and Ag are not qualitatively the same as Ag, one costimulatory signal, and a signal mediated by a natural adjuvant. These data lead us to hypothesize that the third signal (LPS) drives long-term T cell survival and is functionally different from a prototypical costimulatory signal (Fig. 8).

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