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Bystander Virus Infection Prolongs Activated T Cell Survival

Tom Mitchell,* John Kappler,*‡§ and Philippa Marrack2*‡§

In animals, T cells often die rapidly after activation, unless activation occurs in the presence of inflammatory factors. To understand how such activated cells survive to participate in immune responses, we studied the effects of viral infection on T cells responding to an unrelated superantigen. Normal T cells activated by superantigen in uninfected mice died as a result of their activation, whereas T cells that were activated during vaccinia infection survived longer in vivo and in culture. This bystander effect of viral infection on activated T cells was independent of effects on the magnitude of the initial T cell response, on induction of Bcl-2 and Bcl-x, on T cell proliferation, and on Fas killing. The failure of such effects to predict the fate of activated T cells in vivo indicates that virus infections shape T cell responses via mechanisms that differ from those described previously. These mechanisms may contribute to the ability of viral infections to induce autoimmunity. The Journal of Immunology, 1999, 162: 4527–4535.

Mature T cells that are stimulated with soluble Ag in animals mount an abortive response, and most are likely to die as a result of their stimulation. Ag-induced death is avoided, however, when stimulation by Ag is coincident with exposure to adjuvants (1–4). With the recent realization that adjuvants are needed not merely to boost T cell responses, but are critical to sustaining them (5, 6), comes an increased need to understand how the adjuvant effect contributes to T cell immunity.

This laboratory has frequently used staphylococcal enterotoxins, superantigens, as tools to study Ag-induced cell death of mature T cells (1, 7–9). Superantigens, when presented by the appropriate MHC class II molecule, activate T cells bearing particular Vβs as part of their TCRs. Superantigen treatment of mice thus allows for the activation of detectable but limited portions of the available repertoire of normal, primary T cells. Acute exposure of responsive T cells to a superantigen results in activation, expansion, and then deletion of these activated cells by apoptosis. This sequence of events is one postulated mechanism by which self-reactive T cells that have escaped detection during negative selection in the thymus can be removed from peripheral tissues.

Resistance to infections depends upon effective immune responses in which T cells stimulated by the invading organism do not all die rapidly. We and others have therefore reasoned that some property of infectious agents must act as an adjuvant to protect activated T cells against death. Along these lines, we have previously shown that bacterial LPS protects T cells stimulated by a superantigen from death (1). The protective effect of LPS appeared to act via LPS’s ability to induce components of the innate immune system, in particular proinflammatory cytokines, rather than costimulatory molecules, such as B7-1 or B7-2 (1, 9).

With this in mind, we wondered whether infectious agents such as viruses would affect activated T cells in a similar way. We chose vaccinia virus (VV)3 to test this idea. VV is well known for its efficacy as an immunogen, both for smallpox (10) and for protein Ags expressed from recombinant forms of the virus (11, 12), and for its ability to induce cytokine production by its hosts (13, 14). VV infects epithelial cells expressing receptors for epidermal growth factor (15–17), and thus infects many tissues in mice, including those of the ovaries, spleen, liver, and lung. Infection is acute: virus is completely cleared from immunocompetent mice in 7–10 days (15). Infection is also inflammatory by virtue of the inevitable cytotoxicity. VV induces in infected cells (16). Hence, we used infection with VV to determine whether or not infection with this model pathogen can act as adjuvant during superantigen-induced T cell responses, and if so by what means. We found that T cells responding to superantigen during active VV infection showed prolonged survival thereafter in mice and in culture. This enhanced survival of activated T cells was the only one of several adjuvant effects tested that was observed to operate in this model system, and appeared to be independent of cytokines such as IL-2, IL-4, IL-7, and IL-15 that induce Bcl-2 expression.

Materials and Methods

Experimental mice and treatments

Female B10.BR mice (The Jackson Laboratory, Bar Harbor, ME) were maintained in a specific-pathogen-free environment at the Biological Resources Center of the National Jewish Medical and Research Center (Denver, CO). The mice in any given experiment were age-matched, but ranged in age from 8 to 14 wk in various experiments.

CD4 T cells were activated in vivo by injecting mice via the tail vein with 0.1 μg staphylococcal enterotoxin A (SEA; Toxin Technology, Sarasota, FL) in a volume of 0.25 ml balanced saline solution (BSS). VV inoculation was performed by tail vein injection of 105 plaque-forming units (pfu) of the nonrecombinant WR strain of the virus (cultured from stocks provided by J. Bennink, National Institutes of Health, Bethesda, MD). Virus stocks were propagated in cultured cells by exposing 5 × 105 pfu of the virus to 0.125 μg/ml trypsin for 30 min at 37°C before adding the virus to 107–293 cells (American Type Culture Collection [ATCC], Manassas, VA) in medium containing 8% FBS for 72 h. At the

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3 Abbreviations used in this paper: VV, vaccinia virus; SEA, staphylococcal enterotoxin A; BSS, balanced saline solution; pfu, plaque-forming units; CFSE, carboxyfluorescein diacetate succinimidyl ester; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LN, lymph node; LIF, lymphocyte inhibitory factor.
end of this period, the cells were rinsed in BSS, frozen and thawed twice, and then lysed further in BSS by sonication. Stocks of virus were stored at −70°C before use. Cell lysates of uninfected 293 cells were prepared for use as mock inocula. Viral titers were measured by plaque assay on BS-C-1 cells (ATCC CCL 26) as described previously (17).

**Lymphocyte preparation and analysis**

Whole lymph nodes (LN) were removed for analysis from cervically dislocated mice at the specified times. The inguinal, brachial, axillary, and submandibular LN from similarly treated mice were pooled, dispersed by grinding through nylon mesh into culture medium, and counted with a Coulter cell counter (Coulter, Hileah, FL). The cells were resuspended at 1.5 × 10^6 cells per ml culture medium and plated in a volume of 0.2 ml in triplicate in wells of 96-well culture plates for 20 h, or were resuspended in flow cytometric staining buffer (BSS with 2% FBS and 0.01% sodium azide).

Culture with Fas cross-linking Abs was performed by adsorbing Jo2 anti-Fas MAb (PharMingen, San Diego, CA) or polyclonal hamster Ig (Sigma, St. Louis, MO) at 10 μg/well of a 96-well, polystyrene plate for 3 h at 37°C. Before cell culture, the wells were rinsed three times with culture medium. The effectiveness of Fas cross-linking was confirmed in each experiment by culturing L1210.Fas cells in wells coated with Ab (18). Culture of control cells with anti-Fas produced 40% and 60% apoptotic cells after 20 and 40 h, respectively, while culture with control Ig produced 3% apoptotic cells after any period of culture.

Lymphocyte survival was assessed by measuring the proportion of “live-gated” cells that bore a particular Vβ before and after culture. The validity of the live-gate measurement was confirmed by comparison to other assays of apoptosis, including propidium iodide detection of DNA fragmentation (1), propidium uptake (cells stained for cytometry were incubated in 0.5 mg/ml propidium iodide in BSS for 15 min on ice before analysis), and annexin V staining (Sigma; cells stained for cytometry were incubated for 15 min in BSS containing 2.5 mM CaCl2 and the manufacturer’s recommended dilution of annexin V-FITC). Each measure of apoptosis gave similar values (19, and data not shown).

Recombinant mouse cytokines IL-2, IL-4, and IL-7 and human IL-15 were purchased from R&D Systems (Minneapolis, MN). The cytokines were suspended in culture medium and frozen at −70°C before use at the concentrations indicated in Fig. 9.

**Flow cytometry**

Flow cytometric analysis of cell-surface markers was performed with 10^6 uncultured cells or 3 × 10^6 cultured cells per stain, using mAbs specific for CD4, TCR Vβ3, TCR Vβ6, or CD69 (clones GK1.5, KJ25, RR4–7, and H1.2F3, respectively; PharMingen). The fluorochrome conjugates used for 3-color FACS analysis were CyChrome, phycoerythrin, and FITC. Intracellular Bcl-2 was detected by staining in buffer containing 0.03% saponin (Sigma), first with mAb clone 3F11, or human Bcl-2-specific clone 6CS as control, and then with a mixture of anti-hamster IgG clones G70-204 and G94-56 coupled to FITC (PharMingen) essentially as described (20). Bcl-x expression was measured in cells after cell-surface stains were performed after fixation in 1% paraformaldehyde. Polyclonal rabbit anti-Bcl-x (Transduction Laboratories, Lexington, KY) was incubated at ambient temperature with fixed cells in staining buffer containing 0.3% saponin. The cells were washed three times, stained with goat anti-rabbit Ig that was fluoresceinated (Fisher Scientific, Pittsburgh, PA), washed again, and then analyzed flow cytometrically. Flow cytometry was performed on FACScan or FACScalibur instruments, and analysis was with PC-Lysis or CellQuest software (Becton Dickinson, San Jose, CA).

Cells used for mixing experiments were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; FCSF, Molecular Probes, Eugene, OR) immediately before culture. Cells were labeled by incubating 10^7 cells in 0.2 ml BSS containing 0.5 μM CFSE for 10 min at 37°C. After incubation, the cells were washed three times in culture medium supplemented with FBS. Cells used for transfer experiments were labeled by incubating 2 × 10^7 cells per ml BSS in 2 μM CFSE for 10 min at 37°C. The cells were washed once in BSS and then injected into recipient mice iv.

**RNA analysis**

Total cellular RNA was isolated from whole LN using the Rapid Total RNA Isolation Kit (large scale) as directed by the manufacturer (5 Prime → 3 Prime, Boulder, CO). Yields were typically 0.5–1.5 μg RNA per 10^6 LN cells.

RNA transcripts of cytokine genes were detected and quantified using the RiboQuant Multiprobe RNase Protection Assay system, as directed by the manufacturer (PharMingen). Briefly, radiolabeled RNA probes were synthesized in vitro using the RiboQuant In Vitro Transcription kit with incorporation of uridine 5′-triphosphate [α-32P], 6000Ci/mmol (New England Nuclear Life Science Products, Boston, MA). RNA probes were prepared from PharMingen templates and mCK-1, mCK-2b, mCK-3, and mCK-4. For each probe set, 10^6 Cherenkov units were added to 75 μg total RNA obtained from LN of mice after selected treatments and hybridized in solution by heating to 94°C for 2 min and then incubating at 56°C for 12–15 h. Following hybridization, any radiolabeled probe that was not annealed to its complementary cellular transcript was digested with RNases T1 and A. The remaining RNA was resolved by electrophoresis through acrylamide containing urea, transferred to Whatmann filter paper, and dried.

Visualization and quantitation of resolved RNA was performed with a Storm 800 PhosphorImager (Molecular Dynamics, Sunnyvale, CA) after exposing phosphor screens to dried acrylamide gels for 24–48 h. Counts corresponding to each cytokine transcript were normalized from one lane to another by comparison of the sum of the signals from L32 and glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) transcripts in each lane. Each lane of the analysis consisted of one multiprobe set annealed to RNA obtained from one treatment group of mice.

**Results**

**Activated T cells from infected mice survive longer in vivo than activated T cells from uninfected mice**

We tested T cells in VV-infected mice for their susceptibility to superantigen-induced deletion to assess the adjuvant effects of a cytotoxic virus infection on activated T cells. B10.BR mice (H-2b) were either inoculated with VV or not and treated 3 days later with the superantigen SEA, which, when bound to MHC class II proteins, activates CD4 and CD8 T cells bearing the Vβ1, -3, or -11 regions of TCR. Flow cytometric detection of Vβ1 + T cells is currently not possible, and B10.BR mice have very few Vβ1 + T cells (7), so Vβ3 + T cells were examined in all experiments.

Fig. 1 shows that, 2 days after SEA injection, mice contained greatly expanded numbers of Vβ3 + T cells whether they were also infected with VV or not. The enhanced numbers of Vβ3 + cells were found for a much longer time in VV-infected mice, indicating that virus infection sustained the survival of the activated T cells. This pattern was seen with both CD4 and CD8 T cells in spleen (data not shown) and LN (Fig. 1) when tested either for the percent of Vβ3 + T cells, or for the absolute numbers of T cells bearing this TCR. T cells bearing Vβ6, which does not bind SEA, were unaffected (data not shown). Vβ3 + T cells in mice infected with VV alone were also unaffected, demonstrating that VV itself does not stimulate specifically a large percent of Vβ3 + T cells (Fig. 1). These observations showed that T cells activated by a model Ag, SEA, persisted longer in mice when activation occurred during “bystander” virus infection.

**Proliferation of CD4 + Vβ3 + T cells after SEA treatment in uninfected and in VV-infected mice**

CFSE labeling experiments were used to track the proliferative responses of SEA-stimulated cells in VV-infected mice. This was done to determine whether or not CD4 + Vβ3 + T cells proliferated preferentially upon SEA challenge because they had been cross-primed by VV Ags. LN cells from B10.BR mice were loaded with the fluorescent dye CFSE and transferred to syngeneic mice. Groups of recipient mice were then given 10^7 pfu VV on day −3 (VV), 0.1 μg SEA on day 0 (SEA), or 10^7 pfu VV on day −3 and 0.1 μg SEA on day 0 (VV + SEA), as described in Fig. 1. Two days after SEA treatment, CD4 + Vβ3 + cells were gated, and CFSE fluorescence was measured. Fig. 2A shows that the pattern of CFSE loss, indicating successive cellular divisions, was similar in cells from uninfected as in cells from VV-infected mice. Measurement of the percent of CD4 + cells bearing Vβ3 + on subsequent days showed that cells with any amount of CFSE label, those that had been transferred, showed a similar pattern of prolonged survival in vivo as those that had been resident in the recipient mice.
Therefore, transferred cells had not lost all CFSE dye due to extensive proliferation, and had proliferated no more in VV-infected mice than they had in uninfected mice.

**Increased Bcl-x expression in activated cells is insufficient for enhanced survival of activated cells**

Prolonged survival of responding T cells has previously been reported to occur as a result of increased Bcl-x expression following engagement of the CD28 T cell coreceptor by B7 proteins on the surfaces of APC (21–24). We measured Bcl-x in cells activated 2 days previously by SEA in vivo and found that expression of this antiapoptotic protein was increased in activated cells, relative to resting cells, to the same extent whether or not activation had occurred in mice that had been infected with VV (Table I). Hence, SEA-activated cells were destined to die following their stimulation in vivo in spite of having expressed high levels of Bcl-x (Fig. 1), and the increased persistence of activated T cells in infected mice was not correlated with further increases in Bcl-x expression.

This analysis of the expression of Bcl-x did not yield information about its subcellular distribution or its binding partners or other mechanisms of Bcl-x regulation that might contribute to a T cell’s survival. Because increased Bcl-x expression in T cells is due to CD28 signaling, however, (23–25) these results showed that the activated T cells given SEA alone had been exposed to B7 proteins on APCs. The overall amount of co-stimulation available to T cells responding to a superantigen therefore was not limiting, relative to that available during viral infection.

**Activated T cells from infected mice survive longer in culture than activated T cells from uninfected mice**

Analysis of the survival of activated T cells in mice is complicated by cellular proliferation and trafficking and by the rapid clearance of dead cells that occurs in vivo. Previous work from this laboratory and elsewhere has shown that T cells that have been activated and expanded in vivo die more quickly than resting T cells when cultured for short periods of time (1, 26). Measurement of survival in vitro therefore permits an estimate of a cell’s likelihood to die or to live in vivo, in a manner that is not influenced by that cell’s ability to traffic. Moreover, cells whose survival is measured in culture can be analyzed to determine the extent of their proliferation, if any, from the beginning of the culture period to the end.

To determine whether or not T cells that were activated during viral infection survived longer upon culture than other activated cells, B10.BR mice were left uninfected or were inoculated with VV, given SEA, and sacrificed 2 days later. LN cells from treated mice were analyzed by cytofluorometry either immediately after harvest or after culture for 20 h. Survival of SEA-activated cells was assessed by measuring the percent of live CD4 or CD8 T cells that bore Vβ3. CD8 T cells behaved like CD4 T cells whenever tested, however, for simplicity in the rest of this paper, only the results for CD4 cells will be shown.

Live cells were defined by their forward vs side-light scattering properties, which we confirmed gave values that were equivalent to those determined with annexin V and propidium iodide staining (as described in Materials and Methods). A proportion of both the resting and activated T cell populations died during the 20-h culture (data not shown), but a greater proportion of activated than resting cells died, as demonstrated by the fact that after activation with SEA the percentage of live, CD4 T cells bearing Vβ3 fell during culture (Fig. 3A). This increased death rate of the activated T cells was prevented if the T cells came from VV-infected mice. A summary of several experiments in which SEA was given to uninfected or VV-infected mice showed no statistical difference between the starting and ending proportion of live, CD4 T cells bearing Vβ3 during culture (data not shown).

**The enhanced survival of activated T cells is induced on days 3–5 of VV infection**

Infection with VV 3 days before giving SEA was sufficient to confer longer life on the SEA-activated cells, but it was not clear when during the course of infection the effect started and when it ended. A time course of the virally induced protective effect was therefore performed to determine when the effect peaked in vivo. Groups of B10.BR mice were inoculated with VV on successive days and then given SEA, such that Vβ3+ T cells were activated on days 1–7 and on day 14 of infection. LN cells were removed from all mice 2 days after activation and tested for their survival in
FIGURE 2. CFSE measurement of proliferation of CD4\(^+\) V\(\beta\)\(^3\) T cells in VV-infected mice after treatment with SEA. LN cells from B10.BR mice were loaded with the fluorescent dye CFSE and transferred to syngeneic mice. Groups of recipient mice were then given 10\(^7\) pfu VV on day 0 (VV), 0.1 \(\mu\)g SEA on day 0 (SEA), or 10\(^7\) pfu VV on day 3 and 0.1 \(\mu\)g SEA on day 0 (VV + SEA). All injections were i.v. On days 2, 3, 4, and 10, replicate mice were sacrificed for analysis. A, CFSE loss during proliferation. Two days after SEA treatment, CD4\(^+\) V\(\beta\)\(^3\) T cells were assessed for CFSE content to measure their extent of proliferation in response to SEA. Shown are histograms with values summed from mice treated in triplicate. The mean number of divisions was calculated by multiplying the percent of cells in each bin by the number of cellular divisions represented by that bin. The sum of these products was divided by the sum of the percent of cells that had divided at least once. Similar results were observed in the same experiment at day 3 and in an independent experiment. B, CD4\(^+\) V\(\beta\)\(^3\) LN cells were analyzed for CFSE fluorescence at the times indicated and judged to be resident cells (CFSE–) or transferred cells (CFSE+). The percent of CD4 cells bearing V\(\beta\) for each population is shown as a function of time following SEA treatment.

culture. Fig. 4 shows that injection of SEA on day 3, 4, or 5 of infection produced activated T cells with enhanced survival in culture, whereas injection before or after this day 3–5 period had little effect.

**Table 1.** Bystander virus infection and SEA treatment do not raise levels of Bcl-x in activated T cells over that raised by SEA alone\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>CD4(^+) cells bearing Bcl-x</th>
<th>CD8(^+) cells bearing Bcl-x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment in vivo</td>
<td>V(\beta)</td>
<td>V(\beta)6</td>
</tr>
<tr>
<td>None</td>
<td>33.6 ± 3.2</td>
<td>36.2 ± 1.1</td>
</tr>
<tr>
<td>SEA</td>
<td>70.1 ± 1.1</td>
<td>34.9 ± 1.4</td>
</tr>
<tr>
<td>Vaccinia + SEA</td>
<td>70.4 ± 4.7</td>
<td>34.5 ± 0.6</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>42.1 ± 3.8</td>
<td>39.7 ± 2.0</td>
</tr>
</tbody>
</table>

\(^a\)Cells were stained for Bcl-x expression as described in Materials and Methods, and fluorescence levels were measured with flow cytometry. Values shown are the mean and standard error of fluorescence in arbitrary units for cells obtained from three mice in each treatment group. Cells were isolated 5 days after vaccinia infection and 2 days after SEA treatment. Background fluorescence was 15–20 in the same arbitrary units. Embryonic stem cells that were either +/+; +/−, or −/− showed fluorescence values of 42, 29, and 19, respectively.

VV bystander infection does not alter T cell responsiveness to SEA

One explanation for the finding that VV infection increased the life expectancy of SEA-activated T cells was that VV inhibited T cell activation by SEA. The fact that VV did not affect the expansion of V\(\beta\)\(^3\) T cells induced by SEA (Fig. 1), or their increased Bcl-x levels (Table I), suggested that this was not true. However, as an additional test, we measured induction of CD69, an early marker of T cell activation, on V\(\beta\)\(^3\) T cells in VV-infected or normal mice. In normal mice only, 11% of V\(\beta\)\(^3\) CD4\(^+\) T cells were CD69\(^+\) (data not shown). This percentage increased slightly, to 16%, in VV-infected animals on day 5 of infection, probably due to activation of the few CD4\(^+\) V\(\beta\)\(^3\) T cells that were VV-specific. SEA injection caused ~50% of CD4\(^+\) V\(\beta\)\(^3\) T cells to be CD69\(^+\) 1 day later, whether or not the mice were also infected with VV. These results showed that VV infection did not reduce the ability of SEA to activate V\(\beta\)\(^3\) T cells and confirmed that the effects of VV on SEA-activated cells were not due to the fact that the rescued V\(\beta\)\(^3\) T cells were specific for VV as well as SEA.

Also, VV infection did not affect the ability of SEA to reactivate cells after 20 h of culture. In cells that had been cultured overnight following day 2 SEA treatment, all but 2% of CD4\(^+\) V\(\beta\)\(^3\) T cells were CD69\(^+\). Culture of these cells with SEA for an additional 20 h, however, induced CD69 expression on ~75% of the live V\(\beta\)\(^3\) T cells, whether they came from VV infected mice or not.

**Activated T cells that are protected from in vitro death by VV infection remain susceptible to killing via Fas**

Previous results from this laboratory suggested that bacterial LPS functioned as an adjuvant, at least in part, by rendering the activated cells insensitive to cytotoxic signaling via the death receptor Fas (1). To test whether or not this was true for VV as well, T cells that had been activated in mice with or without concurrent VV infection were tested for their susceptibility to death induced by cross-linking Fas. Whole LN populations were removed from mice after having received the treatments indicated in Fig. 5. The lymphocytes were plated in tissue culture wells that had been coated previously with anti-Fas Ab, or with hamster Ig as control. At the outset of culture, and after 20 or 40 h of culture, the proportions of live CD4 cells bearing V\(\beta\) TCR were determined.

VV\(^+\) T cells were isolated 5 days after vaccinia infection and 2 days after SEA treatment. Background fluorescence was 15–20 in the same arbitrary units. Embryonic stem cells that were either +/+; +/−, or −/− showed fluorescence values of 42, 29, and 19, respectively.
VvV whether they came from VV-infected mice or not (Fig. 6). That is, cultured, and assessed for survival. mixed with unlabeled, SEA-activated cells from uninfected mice, were labeled with the intracellular fluorescent dye CFSE (27), culture. To do this, SEA-activated cells from VV-infected mice the adjuvant properties of viral infection operated in trans during Mixing experiments were performed to determine whether or not rapid death is not ongoing during the culture period. The response to infection that protects activated T cells from Fas, but rather by some other mechanism that was manifested as prolonged survival in culture and in animals.

The implications of these results are several. First, the process that is induced by VV infection and that is required to slow the death of activated T cells did not operate during the culture period. Therefore, the rescuing activity induced by VV must change the exposure to Fas Ab. Activated Vβ3+ cells from SEA treated, VV-infected mice, however, died quickly when the culture wells contained Fas Ab. This behavior differed from that of the Vβ3+ cells from mice given SEA and LPS, which were insensitive to killing via Fas. Hence, VV infection did not act as adjuvant by making activated T cells resistant to Fas, but rather by some other mechanism that was manifested as prolonged survival in culture and in animals.

The response to infection that protects activated T cells from rapid death is not ongoing during the culture period. Mixing experiments were performed to determine whether or not the adjuvant properties of viral infection operated in trans during culture. To do this, SEA-activated cells from VV-infected mice were labeled with the intracellular fluorescent dye CFSE (27), mixed with unlabeled, SEA-activated cells from uninfected mice, cultured, and assessed for survival. Coculture had no effect on the survival of SEA-activated cells, whether they came from VV infected mice or not (Fig. 6). That is, Vβ3+ T cells from VV-infected, SEA-treated mice had a slow rate of death, and Vβ3+ T cells from mice given SEA alone died rap-
removed 2 days after SEA treatment for culture and analysis. Cells from Fig. 4 indicated that LN cells removed for analysis of survival at later, on day 5 of infection. The time course of infection shown in giving SEA on day 3 of infection and removing LN cells compared. The previous experiments in this study were performed by prepared. The previous experiments in this study were performed by virtue of having annealed to cellular transcripts were resolved by electrophoresis and quantified by phosphorimaging analysis, as described in Materials and Methods. Each cytokine-specific signal was normalized to the signals specific for L32 + GAPDH transcripts. Shown is the fold-increase of a given transcript following VV infection + SEA treatment vs SEA treatment alone, averaged from two independent experiments.

**FIGURE 6.** Mixed cultures of activated cells from uninfected and VV-infected mice show no influence of one population on the other. B10.BR mice were either treated with SEA (SEA), or infected with VV and then treated with SEA (VV + SEA), as in Fig. 3. LN cells were removed 2 days after SEA treatment for culture and analysis. Cells from VV + SEA-treated mice were loaded with CFSE dye immediately before culture. A, Visualization of mixed cultures of CFSE-labeled and unlabeled cells after culture. B, Percent of live CD4 T cell that expressed Vβ3 before and after culture for each of the T cell populations in each of the cultures. From top to bottom, the cultures contained the proportions of CFSE-labeled and unlabeled cells shown in A. Each culture was plated and tested in triplicate. The results shown are representative of three independent experiments.

![Image](http://www.jimmunol.org/)

phenotype of the activated cells while they are still in the animal. Secondly, the increased rate of death of activated T cells from SEA-treated mice was not due to a trans effect, such as T cell cytotoxicity, manifest in vitro. Thirdly, the increased survival rate in vitro of activated T cells from VV-infected mice is not an artifact of increased cell proliferation since, once isolated from animals, the SEA-activated cells stopped dividing whether they had come from VV-infected animals or not.

**Measurement of cytokine transcripts in LN cells during viral infection**

Because some cytokines rescue activated T cells from death in vivo and in vitro (8, 28–30), cytokine gene expression in LN cells from SEA-treated mice, with or without VV infection, was compared. The previous experiments in this study were performed by giving SEA on day 3 of infection and removing LN cells 2 days later, on day 5 of infection. The time course of infection shown in Fig. 4 indicated that LN cells removed for analysis of survival at this time, day 5, could also be analyzed meaningfully for expression of protective factors. Therefore, RNA was isolated from whole LN 5 days after VV infection and subjected to an RNase protection assay, which is quantitative and allows for measurement of mRNA levels for many cytokines at once. Correlation of gene transcription with virally induced protection required comparison of transcript levels in mice inoculated with VV and then treated with SEA vs levels in mice treated with SEA alone. Fig. 7 shows such a comparison for the 22 cytokine mRNAs out of 34 tested that produced a signal in the RNase protection assay. mRNA levels for the housekeeping genes L32 and GAPDH were used as controls. Of the 22 cytokine mRNAs compared, only 4 showed an increase of >3-fold in VV-infected mice, those for IL-4, IL-10, IFN-γ, and lymphocyte inhibitory factor (LIF). Several others, for IL-1β, IL-1RA, IL-7, IL-12p35, IL-15, IFN-γ-inducing factor, granulocyte-macrophage CSF, and macrophage CSF showed relative increases of <3-fold, and the rest showed no relative increase. The type I IFNs, α and β, which constitute a subfamily of numerous gene transcripts, were not tested in these assays. The expression of these IFNs was presumably induced in VV-infected mice, however, because type I IFN receptor signaling is required for infected mice to survive vaccinia infection (31).

When SEA treatment was compared with no treatment, transcripts of IL-2, IL-10, IL-13, IL-15, LIF, and macrophage CSF genes were found to be increased by a factor of 2–3 (data not shown). We chose to investigate IL-4, IL-10, IFN-γ, and LIF further because transcription of these cytokines correlated best with virally induced protection of activated T cells.

**IL-4 rescues activated cells from death in culture**

IL-4, IL-10, IFN-γ, and LIF were tested for rescuing activity by adding them to cultures of cells that had been activated in vivo by SEA. Of these cytokines, only IL-4 was able to prevent death of activated CD4 T cells (data not shown, and Fig. 9).
showed minimal activity that was not dose-responsive in whole LN cultures (data not shown) and no activity in purified T cell cultures (1). IFN-γ, moreover, was found not to be required for protection in experiments in which IFN-γ was neutralized in vivo with mAb treatment during VV infection, or in which IFN-γ−/− mice were tested for virally induced protection in short-term assays (T.M., unpublished observations). Of the 22 cytokines whose transcript levels were measured, therefore, IL-4 was the only one to increase strongly during VV infection and to protect activated T cells from death when tested in culture.

**VV protects activated T cells from rapid death in culture without increasing Bcl-2 expression**

Increased Bcl-2 expression is one attribute of cytokine-induced survival of lymphoid cells (Refs. 8, 32, and 33, and Fig. 9). If IL-4 secretion were to explain how infection prolongs the survival of activated T cells, the amounts of Bcl-2 per cell might be expected to increase relative to those in cells activated in the absence of infection. This hypothesis was tested by measuring Bcl-2 expression in SEA-activated cells after they were removed from uninfected or VV-infected mice and after they were cultured with IL-4 or IL-15 as positive control. Flow cytometric stains for Bcl-2 under such conditions showed that CD4+Vβ3+ T cells activated 2 days earlier by SEA had decreased their expression of Bcl-2 (Fig. 8). VV infection did not restore Bcl-2 levels, but culture with 100 ng/ml IL-4 or IL-15 did. Hence, IL-4 and IL-15 rescued activated T cells from death in vitro by a mechanism that seemed distinct from that induced by infection in vivo.

**Other cytokines increase Bcl-2 expression as they rescue activated CD4 T cells from death**

The RNase protection assay described in Fig. 7 might have failed to detect other cytokines as candidate survival factors due to insufficient sensitivity of the assay. So, selected cytokines were analyzed for their effects on Bcl-2 expression. IL-2, IL-4, IL-7, and IL-15 have all been reported to rescue activated T cells from death in vitro, and some of these are active in vivo (8, 32, 33). All induced Bcl-2 expression in activated cells when tested in culture (Fig. 9). Each of the responses was dose-dependent and correlative inasmuch as culture doses that caused rescue also induced Bcl-2, while doses insufficient to cause rescue did not induce Bcl-2. Since VV did not induce Bcl-2 in activated CD4+ T cells, exposure of these cells to IL-2, IL-4, IL-7, and IL-15 after the cells had been activated seemed unlikely to explain the adjuvant properties of VV infection.
Discussion

In this report, we show that VV infection increased the life span of activated T cells, even if the T cells were responding to an Ag that was not part of VV. This effect was observed both in vivo and in vitro. The observation that an acute viral infection prevented superantigen-induced death in vivo was reminiscent of other reports in which infectious agents, or factors purified from them, were found to interfere with what would otherwise have been tolerogenic events (34–36). Interference with the induction of peripheral tolerance by “bystander” infections may account for the observation that viral infections are often associated with the onset of autoimmunity, even if the pathogen in question does not share antigenic epitopes with its host (37, 38).

The means by which infectious agents increase the life spans of activated T cells is not clear. Unlike LPS, VV infection did not make activated cells insensitive to Fas killing, since activated T cells from VV-infected mice still succumbed to cytopathic signaling via Fas. This result raised the question of the importance of Fas to the death of superantigen-activated T cells. The data in the literature is contradictory, with some laboratories indicating that Fas is obligatory for superantigen-induced death (39, 40), others showing that Fas is not needed (41), and yet others demonstrating that Fas plays a decisive role at low but not high doses of superantigen (42–44), or with membrane-bound but not soluble Ag (45). Our own experiments with Fas- and TNF receptor-deficient mice indicate that superantigen-activated cells from mutant mice die at least as fast in culture and in vivo as do cells from wild-type or heterozygous mice (D. Hildeman, unpublished observation). A failure of viral infection to confer resistance to Fas-killing is consistent with the view that Fas signaling is not as important as other mechanisms in controlling T cell survival, at least during and after priming in LN.

Bcl-x expression was increased to the same extent in activated cells whether activation occurred in uninfected or in VV-infected mice. Hence, the amount of costimulation available to SEA-responsive cells seemed not to be limiting in uninfected mice, and was not increased further in mice undergoing VV infection. This conclusion was supported by our observations in multiple experiments that VV infection did not increase the expansion of superantigen-specific T cells over that observed after treatment with superantigen alone.

In attempts to discover which cytokines might account for VV’s ability to enhance T cell survival, we found that surprisingly few cytokine transcripts were induced in the LN of VV-infected and SEA-treated mice, relative to mice that had received SEA alone. Since the IL-2 family of cytokines is known to affect T cell survival (8, 28–30, 33) and since a member of the family, IL-15, has recently been reported to affect CD8+ T cells in vivo (46, 47), we paid particular attention to mRNA transcripts for this family. Vaccinia infection caused small increases in the levels of mRNAs for IL-2, IL-7, and IL-15 and a large increase in the level of mRNA for IL-4. These cytokines were unlikely to account for the longer life expectancy of activated T cells in VV-infected mice, however, because these cytokines raised the level of Bcl-2 in T cells (32, 33), and the rescued T cells in VV-infected mice did not have increased amounts of this protein. Moreover, culture of short-lived, activated T cells from mice given SEA alone with T cells from mice given SEA and VV did not rescue the former cells from death. Thus, T cell survival was not affected by a factor produced in vitro by the cells from VV-infected mice. IL-2 family members must be continually available in culture for them to protect T cells from death (T. Kent Teague, unpublished observations), indicating that members of this family must not have been responsible for the increased life expectancy in vitro of activated T cells from VV-infected mice. These conclusions rely, however, on the assumption that T cells respond to cytokines in vivo in the same way that they do in vitro, an assumption that underlies much of immunologic research, but that may inappropriately discount the complexity of immune responses in animals.

The experiments reported here identify several properties of the factor(s) induced by virus infection that protect activated T cells from death. The factor must affect activated but not resting T cells, it must protect cells without inducing proliferation or Bcl-2 or Bcl-x expression, and must not be dependent on continuous secretion for efficacy.

We report elsewhere that type I IFNs satisfy many of these criteria inasmuch as their addition to cultures of activated, but not resting, T cells promotes survival without inducing Bcl-2 or Bcl-x expression, and without inducing proliferation. However, CFSE-transfer experiments with cells from IFNα/β receptor-deficient mice showed that virally induced survival was equivalent whether wild-type or receptor-deficient cells were transferred to infected recipients (our unpublished observation). Thus, type I IFNs can influence T cell survival in vitro, but are apparently not required for prolonged survival in vivo.

Finally, virus infections might be acting to inhibit death pathways, rather than induce rescuing factors. Factors or pathways that are elicited globally in an animal and that induce activated T cell death unless innate immune responses are activated, for example, may be responsible. Examples of mechanisms that might behave in this manner are glucocorticoid-induced death (46), and monocye-induced cytotoxicity of T lymphocytes (47, 48). Whichever factor or pathway is found to support activated T cell survival will likely explain an important part of the mechanism by which viral infections result in dramatically increased numbers of Ag-specific T cells after successful immune responses, and by which self-antigens can become targets in autoimmunity.

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