bcl-xL Is Critical for Dendritic Cell Survival In Vivo

Huiming Hon, Edmund B. Rucker III, Lothar Hennighausen and Joshy Jacob

J Immunol 2004; 173:4425-4432; doi: 10.4049/jimmunol.173.7.4425
http://www.jimmunol.org/content/173/7/4425

References
This article cites 40 articles, 16 of which you can access for free at:
http://www.jimmunol.org/content/173/7/4425.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
bcl-xL Is Critical for Dendritic Cell Survival In Vivo

Huiming Hon,* Edmund B. Rucker III,† Lothar Henninghausen,‡ and Joshy Jacob2*

Dendritic cells (DCs) are important regulators of immune function, transporting Ags from the periphery to draining lymph nodes (dLN) where they prime Ag-specific T lymphocytes. The magnitude of the immune response generated depends upon the longevity of the Ag-bearing DC in lymphoid tissues. We hypothesized that the control of DC survival is regulated by the antiapoptotic factor bcl-xL. Gene gun immunization of dual-expression DNA vaccines into a bcl-xL-/- mouse resulted in the delivery of Ag, as well as selective deletion of the bcl-x gene in directly transfected, skin-residing DC. bcl-x-deficient DC failed to mount effective immune responses, and this corresponded to their rapid disappearance from the dLN due to apoptosis. We confirmed these results using RNA interference to specifically silence the antiapoptotic bcl-xL isoform in targeted skin-residing DC of C57BL/6 mice. In addition, delivery of bcl-xL in trans complemented the bcl-x deficiency in DC of bcl-xL-/- mice, resulting in the maintenance of normal levels of Ag-bearing DC in the dLN. Taken together, our work demonstrates that the bcl-xL isoform is critical for survival of skin-derived, Ag-bearing DC in vivo. The Journal of Immunology, 2004, 173: 4425–4432.

Materials and Methods

Mice

bcl-xL-/- mice were imported from the University of Missouri, and analyzed for genotype by PCR as previously described (18, 19). C57BL/6 mice were obtained from Charles River Labs (Wilmington, MA). Mice used for experiments were between 4 and 10 wk of age, and maintained under standard pathogen-free housing conditions at the Emory Vaccine Center vivarium with the approval of the Institutional Animal Care and Use Committee at Emory University.

PCR and RT-PCR analyses

PCR identification of the recombinant bcl-x locus in bcl-xL-/- mice was performed as previously described (18, 19). To detect bcl-xL and bcl-xS isoforms, primers flanking the C-terminal 189 bp of exon 2 were used in a standard RT-PCR: forward primer, 5'-GGGAGACCGTTCTAGTGATC-3', and reverse primer, 5'-CCAGCCACAGCTGACC-3'.
DNA vaccine plasmids

Cre recombinase-coding cDNA coupled with a SV40-derived nuclear localization sequence was cloned downstream of the CMV immediate-early promoter and upstream of a bovine growth hormone polyadenylation sequence (pCMV-Cre). Similarly, plasmids expressing full-length OVA cDNA (pCMV-OVA) and influenza hemagglutinin (HA) cDNA (pCMV-HA) were constructed. To track Ag-bearing cells, the lacZ gene was cloned downstream of the chicken β-actin (CAG) promoter (pCAG-lacZ). To generate dual-expression DNA vaccines, these Ag expression cassettes were excised and blunt-end ligated into the cre-expressing vector. Dual-expression bcl-xL-small interfering RNA (siRNA) plasmids were assembled by cloning the U6-bcl-xL-siRNA constructs downstream of pCAG-lacZ. Plasmids encoding pBcl-xS, pBcl-xL, and the mt7 pBcl-xL mutant were kind gifts from T. W. Behrens (University of Minnesota, Minneapolis, MN), S. Korsmeyer (Harvard University, Boston, MA), and J. M. Hardwick (Johns Hopkins University, Baltimore, MD), respectively.

Immunizations

Gene gun immunizations were performed on shaved abdominal skin of mice as described previously (3, 20). Briefly, a hand-held Helios gene delivery system (Bio-Rad, Hercules, CA) was used to deliver two doses of 0.5 μg of plasmid DNA coated on 1 mg of gold beads (DeGussa-Huls, Ridgefield Park, NJ) at a helium pressure of 400 psi. In experiments designed to study DC at the immunization site, we immunized ears of mice with one shot each on the dorsal and ventral sides (0.5 μg of plasmid DNA per shot) using the Helios gene gun set at a helium pressure of 600 psi.

T cell proliferation

Mice were immunized with pCMV-OVA, pCMV-Cre-CMV-OVA, or empty DNA vector, and draining superficial inguinal lymph nodes were removed and pooled 5 days later. CD11c+ cells were enriched to >90% purity by anti-CD11c (N418)-coupled magnetic beads (Miltenyi Biotec, Auburn, CA). Responder T cells were isolated from spleens of OT-II TCR transgenic mice by incubation of the single-cell suspension with anti-CD3-FITC (eBiosciences, San Diego, CA) followed by positive selection with anti-CD69-PE (BD Biosciences, San Jose, CA), with a secondary biotin, -I-A b-PE, -annexin V-PE; BD Biosciences), with a secondary biotin, -I-A b-PE, -annexin V-PE; BD Biosciences), with a secondary biotin, -I-A b-PE, -annexin V-PE; BD Biosciences, San Jose, CA), and data were analyzed with Microplate Reader software (Bio-Rad).

Serum ELISA

At days 15 and 30 after gene gun immunization, serum samples were collected from ~50 μl of blood obtained from mice via retro-orbital bleeds, and assayed on ELISA plates coated with sucrose gradient-purified A/PR/8/34 (H1N1) influenza virus. Quantitation of Ab levels was performed by comparison with a standard curve using goat anti-mouse IgG (BD Biosciences, San Jose, CA), and data were analyzed with Microplate Reader software (Bio-Rad).

Influenza challenge

Forty days following DNA vaccination with constructs expressing influenza HA, animals were challenged with a mouse-adapted influenza virus A/PR/8/34 (H1N1), a kind gift from H. Robinson (Emory University, Atlanta, GA). Allantoic fluid containing influenza virus was serially diluted in sterile PBS with 0.2% BSA (Sigma-Aldrich, St. Louis, MO), and a 50-μl volume containing 3 × 10^6 PFU (0.03 HAU) viral dose was given intranasally to anesthetized mice. Following viral challenge, total body weight and survival were recorded over the subsequent 10 days.

siRNA constructs and testing

Candidate siRNA oligos targeted against the 189-bp region at the 3' end of exon 2 of murine bcl-xL were devised according to guidelines established by Tuschl (21). The siRNA oligos were synthesized and cloned into the pSilencer 1.0 vector under control of the U6 RNA Polymerase III promoter per manufacturer’s instructions (Ambion, Austin, TX). Of the two siRNA constructs tested, the greatest knockdown was seen with the oligo targeting bcl-xL-bcl-xl (5’AGAGGAGATGCGAAGTTTGGT-3’). Ability to silence bcl-xL was tested by transient cotransfection with expression vectors encoding murine bcl-xL or bcl-xS into human 293 cells. The control GFP siRNA oligo was constructed following Sui et al. (22).

bcl-xL IS CRITICAL FOR DC SURVIVAL IN VIVO

Epidermal cell preparation

To isolate epidermal cell suspensions, we excised the immunized ears at the base, and split-thickness ears were treated with 0.5% trypsin for 30 min at 37°C. Following incubation, a single-cell suspension was made by disrupting epidermal sheets in a 70-μm cell strainer.

Flow cytometry

For flow cytometric analysis of β-galactosidase (β-gal) expression, collagen-treatet lymph node cells or epidermal cell suspensions from ear skin were hypotonically loaded with 0.5 mM fluorescein di-n-galactopyrano-side (Molecular Probes, Eugene, OR), as previously described (23–25). Aliquots of fluorescein di-n-galactopyranoside-loaded cells were stained with fluorochrome-conjugated Abs (anti-CD11c-allophycocyanin, -CD86-biotin, -I-A<sup>β</sup>-PE, -annexin V-PE; BD Biosciences), with a secondary streptavidin-PerCP (BD Biosciences) Ab staining when needed. Cells were then acquired without fixation with a FACSCalibur (BD Biosciences), and data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Statistical analyses

Statistical differences between experimental groups were analyzed with Prism software (GraphPad, San Diego, CA), with values of p < 0.05 from Student’s t test considered significant.

Results

Targeted deletion of bcl-x in DC

Previously, we had permanently marked and tracked the migration of Ag-bearing DC from the periphery to dLN by biolistic delivery of cre-encoding plasmids into a cre-indicator mouse strain, ROSA26R (3). We showed that DC resident in the skin could be directly transduced in vivo via gene gun immunization. In this study, we used the same approach to permanently delete the bcl-x gene from the genome of mice in bcl-x<sup>x00</sup> mice. These mice have been engineered with two loxP sites flanking the bcl-x gene; in the presence of bacteriophage P1-derived cre recombinase, homologous recombination occurs between these two loxP sites, resulting in the permanent deletion of all bcl-x exons from the genome. bcl-x<sup>x00</sup> mice received, via gene gun delivery to the abdomen, a DNA vaccine encoding cre recombinase (pCMV-Cre). The immunization site was marked, and then excised 6 h later and subjected to genomic DNA extraction. As controls, the same mouse received immunizations at nonoverlapping sites with either pCMV-OVA or gold particles alone. We then analyzed the genomic DNA samples for cre-mediated deletion of the bcl-x gene by PCR, using a primer set that produces a 150-bp fragment only from a deleted bcl-x locus (18, 19). Amplification of the 150-bp recombination product was restricted to the area of skin that had been immunized with cre recombinase (Fig. 1a). The adjacent areas of skin that were immunized with pCMV-OVA or empty gold particles did not produce the 150-bp recombination product, indicating that we could achieve a targeted, cre-dependent deletion of the bcl-x gene. The presence of two copies of the undeleted bcl-x gene was verified by PCR in all control DNA samples (data not shown).

To confirm cre-mediated deletion of the bcl-x gene in transfected DC, we immunized cohorts of bcl-x<sup>x00</sup> mice with DNA vaccines dually encoding cre recombinase and β-gal. In these vectors, the strong hybrid CMV enhancer and CAG promoter drove expression of β-gal to permit tracking of the small percentage of transfected cells. At 2.5 days after immunization, both the CD11c<sup>β-gal</sup> and CD11c<sup>β-gal</sup> populations were sorted from draining superficial inguinal nodes, and subjected to genomic DNA extraction and PCR amplification for the 150-bp recombination product. The CD11c<sup>β-gal</sup> cells, but not the CD11c<sup>β-gal</sup> cells...
munized cohorts of and full-length chicken OVA (pCMV-Cre-CMV-OVA). We im-
constructed a DNA vaccine that dually expresses cre recombinase skin-derived DC to stimulate naive T cell proliferation in vitro. We
ior. CD11c with pCMV-Cre-CMV-OVA, pCMV-OVA, pCMV-Cre, or an empty vec-
mean. Comparable data were obtained in two separate experiments.
positive controls, respectively. Results are shown as the mean [3 H]thymidine
sponder T cells were pulsed with OT-I and OT-II peptides for negative and
naive, OVA-speci
directly in vitro, without exogenous peptide, to stimulate proliferation of
population exhibited the 150-bp recombination product (Fig. 1 b), indicating that biolistic delivery of the dual-expression plasmid deleted the bcl-x gene in skin-residing DC, which could then be tracked to the dLN.

**Decreased T cell stimulation by bcl-x-deficient DC**

We first examined the effect of bcl-x deletion on the ability of skin-derived DC to stimulate naive T cell proliferation in vitro. We constructed a DNA vaccine that dually expresses cre recombinase and full-length chicken OVA (pCMV-Cre-CMV-OVA). We immunized cohorts of bcl-x
mice with this DNA vaccine, or with pCMV-Cre, pCMV-OVA, or an empty vector. Five days later, we magnetically enriched for CD11c
DC from dLN, and used them directly in vitro as APCs, without addition of exogenous peptide, to stimulate proliferation of naive OVA-specific TCR transgenic CD4 T cells (Fig. 2). CD11c
DC isolated from mice immunized with pCMV-Cre-CMV-OVA had significantly reduced Ag-specific T cell proliferation, yielding a lower level of [3 H]thymidine uptake than that achieved from immunization with pCMV-OVA alone (2.05 ± 0.1 vs 18.32 ± 0.6 × 10^3 cpm, respectively). Responder T cells pulsed with cognate OT-II peptide showed maximal amounts of proliferation (25.45 ± 2 × 10^3 cpm), whereas cells pulsed with control OT-I peptide, or stimulated with cells from animals immunized with either an empty vector or pCMV-Cre, showed only background levels of [3 H]thymidine uptake. These data suggest that loss of the bcl-x gene in Ag-bearing DC resulted in a drastic loss of their T cell-priming capacity.

bcl-x-deficient DC elicit reduced Ab responses

To determine whether humoral immune responses would be blunted by the loss of bcl-x in Ag-bearing DC, we used the mouse influenza infection model system. Protection from influenza infection depends upon the generation of Abs against the viral HA protein (26). DNA vaccines encoding HA induce long-lasting and high titers of specific Abs (27). We immunized groups of bcl-x
mice with a DNA vaccine coexpressing cre recombinase and influenza HA (pCMV-Cre-CMV-HA), or with controls pCMV-HA or empty vector. At days 15 and 30 postimmunization, we collected sera from each mouse and measured the concentration of anti-HA Abs by a standard ELISA (Fig. 3 a). Mice immunized with pCMV-HA exhibited 24.37 ± 2.4 μg/ml anti-HA IgG at day 15, and 77.39 ± 7.4 μg/ml at day 30 after vaccination. In contrast, mice immunized with pCMV-HA-CMV-Cre produced significantly lower levels of influenza-specific Abs at both time points (7.23 ± 2.6 and 9.3 ± 3 μg/ml; p < 0.05), whereas the response of control mice immunized with an empty DNA vector remained at baseline levels.

---

**FIGURE 1.** Specific deletion of bcl-x gene in bcl-x
mice following gene gun immunization with cre recombinase. a, Mice (n = 3) were gene gun immunized with nonoverlapping shots of pCMV-Cre (site 1), pCMV-OVA (site 2), or empty bullets (site 3). The immunization sites were marked and excised 6 h later. Genomic DNA isolated from excised skin sections at each site and from the tail was subjected to PCR analysis for cre-mediated bcl-x deletion. Amplification of the 150-bp recombination product occurred only with DNA from the area immunized with pCMV-Cre. Amplification of β-actin was used as a PCR positive control. Data from one mouse are shown; comparative data were obtained in all experiments. b, Mice (n = 5) were immunized with pCAG-lacZ-CMV-Cre, and dLN were pooled at 2.5 days. The CD11c
β-gal and CD11c
β-gal populations were isolated by cell sorting, and subjected to genomic DNA extraction. PCR analysis showed the bcl-x recombination product in the CD11c
β-gal (lane 1), but not in the CD11c
β-gal (lane 2) population.

**FIGURE 2.** Reduced T cell stimulatory capacity of CD11c
DC after loss of the bcl-x gene. Groups of bcl-x
mice (n = 5) were immunized with pCMV-Cre-CMV-OVA, pCMV-OVA, pCMV-Cre, or an empty vector. CD11c
DC isolated from dLN 5 days later were pooled and used directly in vitro, without exogenous peptide, to stimulate proliferation of naive, OVA-specific, OT-II TCR transgenic T cells. In control wells, responder T cells were pulsed with OT-I and OT-II peptides for negative and positive controls, respectively. Results are shown as the mean [3 H]thymidine uptake of duplicate wells after 72-h culture; error bars indicate range from the mean. Comparable data were obtained in two separate experiments.

**FIGURE 3.** Cre-mediated excision of the bcl-x gene in DC leads to lowered humoral responses. a, Cohorts (n = 5) of bcl-x
mice were immunized with pCMV-Cre-CMV-HA, pCMV-HA, or an empty vector. Serum Ab levels of anti-HA IgG at day 15 and 30 after gene gun immunization were quantified by ELISA for each individual mouse. The mean IgG concentrations (±SEM) are plotted. b, The same cohorts of mice were challenged intranasally with 3× LD_{50} of mouse-adapted influenza virus A/PR/8/34 at day 40 after immunization, and monitored for weight loss over the subsequent 10 days. Mean percent baseline weight (±SEM) at day 5, the peak of viral infection, is graphed. Comparable data were obtained in two separate experiments. *p < 0.05.
We then infected the same groups of mice with 3× LD₅₀ of a mouse-adapted influenza strain A/PR/8/34 at day 40 after immunization, and monitored them daily for weight loss (Fig. 3b). At the peak of infection (day 5), the pCMV-HA-CMV-Cre-immunized mice had dropped to an average of 79.8 ± 1.2% of their baseline weight, and the control empty vector group weighed in at 78.9 ± 1.1% of baseline. Both of these cohorts quickly succumbed to the viral infection. However, mice that received pCMV-HA showed a higher level of protection from viral challenge, as would be expected from their high anti-HA Ab titers, maintaining 89.7 ± 0.3% of their baseline weight at day 5 postinfection. The failure of the bcl-x-deficient DC to induce anti-HA Abs was reflected in the drastic weight loss of the pCMV-Cre-CMV-HA cohort upon viral challenge. Thus, Ag-bearing DC that had undergone cre-mediated deletion of bcl-x demonstrated a diminished capacity to elicit humoral immune responses.

**bcl-x is needed for survival of DC**

The loss of immunostimulatory capacity in DC established by the loss of bcl-x could be caused by a failure of these Ag-bearing DC to migrate to the dLN, or by their decreased survival in secondary lymphoid tissues. To track the ability of transduced DC to migrate to the dLN and mature, we genetically tagged the DC with lymphoid tissues. To track the ability of transduced DC to migrate to the dLN, or by their decreased survival in secondary loss of bcl-x.

The loss of immunostimulatory capacity in DC established by the deletion of bcl-x demonstrated a diminished capacity to elicit humoral immune responses. The loss of immunostimulatory capacity in DC established by the loss of bcl-x could be caused by a failure of these Ag-bearing DC to migrate to the dLN, or by their decreased survival in secondary lymphoid tissues. 

FIGURE 4. bcl-x deletion in DC leads to normal maturation, but enhanced apoptosis in dLN over time. a, bcl-x⁺/⁻ mice (n = 2) were immunized with pCMV-Cre-CAG-lacZ or pCAG-lacZ, and dLN were analyzed by flow cytometry for β-gal expression at various time points. Mice immunized with pCMV-Cre-CAG-lacZ had a consistently diminished absolute number of CD11c⁺β-gal⁺ cells that migrated to the dLN, compared with bcl-x⁺/⁻ mice immunized with pCAG-lacZ alone. Representative data from two independent experiments are shown, and have been gated on the early apoptotic 7-aminoactinomycin D (7AAD)⁺CD11c⁺ population. 

b, Enhanced apoptosis in the bcl-x-deficient, β-gal⁺ population was observed as a higher percentage of annexin V⁺ cells. The data are represented as percent change in frequency of CD11c⁺β-gal⁺ annexin V⁺ DC in experimental mice vs pCAG-lacZ-immunized control mice. c, At day 2.5, bcl-x-deficient DC exhibited comparable expression of activation markers, MHC class II-A⁺ and CD86, as control cells. The flow cytometry histograms plot the CD11c⁺β-gal⁺ DC from animals immunized with pCMV-Cre-CAG-lacZ (solid lines) or pCAG-lacZ (dotted lines). Shaded region represents the isotype Ab control.
bcl-x\textsubscript{L} isoform is predominant in CD11c\textsuperscript{+} DC

Multiple isoforms of the bcl-x gene have been described to date, chief among them being the antiapoptotic bcl-x\textsubscript{L} and the proapoptotic bcl-x\textsubscript{S}. Because the loxP sites engineered into the genome of mice, and subjected to mRNA extraction. RT-PCR analysis reveals the presence of the bcl-x\textsubscript{L}, but not the bcl-x\textsubscript{S} isoform (lane 1). As a reference, RT-PCR analyses of mRNA from human kidney cell line 293 transfected with pBcl-x\textsubscript{L} (lane 2) or pBcl-x\textsubscript{S} (lane 3) are shown. Data are representative of three separate experiments.

FIGURE 5. CD11c\textsuperscript{+} DC express the bcl-x\textsubscript{L}, but not the bcl-x\textsubscript{S} isoform. Schematic of the murine bcl-x gene. The 189-bp segment that is alternatively spliced from the bcl-x\textsubscript{S} isoform is indicated by crosshatches. Amplification of mRNA using a primer set (small arrows) flanking the segment missing in bcl-x\textsubscript{S} can distinguish between the bcl-x\textsubscript{L} isoform (343-bp product) and the bcl-x\textsubscript{S} isoform (154-bp product). CD11c\textsuperscript{+} DC were isolated by cell sorting from the superficial inguinal lymph nodes of naïve C57BL/6 mice, and subjected to mRNA extraction. RT-PCR analysis reveals the presence of the bcl-x\textsubscript{L}, but not the bcl-x\textsubscript{S} isoform (lane 1). As a reference, RT-PCR analyses of mRNA from human kidney cell line 293 transfected with pBcl-x\textsubscript{L} (lane 2) or pBcl-x\textsubscript{S} (lane 3) are shown. Data are representative of three separate experiments.

RNA interference silencing supports the role of bcl-x\textsubscript{L} in DC survival

Although the data in Fig. 5 indicate that bcl-x\textsubscript{L} is the predominant isoform in DC in the lymph node, bcl-x\textsubscript{S} could still be present at levels undetectable by standard RT-PCR, or it could be critical in regulating immature DC survival in the periphery. Therefore, we sought to specifically silence the bcl-x\textsubscript{L} isoform in skin-resident DC using siRNA oligonucleotides. DNA vectors based on the pSilencer backbone (Ambion) were engineered to express RNA hairpin oligonucleotides complementary to 21-nt targets. We directed the siRNA oligonucleotides against the 189-bp region of bcl-x exon 2 that is alternatively spliced from bcl-x\textsubscript{S} mRNA, and tested them for their ability to specifically reduce expression of bcl-x\textsubscript{L} by

FIGURE 6. siRNA-mediated silencing of bcl-x\textsubscript{L} isoform supports the role of bcl-x\textsubscript{L} in maintenance of Ag-bearing DC in dLN. RT-PCR analyses of 293 cells cotransfected with a plasmid expressing a bcl-x\textsubscript{L}-siRNA and either pBcl-x\textsubscript{L} or pBcl-x\textsubscript{S} showed specific silencing of bcl-x\textsubscript{L} expression (a). No change in expression was seen in parallel cotransfection experiments with a GFP-siRNA plasmid (data not shown). Densities were normalized to β-actin levels (data not shown) and are representative of two separate experiments. C57BL/6 mice (n = 3) were immunized with plasmids dually expressing β-gal with or without bcl-x\textsubscript{L}-siRNA, or control GFP-siRNA, and analyzed for expression of β-gal (b) and annexin V (c) by flow cytometry at 2.5, 4.5, and 6.5 days postvaccination. Cells were gated on the early apoptotic 7-aminoactinomycin D (7AAD)\textsuperscript{+}CD11c\textsuperscript{+} population (±SEM). The percentage of annexin V\textsuperscript{+} cells in the pCAG-lacZ-immunized group was used as a baseline to calculate the relative frequency of apoptotic cells in the pCAG-lacZ-bcl-x\textsubscript{L}-siRNA and pCAG-lacZ-GFP siRNA cohorts.
transient cotransfection with bcl-xL and bcl-xS expression constructs in human 293 cells. One of two selected siRNAs consistently reduced bcl-xL message by >96%, whereas levels of bcl-xS were unaffected (Fig. 6a). We cloned this siRNA hairpin molecule, along with the RNA Polymerase III U6 promoter, into the pCAG-lacZ vector (pCAG-lacZ-bcl-xL-siRNA). As a control, we similarly produced DNA vaccines containing siRNA oligos targeted against GFP (pCAG-lacZ-GFP-siRNA).

To confirm that the defect in Ag-bearing DC survival was due to loss of the bcl-xL isoform, we immunized cohorts of C57BL/6 mice with pCAG-lacZ, pCAG-lacZ-bcl-xL-siRNA, or pCAG-lacZ-GFP-siRNA, and analyzed dLN cells for survival of β-gal-marked DC at day 4.5. The frequency of β-gal-marked CD11c+ cells in the dLN decreased 4-fold in the pCAG-lacZ-bcl-xL-siRNA-immunized group in comparison with the pCAG-lacZ-immunized group (Fig. 6b). In contrast, the pCAG-lacZ-GFP-siRNA-immunized animals exhibited a similar amount of β-gal marking as the positive control (pCAG-lacZ), indicating that effects seen by silencing with the bcl-xL-siRNA are not due to a generalized IFN response to the presence of the siRNA (28).

Posttranscriptional silencing of bcl-xL also resulted in a significant increase in the frequency of β-gal-marked cells undergoing apoptosis in the dLN. At 2.5 days postimmunization, there was a 15.2 ± 3.1% increase in the number of CD11c+β-gal” annexin V+ cells in the pCAG-lacZ-bcl-xL-siRNA-immunized group over baseline levels in the pCAG-lacZ-immunized group (Fig. 6c). This percentage rapidly increased such that, by day 6.5, the bcl-xL-silenced CD11c+β-gal+ DC population had a 6-fold higher frequency of apoptotic cells. By contrast, the untransfected CD11c+β-gal+ DC population in the pCAG-lacZ-bcl-xL-siRNA-immunized group exhibited similar levels of annexin V+ cells as controls. This signified that the poor rate of survival was unique to the directly transfected DC that had migrated from the site of immunization, and not due to a systemic phenomenon. These results are in full agreement with the data obtained from cre-mediated bcl-x excision in DC of bcl-x<sup>fl/fl</sup> mice, indicating that it was the loss of the antiapoptotic bcl-xL isoform that produced the survival defect in bcl-xL-deficient, migrating CD11c+ DC.

Migration of bcl-xL-deficient DC is not impaired

The data presented in Figs. 4 and 6 suggest that the inability to elicit Ag-specific immune responses is due to the heightened sensitivity of bcl-x-deficient DC to apoptosis. However, it is also possible that the low numbers of CD11c+β-gal+ cells in the dLN may also be due to a defect in migration from the skin. To address this issue, we analyzed bcl-xL-deficient DC at the immunization site. We chose to immunize the ears, because isolation of DC from ear skin as well as from the draining auricular lymph nodes is highly tractable. Briefly, we gene gun immunized cohorts of C57BL/6 mice with pCAG-lacZ, pCAG-lacZ-bcl-xL-siRNA, or pCAG-lacZ-GFP-siRNA. At 1.5 and 2.5 days after vaccination, we quantified the absolute number of CD11c+β-gal+ cells that remained at the immunization site or that migrated to the dLN. There was no significant difference among the three groups in the absolute numbers of Ag-bearing cells per ear at either time point. Although ~1000 CD11c+β-gal+ cells were still present at the immunization site at day 1.5, only ~250 remained by day 2.5 (Fig. 7a). We also analyzed the DC at the immunization site for evidence of apoptosis by annexin V staining. We found no difference among the three groups in levels of annexin V in the CD11c+β-gal+ population isolated from the ear at both time points (data not shown), suggesting that DC were not dying at the immunization site, but had migrated away from the skin.

To confirm the migration of the CD11c+β-gal+ cells, we analyzed the draining auricular lymph nodes for the presence of Ag-bearing DC. Similar to the results observed with immunization of the abdominal skin, we found reduced numbers of bcl-xL-deficient, CD11c+β-gal+ DC in the dLN at both time points (Fig. 7b). In comparison, we enumerated ~500 and 1500 CD11c+β-gal+ DC per dLN at days 1.5 and 2.5, respectively, from mice immunized with pCAG-lacZ or pCAG-lacZ-GFP-siRNA. This increase in CD11c+β-gal+ DC in the dLN mirrored a concomitant decrease in CD11c+β-gal+ DC at the immunization site of both control groups. The corresponding bcl-xL-deficient population showed a similar reduction in numbers of Ag-bearing DC at the immunization site at day 2.5; however, there were persistently low numbers of CD11c+β-gal+ cells in the dLN, due to higher levels of apoptosis. Taken together, these data indicate that bcl-xL-deficient DC are capable of migration from the periphery, and support the hypothesis that impaired longevity of the Ag-bearing DC in the dLN causes the failure to elicit immune responses.

Delivery of bcl-x<sub>L</sub> in trans complements bcl-x deficiency

To further confirm that the bcl-xL isoform is indeed critical for mature DC survival, we attempted to rescue the phenotype observed with bcl-x-deficient DC by providing bcl-x<sub>L</sub> in trans. bcl-x<sup>fl/fl</sup> mice were immunized with gene gun bullets that had been cocrated with the pCMV-Cre-CAG-lacZ DNA vector, along with a second plasmid encoding either bcl-xL cDNA, or a mutant bcl-xL (bcl-xL<sub>M7</sub>) modified in the BH1 domain to abrogate its antiapoptotic function (29). At 4.5 days after immunization, we evaluated CD11c+ cells from the dLN for their expression of β-gal and annexin V. Complementation of cre-mediated, bcl-x deficiency with wild-type bcl-xL, but not bcl-xL<sub>M7</sub>, restored the numbers of β-gal-marked CD11c+ DC in the dLN (Fig. 8a). Furthermore, the
addition of wild-type bcl-xL was sufficient to protect the transplanted DC from apoptotic death, showing a 21.55 ± 6.8% reduction in the percentage of annexin V+ cells in the CD11c−β-gal+ population (Fig. 8b). However, complementation with the mutant bcl-xL did not produce any significant change in the level of apoptotic cells. Thus, rescue of bcl-x deficiency in CD11c+ DC was accomplished by delivery of wild-type bcl-xL in trans.

Discussion

The longevity of Ag-bearing DC in secondary lymphoid tissues is thought to be a major determinant of the potency of the immune response generated. Prolonged survival would increase the chance that the DC will make contact with and stimulate the small number of Ag-specific naive T cells. Previous work has implicated antiapoptotic factors such as bcl-2 and bcl-xL in vivo enhances mature DC longevity in secondary lymphoid tissues, and correspondingly increases B and T cell responses (30, 31). In DC, TLR signaling induced by inflammatory stimuli has been shown to up-regulate expression of both bcl-2 and bcl-xL, and other antiapoptotic proteins (33). Furthermore, the addition of T cell-derived factors, such as TRANCE and CD40L, to DC cultures can also promote their survival, thus implicating the DC-T cell interaction in the cellular homeostasis of Ag-bearing DC (10, 11, 32). TRANCE ligation inhibited apoptosis by selective up-regulation of bcl-xL expression, and concomitantly improved the T cell stimulatory capacity of DC (34). In contrast, CD40 signaling increased levels of the antiapoptotic protein bcl-2, and has also been shown to prolong the longevity and Ag-presenting capacity of DC (12, 32). Although it is possible that both bcl-xL and bcl-2 fulfill redundant roles in protection from cell death, gene microarray studies detected a significant increase in bcl-xL expression immediately after DC maturation, whereas bcl-2 remained at basal levels (13). Additionally, Kim et al. (29) found that Ag-specific immunostimulation by DNA vaccines was most optimally enhanced by engineering constructs to coexpress bcl-xL, rather than other antiapoptotic proteins such as bcl-2. Indeed, our studies show that bcl-x-deficient DC are unable to maintain their survival in the dLN, implying that the loss of this antiapoptotic gene is sufficient to drive these cells to an accelerated death.

Recent reports (35–37) have suggested that the initiation of T cell responses may not require prolonged interaction with Ag and APC. This implies that DC longevity may not play such a crucial role in the initiation of T cell responses, and that, after the initial interaction between T cell and DC, the T cell may be able to autonomously differentiate and exert effector functions. However, our studies seem to suggest that survival of Ag-bearing DC is critical for the generation of immune responses. bcl-x-deficient DC were still capable of migration from the immunization site, although they were present in the dLN at much fewer numbers due to higher levels of apoptosis. At the peak of migration (2.5 days after gene gun immunization) (3), there were only ~1600 Ag-bearing skin-derived DC per draining superficial inguinal lymph node. Indeed, not only were the frequencies of marked cells in the dLN consistently lower, the rate of decline of the bcl-x-deficient DC population was much more rapid than that seen in the mice immunized with the tracking Ag alone. By day 6.5, the absolute number of CD11c−β-gal+ cells had dropped to 250 per lymph node, whereas >5000 could be detected in the control group. The fact that immune responses were so markedly diminished by the decrease in the number of β-gal-marked, bcl-x-deficient DC in the dLN suggests that there may be a threshold number of Ag-bearing DC required to induce acquired immunity. This may be due to the inability of the few DC to find the small number of Ag-specific T cells. Recent work by our laboratory has shown that previous reports enumerating the peak frequencies of Ag-bearing DC had underestimated the actual numbers by ~100-fold (3), which raises the possibility that optimal immune responses require a higher number of DC than was achieved in the environment of bcl-x deficiency.

The results presented in this study have implications for the rational design of DC-based vaccine regimens. As vaccine-mediated immunity is thought to rely upon APC, especially DC, it is important to find ways by which DC-driven Ag-specific responses can be heightened. Previous studies have attempted to ameliorate mediated bcl-x excision in the bcl-x(+/−) mouse indicates that bcl-xL plays a pivotal role in maintaining the longevity of Ag-bearing DC after migration to the dLN.

Our data supplement findings that overexpression of antiapoptotic factors such as bcl-2 and bcl-xL in vivo enhances mature DC longevity in secondary lymphoid tissues, and correspondingly increases B and T cell responses (30, 31). In DC, TLR signaling induced by inflammatory stimuli has been shown to up-regulate expression of both bcl-2 and bcl-xL, and other antiapoptotic proteins (33). Furthermore, the addition of T cell-derived factors, such as TRANCE and CD40L, to DC cultures can also promote their survival, thus implicating the DC-T cell interaction in the cellular homeostasis of Ag-bearing DC (10, 11, 32). TRANCE ligation inhibited apoptosis by selective up-regulation of bcl-xL expression, and concomitantly improved the T cell stimulatory capacity of DC (34). In contrast, CD40 signaling increased levels of the antiapoptotic protein bcl-2, and has also been shown to prolong the longevity and Ag-presenting capacity of DC (12, 32). Although it is possible that both bcl-xL and bcl-2 fulfill redundant roles in protection from cell death, gene microarray studies detected a significant increase in bcl-xL expression immediately after DC maturation, whereas bcl-2 remained at basal levels (13). Additionally, Kim et al. (29) found that Ag-specific immunostimulation by DNA vaccines was most optimally enhanced by engineering constructs to coexpress bcl-xL, rather than other antiapoptotic proteins such as bcl-2. Indeed, our studies show that bcl-x-deficient DC are unable to maintain their survival in the dLN, implying that the loss of this antiapoptotic gene is sufficient to drive these cells to an accelerated death.

Recent reports (35–37) have suggested that the initiation of T cell responses may not require prolonged interaction with Ag and APC. This implies that DC longevity may not play such a crucial role in the initiation of T cell responses, and that, after the initial interaction between T cell and DC, the T cell may be able to autonomously differentiate and exert effector functions. However, our studies seem to suggest that survival of Ag-bearing DC is critical for the generation of immune responses. bcl-x-deficient DC were still capable of migration from the immunization site, although they were present in the dLN at much fewer numbers due to higher levels of apoptosis. At the peak of migration (2.5 days after gene gun immunization) (3), there were only ~1600 Ag-bearing skin-derived DC per draining superficial inguinal lymph node. Indeed, not only were the frequencies of marked cells in the dLN consistently lower, the rate of decline of the bcl-x-deficient DC population was much more rapid than that seen in the mice immunized with the tracking Ag alone. By day 6.5, the absolute number of CD11c−β-gal+ cells had dropped to 250 per lymph node, whereas >5000 could be detected in the control group. The fact that immune responses were so markedly diminished by the decrease in the number of β-gal-marked, bcl-x-deficient DC in the dLN suggests that there may be a threshold number of Ag-bearing DC required to induce acquired immunity. This may be due to the inability of the few DC to find the small number of Ag-specific T cells. Recent work by our laboratory has shown that previous reports enumerating the peak frequencies of Ag-bearing DC had underestimated the actual numbers by ~100-fold (3), which raises the possibility that optimal immune responses require a higher number of DC than was achieved in the environment of bcl-x deficiency.

The results presented in this study have implications for the rational design of DC-based vaccine regimens. As vaccine-mediated immunity is thought to rely upon APC, especially DC, it is important to find ways by which DC-driven Ag-specific responses can be heightened. Previous studies have attempted to ameliorate...
the level of immune responses by inducing apoptosis in the Ag-bearing cell (38–40), such that many other APC can take up the apoptotic body and initiate cellular immunity to a greater degree than could the small number of DC targeted by the vaccine. However, shortening the life span of Ag-bearing DC in the dLN by apoptotic body and initiate cellular immunity to a greater degree

Wick for DNA constructs; H. Robinson for in

Acknowledgments

We gratefully acknowledge T. W. Behrens, S. Korsmeyer, and J. M. Hardwick for DNA constructs; H. Robinson for influenza virus; K. A. Smith for mouse colony management; and A. Lukacher and J. Macke for helpful comments and editing.

References


