Immunological adjuvants promote activated T cell survival *via* induction of Bcl-3

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Injection of soluble protein antigen into animals causes abortive proliferation of the responding T cells. Immunological adjuvants boost T cell responses at least in part by increasing the survival of activated T cells during and after the initial proliferative phase of their clonal expansion. To understand how adjuvants promote T cell survival, we used gene microarrays to analyze gene expression in T cells activated either with antigen alone or in the presence of two different adjuvants. Among the genes whose expression was increased by both adjuvants was the IκB family member Bcl-3. Retroviral infection experiments showed that expression of Bcl-3 increased survival of activated T cells *in vitro* and *in vivo*. Adjuvants may therefore improve survival of activated T cells *via* induction of Bcl-3.

Injection of purified protein antigens into animals causes rapid proliferation followed by rapid death of antigen-specific T cells[1–4]. Consequently, animals exposed to these protein antigens often have fewer antigen-specific T cells at the end of a response than they had before the antigens were administered. This process of activation-induced cell death (AICD) is probably important to the animal because it quickly reduces the load of activated effector T cells and also allows the removal of T cells that are responding to host antigens, thus reducing the risk of autoimmune responses.

Activation-induced death, however, could be deleterious to the animal because it may drive too rapid an elimination of T cells that are responding to antigens expressed by invading organisms, thus reducing the ability of the host to resist infections. It is now clear that materials associated with infections reduce the rate of AICD and help to maintain the numbers of responding T cells in the animal. For example, the death of activated T cells is reduced in animals infected with vaccinia virus (VV) even though the activated T cells themselves are not necessarily responding to viral antigens[5]. Likewise, the rate of death of T cells responding to protein antigens or superantigens is reduced if animals are simultaneously injected with heat-killed mycobacteria in complete Freund's adjuvant or with bacterial lipopolysaccharide (LPS)[4,6]. Thus, materials associated with infections, called adjuvants, help to preserve responding T cells and allow productive immune responses to occur.

Little is known about the mechanism by which adjuvants increase the life expectancy of activated T cells. Some studies have shown that the activated T cells die by caspase action after Fas and tumor necrosis factor receptor (TNFR) engagement[7]. However, because AICD induced *in vivo* by superantigen is only dependent on Fas and TNF signaling to a small degree[8,9], we do not believe that adjuvants act by interfering with Fas- or TNF-induced death. Activated T cells appear to die, directly or indirectly, from the effects of reactive oxygen species (ROS), species that are increased

Table 1. Properties of sorted T cells used for Affymetrix genechip experiments

Cell properties	T cell treatment		
	Activated[a]	Activated + VV[b]	Activated + LPS[c]
Sorted cells bearing $V_\beta8^+$ TCR[d]	97.4%	98.2%	97.1%
Sorted cells bearing MHC class II	0.5%	0.2%	0.2%
$CD4^+$ and $CD8^+$ cells surviving 24 h of culture[e]	23.4%	35.3%	44.4%
$CD4^+V_\beta8^+$ T cells present after SEB treatment[f]	7.0±0.3	10.7±1.3	54.7±10.9
$CD8^+V_\beta8^+$ T cells present SEB treatment[f]	3.6±0.4	11.5±1.6	25.7±2.0

All treatments were given intravenously and all cells were collected on day 2, except when cells were analyzed 12 days after administration of SEB. [a]T cells from mice given SEB (150 μg) on day 0. [b]T cells from mice given SEB (150 μg) on day 0 and VV (10^7 PFUs) three days prior to this. [c]T cells from mice given SEB (150 μg) day 0 and LPS (5 μg) + anti-CD40 (300 μg) on day 1. [d]Cells were sorted based on expression of $V_\beta8$ and CD4 or CD8. [e]Survival was determined by culture for 24 h in minimal medium supplemented with serum. Live cells were defined as those able to exclude propidium iodide. The s.e.m. from triplicate measurements was less than 5% of the values shown. [f]Twelve days after SEB treatment three animals from each treatment group were killed and the total numbers of $CD4^+$ or $CD8^+$ $V_\beta8$ TCR-bearing T cells in the spleen and inguinal, axillary, brachial, submandibular, periaoritc and mesenteric lymph nodes were measured. All values are $\times10^{-6}$.

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Table 2. Genes scored as increased in T cells after activation and adjuvant treatment, relative to activation alone

Genbank accession number	Protein encoded	Fold increase compared to activation alone +VV	+LPS
M90397	Bcl-3	9.8	17
U15636	GTP-binding (clone U2) T cell–specific protein	2.2	6
M63630	GTP binding protein (IRG-47)	1.9	4.9
U06924	STAT1	2.0	4.8
U60150	VAMP-2	4.7[a]	3.8[a]
M13226	Granzyme A	2.0	2.7
X04072	Granzyme B	3.1	2.6
U43678	Ataxia telangiectasia gene homolog (Atm)	1.9	2.5[a]
X58438	MP4 gene for a proline-rich protein	2.9[a]	2.4[a]
X60452	Cytochrome P-450IIIA	2.3	2.4[a]
L00919	Protein 4.1	2.0[a]	2.3[a]
M83380	RelB	1.9	2.0
X57800	DNA polymerase δ auxiliary protein	1.8	2.0
L36829	AlphaA-crystallin-binding protein I	2.0	1.8
M95564	Protein PC326	1.7[a]	1.8[a]
D29639	3-hydroxyacyl CoA dehydrogenase	1.5	1.8
U70674	B2 element (Rn18s) and 18S ribosomal RNA	1.3[a]	1.8[a]
J00361	Amylase isozyme B-1	2.4[a]	1.6[a]
U60328	Proteasome activator PA28 α subunit	1.6	1.7
X13460	p68 protein of the lipocortin family"	2.0	1.6
U24674	Max interacting protein 1 (Mxi1)	1.5	1.6
M31585	ICAM-1	1.3	1.6
M29697	IL-7R	1.2	1.6

Increases in gene expression were determined using Affymetrix Genechip Analysis software. [a]Negligable expression was observed for the reference sample (SEB alone), therefore the increase in this value is approximate.

in activated *versus* resting T cells[7]. Adjuvants may therefore act by lowering the amount of ROS. However, our preliminary experiments indicate that, if they have any effect at all, adjuvants act to increase rather than decrease ROS content in activated T cells.

Members of the Bcl-2 family of proteins are often involved in the life and death of cells. The increased survival of activated T cells could be caused either by increased expression of anti-apoptotic proteins such as Bcl-2 and Bcl-x_L or decreased expression of pro-apoptotic proteins such as Bim, Bad and Bax. However, our published and preliminary experiments have shown that expression of these proteins is not affected by exposure to adjuvants[5]. These observations do not eliminate the possibility that Bcl-2 family members are involved in the effects of adjuvants on T cells because the activity of such proteins is controlled not only by the amount of expression but also by post-translational phosphorylation, proteolytic cleavage and changes in cellular localization[10]. Nevertheless, superantigen-induced AICD is inhibited by cyclohexamide (D. Hildeman, unpublished observation), which indicates that new protein translation must play a necessary role in regulating T cell death after activation. Therefore, we continued to seek factors whose sustained expression was correlated with T cell survival effects.

Accordingly, we used microarray technology to compare gene expression in activated T cells that had or had not been exposed to adjuvants. To eliminate effects of the adjuvants that might be specific to a particular material and not general to all adjuvants, we searched for genes that were similarly affected by exposure to two different types of adjuvants. T cells were activated in mice in the presence or absence of the adjuvants VV or LPS. RNA was isolated from the activated cells and their gene expression compared with the use of Affymetrix GeneChip microarrays. We found that 23 genes, whose sequences have been deposited in the Genbank database, were increased in both VV- and LPS-exposed cells, as compared to T cells activated in the absence of adjuvant. These included the gene encoding Bcl-3, which is a member of the nuclear factor NF-κB–IκB protein family. Because it has been reported that some members of this family of transcription factors have anti-apoptotic activity, we tested the effect these family members (including Bcl-3) had on AICD using retroviral vectors. Transduction experiments showed that Bcl-3 expression increased the survival of activated T cells both *in vitro* and *in vivo*. Thus, we conclude that adjuvants increase T cell life expectancy, at least in part, by increasing Bcl-3 expression in these cells, which may alter the balance of NF-κB transcriptional activities during immune responses.

Results

RNA preparation and analysis

We used Affymetrix gene arrays to broaden our search for adjuvant-induced factors. This required large numbers of purified T cells (>5×10[7]) that have been activated under various conditions. To obtain these cells, normal C57BL/10 mice were injected with the superantigen staphylococcal enterotoxin B (SEB), which reacts with T cells bearing $V_\beta 8$ as part of their T cell antigen receptor (TCR). Two days after SEB injection, activated $V_\beta 8^+$ T cells start to undergo rapid apoptotic death that is partially prevented in adjuvant-treated mice[11]. For gene array comparisons, therefore, T cells were given SEB alone, SEB + bystander VV infection or SEB + LPS + anti-CD40. Anti-CD40 was added

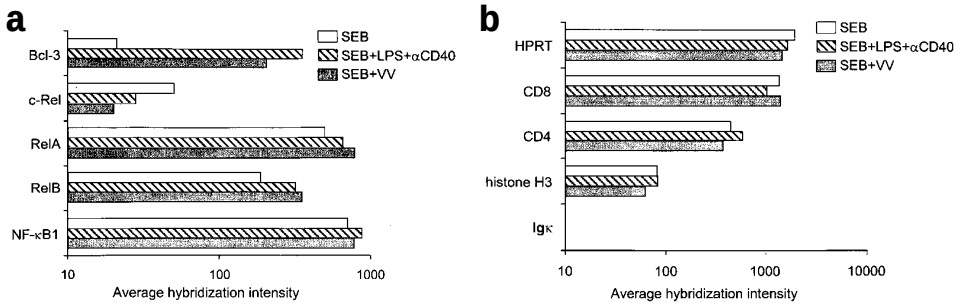

Figure 1. RNA transcripts of the genes encoding Bcl-3 and RelB are increased in T cells exposed to adjuvant. Affymetrix mu6400 gene microarrays were used to assess gene expression in activated CD4 and CD8 T cells that had been collected, 2 days after injection of SEB, from mice treated with SEB alone, SEB + LPS + anti-CD40 or SEB + VV. Cellular RNA from $CD4^+$ and $CD8^+$ $V_\beta 8$ TCR^+ MHC class II^- cells were analyzed with Affymetrix genechips (see Methods). The microarray signals shown are Affymetrix average differences, which were calculated with Affymetrix GeneChip Analysis software and are expressed in arbitrary units. (**a**) RNA transcript levels of genes encoding NF-κB family members. (**b**) RNA transcript levels of selected control genes.

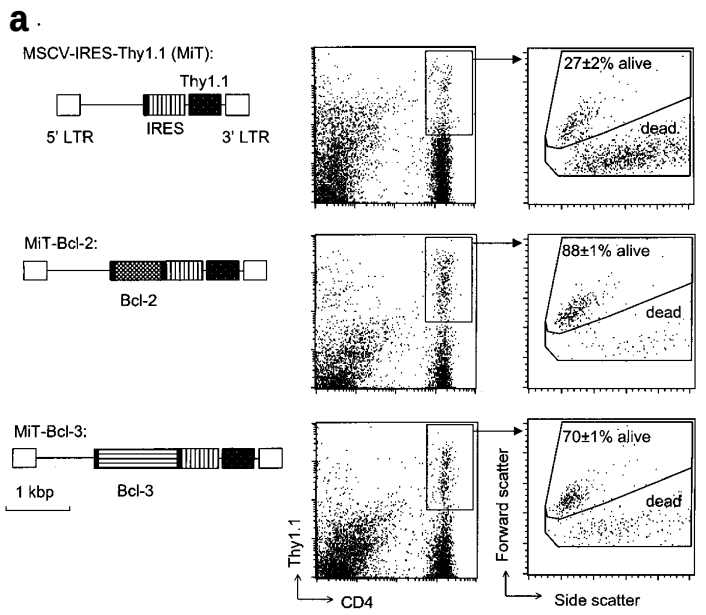

Figure 2. Survival of activated T cells can be measured with Thy1.1⁺ retroviral vectors. The retroviral vector MSCV-IRES-Thy1.1 (MiT) was used to measure T cell survival after expression of either Bcl-2 or Bcl-3. (**a**) Activated T cells were collected from V_βDO mice 48 h after injection of 100 μg of SEB. The survival of T cells that had been infected either with control MiT, MiT–Bcl-2 or MiT–Bcl-3 viruses was assessed by flow cytometric detection of the light scatter properties of CD4⁺ Thy1.1⁺ (gate shown as inset boxes) cells. Identical results were obtained by scoring live cells as those that failed to bind FITC–annexin V (data not shown). The values shown for percentage of gated cells alive are the mean±s.e.m from triplicate cultures of retrovirus-infected cells and are typical of six experiments in which control vector, MiT–Bcl-2 or MiT–Bcl-3 were tested. (**b**) Time-course of *in vitro* survival after mock *versus* MiT–Bcl-3 retroviral transduction. Activated T cells from V_βDO mice were prepared, infected with retrovirus, cultured for 18, 24, 36 or 48 h and analyzed in triplicate as described above. (Mock-infected, CD4⁺Thy1.1⁻ T cells. Thy1.1⁻, uninfected CD4⁺Thy1.1⁻ cells from MiT–Bcl-3-infected cultures. MiT-Bcl-3, virus-infected CD4⁺Thy1.1⁺ T cells.)

because it potentiates the adjuvant effects of LPS[12] (and G. Marshall and T. Mitchell, unpublished observations). Cells from the lymph nodes and spleens of treated mice were isolated at the peak of their expansion, just before they began to die. T cells from the three groups of mice were purified by high-speed cell sorting to yield populations that were >97% $V_\beta8^+$ and <0.5% major histocompatibility complex (MHC) class II⁺ (**Table 1**). Samples of these cells were tested to check that the adjuvant treatments had indeed prolonged their life expectancy (**Table 1**). RNA was prepared from these cells and analyzed with the mu6400 Affymetrix gene array system, as described[13].

Of the genes and expressed sequence tags (ESTs) represented on the mu6400 genechip, expression of 288 genes increased in T cells after SEB + VV treatment and expression of 141 genes increased after SEB + LPS + anti-CD40 treatment, compared to T cells stimulated with SEB alone. Of these, expression of 23 previously described genes was increased by both the viral and bacterial adjuvants (**Table 2**). Several of the 23 genes, such as those encoding the granzymes A and B, interleukin 7 receptor α (IL-7Rα), signal transducers and activators of transcription 1 (STAT1) and intercellular adhesion molecule 1 (ICAM-1), were likely to be relevant to T cell immune responses. Expression of two members of the NF-κB family, RelB and the IκB subfamily member Bcl-3, also increased by about three- and tenfold, respectively (**Fig. 1**). Independent quantification of Bcl-3 expression by "real-time" polymerase chain reaction (PCR) confirmed these results by showing increases of similar magnitudes. Because the adjuvant-induced increases in Bcl-3 were large and because NF-κB factors are reported to have anti-apoptotic activity[14–16], this family was studied further.

Bcl-3 prevents the death of activated T cells

To test for their effects on activated T cell survival, cDNAs encoding Bcl-3 and other NF-κB–related genes were cloned into the retroviral vector MSCV-IRES-Thy1.1 (MiT)(**Fig. 2**). This vector allows bicistronic expression of an introduced gene and of the T cell antigen Thy1.1 (CD90.1) as a cell-surface marker of retroviral transduction. When Thy1.1 was compared to green fluorescent protein (GFP) it proved to a superior marker for the analysis of activated T cell death. This is because GFP is rapidly lost from dead cells, whereas Thy1.1 is retained for at least 48 h after cell death (data not shown). Use of Thy1.1 also allowed

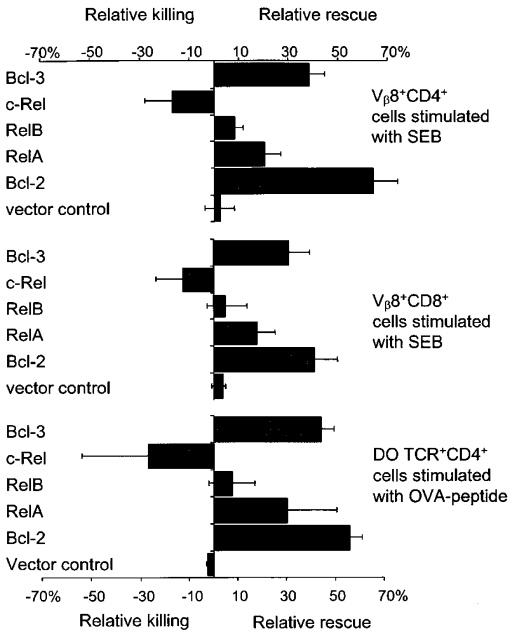

Figure 3. Bcl-3 increased survival of activated T cells. Activated T cells were collected either from V_βDO mice 48 h after treatment with 100 μg of SEB or DOTgR mice 48 h after injection of stimulatory OVA peptide. Cells were infected with retroviruses expressing genes encoding Thy1.1 alone or Thy1.1 + Bcl-3, c-Rel, RelB, RelA or Bcl-2 and tested for survival *in vitro* 24 h later. The survival profiles for retrovirally transduced CD4⁺ or CD8⁺ T cells from V_βDO mice after activation with superantigen and for CD4⁺ DO TCR⁺ cells from DOTgR mice after activation with OVA peptide are shown. Relative killing and relative rescue were calculated from the percentage of infected cells left alive after infection with each retroviral vector compared with the MiT vector control (see Methods). Data are mean±s.e.m. from four to six independent experiments done with each vector.

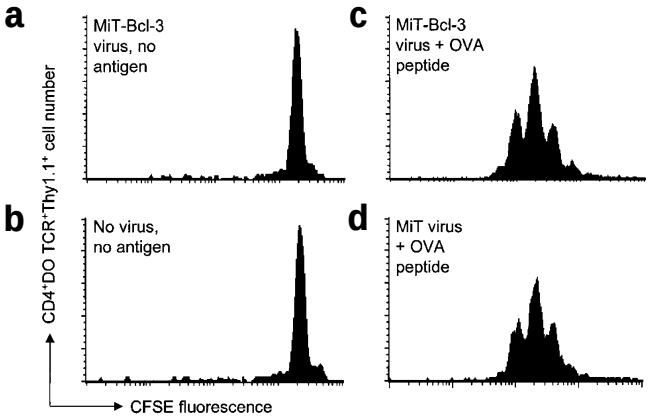

Figure 4. Bcl-3 did not affect T cell proliferation. *In vitro* proliferation of T cells after retroviral infection with MiT control or with MiT–Bcl-3. Activated T cells were collected from DOTgR mice 24 h after administration of OVA peptide, exposed to control or MiT–Bcl-3 retrovirus and cultured. Two days later, the surviving cells were loaded with CFSE, restimulated for 4 days with 1.0 μM of peptide antigen + splenic antigen-presenting cells and the green fluorescence per live $CD4^+$ DO TCR^+ $Thy1.1^+$ cell visualized. The histograms shown are typical of four independent experiments that were used to calculate the mean±s.e.m. cellular divisions. (**a**) MiT–Bcl-3–infected cells with no antigen added**.** (**b**) Uninfected cells with no antigen added. (**c**) MiT–Bcl-3–infected T cells with 1.0 μM of OVA peptide, for which the average number of cell divisions recorded was 2.94±0.01. (**d**) MiT control virus–infected T cells + 1.0 μM of OVA peptide, for which the number of cell divisions recorded was 2.69±0.01.

detection of cells that had been retrovirally transduced with minimal effects on the cell because the Thy1.1 allele differs from the more commonly expressed Thy1.2 allele by a single amino acid residue[17].

$V_\beta8^+$ T cells were activated with SEB in mice that were transgenic for a TCR $V_\beta8^+$ chain (V_βDO mice[18]). Two days later, when they were about to die, the $V_\beta8^+$ T cells were isolated, infected with various retroviruses and cultured overnight. Flow analysis was then used to assess survival of the retrovirally transduced cells, which were identified by surface expression of Thy1.1 (**Fig. 2**). Cultured cells were stained with fluorescein isothiocyanate (FITC)–anti-CD4 and phycoerythrin (PE)–anti-Thy1.1. The viability of $CD4^+Thy1.1^+$ cells was determined by analysis of forward and side light-scattering properties. In one experiment, 27% of activated $CD4^+$ cells transduced with a Thy1.1-expressing control virus were alive after overnight culture (**Fig. 2a**). As expected, transduction with a virus expressing *Bcl2* increased the percentage of surviving cells to 88% of activated $CD4^+$ cells. Transduction with a virus expressing *Bcl3* also increased the percentage of surviving cells, although this time it was to 70%. This ability of *Bcl3* expression to slow the death rate for activated T cells was not limited to a particular time during the assay, as shown by a time-course in which MiT–Bcl-3–infected or mock-infected cells were analyzed for death at various times after infection. The percentage of activated T cells that were alive was consistently higher in cells expressing Bcl-3 compared to controls (**Fig. 2b**).

To compare the effects of different members of the NF-κB family, T cells were activated *in vivo*, purified 2 days later, transduced with viruses expressing genes encoding the indicated NF-κB family members and analyzed for survival after overnight culture (**Fig. 3**). The overall patterns seen in $CD4^+$ T cells were also seen in $CD8^+$ T cells that had been activated in normal mice with superantigen. In addition, they were seen in $CD4^+$ T cells from recombination activating gene (RAG-2)–deficient mice that were transgenic for the chicken ovalbumin (OVA) and $I\text{-}A^d$–reactive TCR from the T cell hybridoma line DO-11.10[19] (DOTgR) mice that had been activated *in vivo* with stimulatory peptide. Bcl-3 gave the strongest and most consistent survival effect, second only to Bcl-2 (**Fig. 3**, top). RelA (also called p65) enhanced survival to a lesser extent, whereas RelB had no significant effect. c-Rel, however, impaired T cell survival in most experiments. This is in contrast to the reported role of c-Rel in lymphocytes, in which endogenous c-Rel expression is required for survival during antigen-induced proliferation or exposure to mitogens *in vitro*[20]. Our observation that T cell death occurs after enforced expression may therefore result from nonphysiological c-Rel expression or from inappropriately timed stimulation of its transcriptional activity.

T cells that have been activated *in vivo* and then purified and cultured in the absence of antigen do not divide (**Fig. 4** and data not shown). However, NF-κB family members are known to affect cellular proliferation. To test whether Bcl-3 affected T cell survival or proliferation, activated T cells from DOTgR mice were transduced with viruses expressing the genes encoding Bcl-3 + Thy1.1 or Thy1.1 alone, labeled with 5-(6)-carboxyfluorescein diacetate succinimidyl diester (CFSE) and cultured with or without antigen. CFSE binds irreversibly to intracellular proteins and can be used to count divisions of labeled cells because its fluorescence per cell is halved with each cellular division[21].

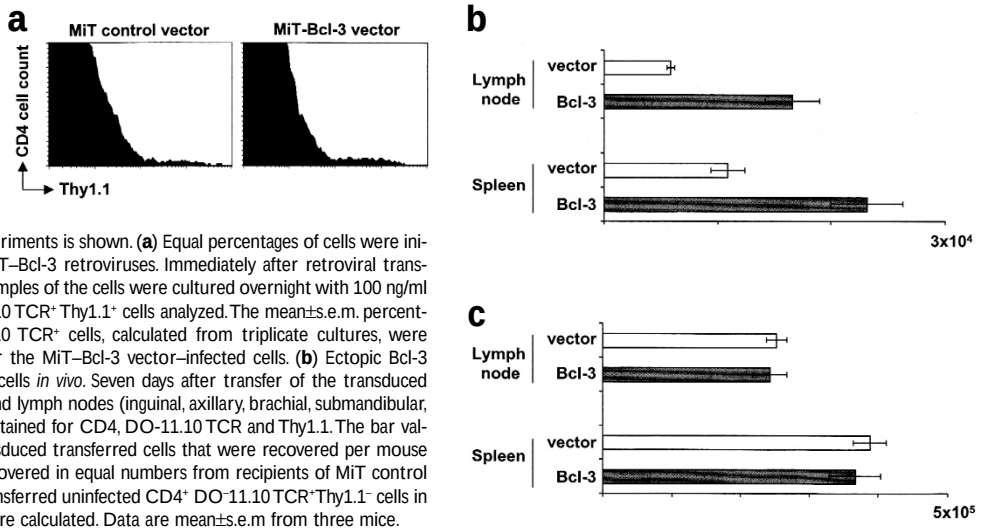

Figure 5. Bcl-3 increased activated T cell survival *in vivo*. Activated T cells were collected from DOTgR mice that had been injected with stimulatory OVA peptide 2 days before. T cells were exposed to control or *Bcl3*-encoding retrovirus by low-speed centrifugation and washed three times in serum-free saline solution. Cells (10^7) were injected intravenously into four BALB/c recipients for each retrovirus tested. One of three representative experiments is shown. (**a**) Equal percentages of cells were initially transduced with the MiT control or MiT–Bcl-3 retroviruses. Immediately after retroviral transduction, at a multiplicity of infection of 0.05, samples of the cells were cultured overnight with 100 ng/ml of IL-4 and the percentages of $CD4^+$ DO-11.10 TCR^+ $Thy1.1^+$ cells analyzed. The mean±s.e.m. percentages of $Thy1.1^+$ cells among $CD4^+$ DO-11.10 TCR^+ cells, calculated from triplicate cultures, were 2.52±0.06 for the control- and 2.41±0.08 for the MiT–Bcl-3 vector–infected cells. (**b**) Ectopic Bcl-3 expression promotes survival of activated T cells *in vivo*. Seven days after transfer of the transduced cells, T cells were isolated from the spleens and lymph nodes (inguinal, axillary, brachial, submandibular, mesenteric and periaortic) of recipients and stained for CD4, DO-11.10 TCR and Thy1.1. The bar values indicate the mean±s.e.m. number of transduced transferred cells that were recovered per mouse from four mice. (**c**) Uninfected cells were recovered in equal numbers from recipients of MiT control or MiT-Bcl-3–based cells. Total numbers of transferred uninfected $CD4^+$ DO^-11.10 $TCR^+Thy1.1^-$ cells in the lymph nodes and spleens of recipients were calculated. Data are mean±s.e.m from three mice.

Cells cultured without antigen did not divide, whether or not they expressed Bcl-3 (**Fig. 4a,b**). Conversely, cells cultured with peptide antigen for 4 days divided to the same extent whether or not they expressed Bcl-3 (**Fig. 4c,d**). Therefore, the major effect of Bcl-3 on T cells was the inhibition of activated T cell death. This deduction is similar to that of a previous report, which showed that Bcl-3 prevented death of a T lymphoid cell line withdrawn from growth factor[22].

Different factors may affect T cell survival *in vivo versus in vitro*. Therefore, we tested the effect of Bcl-3 expression on activated T cell survival in adoptively transferred mice. Activated T cells from DOTgR mice were exposed to MiT–Bcl-3 or control MiT retrovirus for 2 h by low-speed centrifugation. Some of the transduced cells were cultured overnight and the efficiency of infection analyzed by staining for Thy1.1 expression. We found that equal proportions of cells (~2.5%) had been transduced with MiT–Bcl-3 or MiT (**Fig. 5a**).

Immediately after virus exposure, the remaining transduced and untransduced cells were transferred into untreated recipient mice. Seven days later, the recipients were killed and their spleen and lymph node cells analyzed for DO TCR expression with or without concommitant Thy1.1 expression. Because normal mice contain undetectable numbers of cells bearing the DO TCR, we could measure the number of surviving transferred cells that had or had not been transduced with MiT–Bcl-3 or MiT. The results showed that T cells transduced with Thy1.1 and Bcl-3 were recovered in greater numbers from the recipients than cells transduced with Thy1.1 alone (**Fig. 5b**). This effect was not due to the inadvertant transfer of more cells from the MiT–Bcl-3–transduced mixture because nontransduced cells in the MiT–Bcl-3–transduced and the control MiT–transduced mixtures were recovered in equal numbers (**Fig. 5c**). Therefore, Bcl-3 conferred a survival advantage on activated T cells both *in vivo* and thus ectopic expression of Bcl-3 was sufficient for the prolonged survival of previously activated T cells in animals.

Discussion

The experiments reported here represent our continuing efforts to understand how immunological adjuvants affect T cells. Data obtained from these and previous experiments show that, among other effects, adjuvants certainly increase the lifespan of activated T cells[4–6,23–25]. Gene expression in T cells activated in the absence of adjuvants, when compared to activation in the presence of either of two different adjuvants, showed that few genes were similarly affected. Among these, the increase in cellular Bcl-3 expression after adjuvant treatment was of particular interest because the increase was large, *Bcl3* is a relatively little-studied proto-oncogene and other members of the NF-κB family affect the life expectancy of cells[14–16,26].

The gene encoding Bcl-3 was first identified as the gene altered by t(14;19) translocation in some cases of chronic B cell lymphocytic leukemia and was later determined to belong to the IκB subfamily of inhibitors of NF-κB transcription factors[27,28]. Unlike other IκB-related proteins, however, Bcl-3 is primarily nuclear in localization and appears not to be regulated by degradation[29]. Some groups have reported that it is capable of transcriptional transactivation, which makes it unique among IκB members[30]. Mice with targeted disruption of *Bcl3* have a variety of immunological defects including abnormal germinal center formation, reduced production of T cell–dependent antibodies and failure to clear selected pathogens[30–32]. T cell function in these mice has not been analyzed in detail. However, it is likely that the gross disruption of their lymphoid architecture will make it difficult to distinguish the direct effects of absence of *Bcl3* on T cells *versus* indirect effects on, for example, the conditions of T cell exposure to antigen. We do not yet know how the gene encoding Bcl-3 is induced by adjuvants. Because its expression is induced by adaptor protein AP-1 transcription complexes[22], it is possible that the inflammatory environment induced by adjuvants activates AP-1 in activated T cells, which in turn contributes to the induction of Bcl-3.

Bcl-3 does not have DNA-binding activity, although it has been reported to interact with NF-κB1 (also called p50) or NF-κB2 (also called p52) homodimers, the DNA binding of which may otherwise repress gene transcription[33]. Bcl-3 may thus prevent the death of activated T cells by promoting gene expression that is repressed by other NF-κB family members. Our observation that c-Rel expression was often lethal in T cells suggests another role for Bcl-3 in preventing activated T cell death, however. Because c-Rel, RelA and Bcl-3 all have binding affinity for p50 and p52[34], it is possible that RelA and Bcl-3 promote T cell survival by opposing an apoptotic side-effect of c-Rel–driven cellular proliferation. This could occur either by competition for the heterodimeric binding partners of c-Rel (p50 and p52) or by induction of new gene expression. If true, this scenario would mean that activated T cell survival is regulated by a balance in the concentration of various NF-κB family members.

Whatever the mechanism by which Bcl-3 acts, we have shown that an IκB subfamily member may be involved in the action of adjuvants on T cells. Thus Bcl-3 may be an essential component of the means by which adjuvants, inflammation and innate immunity control the adaptive immune response.

Methods

Use of mice. Mice were maintained under specific pathogen-free conditions at the Biological Resources Center of the National Jewish Medical and Research Center (NJMRC). Mice in any given experiment were age-matched, although they ranged from 8- to 14-weeks-old in various experiments. Female BALB/cByJ, B10.BR and C57BL/10J mice were from The Jackson Laboratory (Bar Harbor, ME). Female V_βDO and DOTgR mice were collected from a colony maintained at the NJMRC. DOTgR mice were generated by backcrossing DO-11.10 TCR transgenic (DOTg[19]) RAG-2$^{+/-}$ mice 11 times to the BALB/cByJ background. Brother-sister matings produced RAG-2$^{-/-}$ mice with the DO TCR transgene. In all experiments, mice were injected intravenously *via* the tail vein after being warmed briefly under heat lamps.

Toxins and viruses used to treat mice. SEB and *Salmonella typhosa* LPS were from Sigma-Aldrich (St. Louis, MO). Anti-CD40 (clone 1C10[35]) was collected as hybridoma supernatant and purified with agarose–protein G affinity columns. OVA peptide, consisting of amino acids 323–339 (ISQAVHAAHAEINEAGR), was synthesized by the Molecular Resource Center of the NJMRC. All agents were resuspended and injected intravenously in saline solution. VV inoculation was done by tail-vein injection of 10^7 plaque forming units (PFUs) of the nonrecombinant WR strain of the virus (cultured from stocks provided by J. Bennink, NIH). VV culture and measurement of its plaque-forming activity were done as described[5].

Preparation and culture of lymphocytes. Whole lymph nodes and spleens were removed for analysis at specified times from cervically dislocated mice. Lymphocytes were dispersed by grinding through nylon mesh into culture medium. The cells were resuspended in staining buffer (Hanks' balanced salt solution with 2% fetal bovine serum and 0.1% sodium azide) for flow cytometric analysis. For *in vitro* survival assays, cells were instead resuspended at a concentration of 3×10^5 cells per 0.2 ml culture medium with no added growth factors, then aliquoted in 96-well culture plates and left for 20 h before analysis by flow cytometry.

To measure T cell proliferation, cells from DOTgR mice were labeled with 1.0 μM CFSE (Molecular Probes, Eugene, OR) for 10 min at 37 °C. After incubation, cells were washed three times in culture medium and recultured for 4 days in the presence or absence of 1.0 μM OVA peptide with 10^6 unlabeled, adherent splenocytes from BALB/c mice used as antigen-presenting cells. CFSE fluorescence per cell was measured cytofluorimetrically.

Flow cytometric staining and sorting. Flow cytometric analysis of cell-surface markers was done by incubating cells with monoclonal antibodies (mAbs) specific for CD4 (clone RM4-5), CD8α (53-6.7), V_β3 TCR (KJ25), V_β8 TCR (F23.1), Thy1.1 (OX-7), MHC class II (M5/114.15.2) and FcγIII/II (2.4G2) (all from BD PharMingen, San Diego, CA). All flow cytometry was with FACScan or FACscalibur instruments and analysis with PC-Lysis or CellQuest software (BD Immunocytometry, Mountain View, CA). High-speed cell sorting was done on a MoFlo fluorescence-activated sorter (Cytomation, Fort Collins, CO) with chilled cells stained for CD4, CD8, MHC class II and Fc receptor. $V_\beta8^+CD4^+$ or $CD8^+$, MHC class II^- and FcR^- cells were collected, washed in saline buffer, lysed in guanidium hydrochloride and frozen until required for mRNA isolation.

RNA analysis. Total cellular RNA was isolated from purified T cells with the Rapid Total

RNA Isolation Kit as directed by the manufacturer (5 Prime→3 Prime, Boulder, CO). Yields were typically 0.5–1.5 μg RNA per 10^6 lymph node cells. Poly(A)$^+$ mRNA was purified with the Oligotex mRNA Purification System (Qiagen, Valencia, CA), as directed by the manufacturer. From mRNA, double-stranded DNA was synthesized with the SuperScript Choice System (Life Technologies, Grand Island, NY, Gibco-BRL, Gaithersburg, MD), as directed by the manufacturer. Double-stranded DNA was then amplified as cRNA, labeled and hybridized to Affymetrix mu6400 GeneChips (Affymetrix), as directed by the manufacturer.

RNA quantification by "real-time" PCR detection was done with the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Norwalk, CT), according to the manufacturer's directions. $V_\beta8^+$ T cells were sorted as described in Table 1 from mice given SEB alone, SEB + LPS + anti-CD40 or SEB + VV with the following treatment schedules. SEB: 150 μg of SEB on day 0. SEB + LPS + anti-CD40: 150 μg of SEB on day 0 and 5 μg of LPS + 300 μg of 1C10 mAb on day 1. SEB + VV: 10^7 PFUs of VV 3 days before treatment and 150 μg of SEB on day 0. Total cellular RNA was prepared with a Large Scale RNA isolation kit (5 Prime→3 Prime) and used as template to prepare 1st-strand cDNA with the SuperScript Choice System (Life Technologies, Gibco-BRL). Forty cycles of Real-Time PCR were done in optical quality PCR tubes to measure relative copy numbers of mouse Bcl-3, with measurement of hypoxanthine ribosyl transferase (HPRT) used as a control. Signals from two separate probe sets specific for mouse Bcl-3 were analyzed. The probe sets were as follows. Bcl-3 set A: forward primer, 5′–GCGAAGTAGACGTCCATAACAACC–3′; reverse primer, 5′–ACCAAGAGCCGGACCATGT–3′; FAM-TAMRA-fluorogenic probe: 5′–CACCTGGCTGTCATCACCACATTACCA–3′. Bcl-3 set B: forward primer 5′–ACGCCGTGGAGAACAACAG–3′; reverse primer 5′–AGAATACATCTGAGCGTTCACGTT–3′; FAM-TAMRA probe 5′–AACTCCTGCTGCTGCACGGCG–3′. HPRT: forward primer 5′–CCTGTGGCCATCTGCCTAGT–3′; reverse primer 5′–CGCAGCAACTGACATTTCTAAAA–3′; FAM-TAMRA probe 5′–AAGCTTTTTGCATGAACCTTCTATGAATGTTACTGTTT–3′. Signals specific for Bcl-3 from each RNA sample were normalized to that sample's signal for HPRT. The fold change relative to the signal observed for SEB treatment alone was calculated for the SEB + VV and SEB + LPS + anti-CD40 samples, averaged for the two Bcl-3 probe sets used and found to be 11.5-fold and 2.6-fold increased, respectively.

Retroviral constructs. The MiT retroviral vector was derived from GFP-RV[36] (also called MSCV2.2-IRES-GFP) by replacing the *Nco*I-*Eco*RI DNA fragment that contained the GFP open reading frame (ORF) with an *Nco*I-*Eco*RI fragment from pBSXCThy1.1 (a gift of D. S. An and I. Verma, The Salk Institute) that contained the mouse Thy1.1 ORF. MiT derivatives containing RelA, RelB and c-Rel were constructed from the corresponding pMSCV2.2-IRES-GFP vectors containing the indicated cDNA (gifts of D. Battacharya and W. Sha, University of California at Berkeley). MiT–Bcl-3 was constructed by ligating the *Bst*YII-*Bgl*II restriction fragment of pGEM4Z-Bcl3[37] into the *Bgl*II cloning site of MiT. DNA sequencing was done to confirm that this mouse *Bcl3* matched the correct Genbank sequence (AF067774). MiT–Bcl-2 was constructed from a PCR product of cDNA made from BALB/cByJ lymphocytes. The Bcl-2 cDNA was ligated to MiT at its *Bgl*II-*Not*I cloning sites and sequenced before use.

Production of retroviruses. MiT retroviral genomes were packaged into retroviruses by cotransfecting 293 human embryonic kidney cells with MiT vector plasmid + pCL-eco packaging plasmid, which expresses structural proteins of the Moloney murine leukemia virus[38,39]. Culture medium was replaced the next day and collected 18 h later. Culture supernatants containing packaged retroviruses were kept on ice until use. In some experiments, titers of infectious retrovirus were measured before the culture supernatants were used to transduce T cells with genes.

To measure retroviral titers, 10^5 NIH3T3 cells were plated in each 35 mm well in six-well plates, cultured for 1 day, the culture medium exchanged for 1.0 ml culture medium containing 8 μg/ml of polybrene (Sigma-Aldrich) and 1, 10 or 100 μl of retrovirus culture supernatant added. The average number of NIH3T3 cells per well exposed to retrovirus at that time was measured by counting trypsinized cells. NIH3T3 cells were treated with trypsin 40–48 h later and stained for cytofluorimetric detection of Thy1.1 expression. The number of infectious units per ml was calculated using:

positive cells (%) × the number of cells initially exposed to virus × the dilution factor.

Retroviral infection of T cells. For retroviral infections, T lymphocytes from treated mice were enriched by passage over sterile nylon wool, mixed with retrovirus culture supernatant containing 8 μg/ml of polybrene and centrifuged in sealed (10% CO_2 atmosphere) six-well plates at 1000*g* for 2 h. After centrifugation, cells were placed in medium containing serum but no added growth or survival factors and cultured for 24h. Alternatively they were washed extensively in serum-free saline solution and injected into recipient mice. The relative killing or rescue of T cells transduced with different genes was calculated from the percentage of infected cells left alive after infection with each retroviral vector, compared with the MiT vector control, as follows. Relative killing was calculated as:

$$[(\text{vector control} - \text{experimental}) / \text{vector control})] \times 100$$

and relative rescue as:

$$[(\text{experimental} - \text{vector control}) / 100 - \text{vector control}] \times 100.$$

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