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Evidence for the Involvement of Fas Ligand and Perforin in the Induction of Vascular Leak Syndrome

Asimah Q. Rafi,² * Ahmet Zeytin, * Michael J. Bradley, * D. Phillip Sponenberg, † Randolph L. Grayson, ‡ Mitzi Nagarkatti, † and Prakash S. Nagarkatti³ *

Endothelial cell injury resulting in vascular leak syndrome (VLS) is one of the most widely noted phenomena in a variety of clinical diseases. In the current study we used IL-2-induced VLS as a model to investigate the role of cytolytic lymphocytes in the cytotoxicity of endothelial cells. Administration of IL-2 (75,000 U/mouse, three times a day for 3 days) into BL/6 wild-type mice triggered significant VLS in the lungs, liver, and spleen. Interestingly, perforin-knockout (KO) mice exhibited a marked decrease in IL-2-induced VLS in all three organs tested. Also, Fas ligand-defective (gld) mice and Fas-deficient (lpr) mice exhibited decreased VLS in the liver and spleen, but not in the lungs. The decreased VLS seen in perforin-KO, gld, and lpr mice was not due to any defect in lymphocyte migration or homing to various organs because histopathologic studies in these mice demonstrated significant and often greater perivascular infiltration of lymphocytes compared with the IL-2-treated wild-type mice. Ultrastructural studies of the lungs demonstrated significant damage to the endothelial cells in IL-2-treated wild-type mice and decreased damage in perforin-KO mice. IL-2 administration caused up-regulation of CD44 in all strains of mice tested and triggered increased LAK activity against an endothelial cell line in wild-type and gld mice, but not in perforin-KO mice. The current study demonstrates for the first time that perforin and Fas ligand may actively participate in endothelial cell injury and induction of VLS in a variety of organs. The Journal of Immunology, 1998, 161: 3077–3086.

A t sites of chronic inflammation as seen in a variety of infections, autoimmune diseases, graft-vs-host disease, and during treatment of cancer patients with high doses of IL-2, significant damage to the endothelial cells has been known to occur, which leads to severe toxicity or pathogenesis associated with the disease. For example, in murine lymphocytic choriomeningitis viral infection, massive delayed-type hypersensitivity reaction occurs in the cerebrospinal fluid. It has been speculated that virally activated CD8⁺ T cells kill the endothelial cells, leading to massive extravasation of monocytes and CD4⁺ T cells via the subarachnoid space (1). In multiple sclerosis, damage to the blood-brain barrier has been known to occur after injury to the endothelial cells by cytotoxic T cells (2). Also, in autoimmune disease models involving vasculitides, the lesions have been associated with infiltration of lymphocytes and macrophages at the vascular wall structure (3). Such types of vasculitides have been described in human and murine autoimmune diseases (4, 5). Similarly, in atherosclerosis, endothelial cell damage and inflammatory cell activation have been shown to contribute to the further development of the cardiovascular disease (6, 7).

Although IL-2 therapy has yielded encouraging results in the treatment of certain types of cancer, its use is limited by dose-dependent toxicity characterized by weight gain, dyspnea, ascites, and pulmonary edema (8, 9). Such toxicity results from increased capillary leak, also known as vascular leak syndrome (VLS). A number of cytokines used as hemopoietic growth factors are also known to trigger VLS (9). The exact mechanism of cytokine-triggered endothelial cell damage and induction of VLS is not clear.

Several recent studies have suggested that VLS may result from actual damage to the endothelial cells caused by cytotoxic lymphocytes (10, 11). In contrast, some types of endothelial cell damage may result from participation of neutrophils and complement components (12). The fact that CTL are involved in the induction of VLS was demonstrated in a recent study from our laboratory in which administration of a CTL clone into a syngeneic irradiated mouse along with IL-2 led to a significant induction of VLS in vivo (13).

In the current study we used VLS as a model to study the endothelial cell damage seen after IL-2 administration. To directly test the involvement of cytotoxic lymphocytes in endothelial cell injury leading to the induction of VLS, we used perforin-knockout (KO) and Fas ligand (FasL)-defective, gld mice, inasmuch as perforin and FasL have been characterized as two important effector molecules involved in cytotoxicity mediated by CTL and NK/LAK cells (14). The results demonstrated that the VLS was markedly decreased in all organs tested in perforin-KO mice and was significantly decreased in the liver and spleen of gld mice, thereby supporting the hypothesis that VLS results from direct cytotoxicity of endothelial cells by cytotoxic lymphocytes.

Materials and Methods

Mice

Four to six-week-old female C57BL/6 mice were purchased from the National Institutes of Health (Bethesda, MD). Age-matched B6/gld/gld B6/lpr/lpr and perforin-KO mice were bred in our animal facilities. The perforin-KO mice were provided by W. K. Clark (University of California,
Los Angeles, CA) (15). It should be noted that gld and lpr mice used at 4 to 6 wk of age did not exhibit any lymphoproliferative disease.

Cell lines

YAC1, a NK-sensitive Moloney virus-induced lymphoma, and TME-1H3, an SV40-transformed endothelial cell line, were maintained in vitro by serial passages, as previously described (13, 16).

Antibodies

Monoclonal MEL-14 (lymphocyte homing receptor; rat IgG) and anti-LFA-1 (M17/4; rat IgG) were grown in vitro as described previously (17). The FITC-CD3, PE-CD4, PE-CD8, FITC-CD4, and Jo2 (anti-Fas) mAbs were purchased from PharMingen (San Diego, CA). FITC-conjugated F(ab`)2 goat anti-syrian hamster IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). [125I]BSA was purchased from ICN (Costa Mesa, CA).

ILs

Recombinant IL-2 was provided by Hoffmann-La Roche (Nutley, NJ) and Dr. C. Reynolds (National Institutes of Health).

Detection of surface molecules using immunofluorescence analysis

Splenic T cells and LN cells were analyzed for CD3 and CD44 expression by staining the cells with FITC-CD3- and PE-CD44-labeled Abs for 30 min on ice followed by washing three times. LFA-1 and MEL-14 expression was detected by staining the cells with unlabeled primary Abs against these markers for 30 min on ice followed by washing three times. After washing, FITC-conjugated secondary Ab was added to detect the presence of LFA-1 and MEL-14. The secondary Ab consisted of FITC-conjugated anti-rat IgG (Fab`)2 (Cappel Laboratories, Durham, NC). Negative controls consisted of fluorescence obtained by staining cells with FITC-conjugated secondary Ab. Fas expression was detected by staining the cells with Jo2 mAbs (anti-Fas) for 30 min on ice followed by washing three times. After washing, FITC-conjugated F(ab`)2 goat anti-syrian hamster IgG was added to detect for the presence of Fas. Negative controls consisted of fluorescence obtained by staining cells with FITC-conjugated secondary Ab. The cells were washed twice and analyzed flow cytometrically. CD3, CD4, CD44, and CD8 expression was detected by staining the cells with fluorescein-labeled Ab against these markers for 30 min on ice followed by washing three times. Nonspecific staining was blocked by incubation of cells with 0.5% normal mouse serum for 30 min before staining with labeled Ab. Next, 10,000 cells were analyzed by a flow cytometer (EPICS V, model 752; Coulter, Miami, FL).

Quantitation of VLS

Vascular leak was studied by measuring the extravasation of [125I]BSA into various organs as described previously (18). Groups of five mice were injected i.p. with 75,000 U rIL-2 or PBS as a control, three times a day for 3 days. On day 4, they received one injection in the morning and 2 h later were injected i.v. with 0.5 μCi of [125I]BSA in 0.5 ml of PBS. After 2 h, the mice were bled to death under anesthesia, and the heart was perfused with heparin in PBS as described previously (18). The lungs, liver, and spleen were harvested, placed in vials, and measured in a gamma counter. The VLS seen in IL-2-treated mice was expressed as the percent increase in extravasation compared with that in PBS-treated controls and was calculated as: [(cpm in the organs of IL-2-treated mice − cpm in the organs of PBS-treated controls)/cpm in the organ of PBS treated control] × 100.

Each mouse was individually analyzed for vascular leak, and the data from five mice were expressed as the mean ± SEM percent increase in VLS in IL-2-treated mice compared with that in PBS-treated controls.

Cytotoxicity

The ability of splenic T cells to lyse various tumor targets was tested using 51Cr release assays, described previously (13). Briefly, 5 × 10^6 target cells (YAC-1 or TME-3H3) were labeled with 51NaCrO by incubation at 37°C for 1 h. Varying E:T cell ratios in triplicate were added in 96-well round-bottom plates (Falcon 3910, Becton Dickinson, Lincoln Park, NJ) and incubated for 4 h at 37°C. Spontaneous release was measured by incubating the 51Cr-labeled targets alone, and total release was determined by incubating the labeled target cells with 0.1% SDS. The supernatants were harvested after 4 h, and radioactivity was measured with a gamma counter (TriAnalytic, Elk Grove Village, IL).

Histology

For histopathologic studies, groups of five separate mice were injected with IL-2 or PBS as described above, and on day 4, lungs and liver were fixed in 10% formalin solution. The organs were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Perivascular infiltration was scaled by counting the number of lymphocytes infiltrating the vessel and averaging the minimum and maximum ranges for each group. Three samples were used for lung, and 10 samples were used for the liver.

Electron microscopy studies

Tissue samples were fixed in 5% glutaraldehyde/4.4% formaldehyde/2.75% picric acid in 0.05 M sodium cacodylate buffer, pH 7.4; washed in a sodium cacodylate buffer; postfixed in osmium tetroxide; embedded in Polybed 812 resin (Polysciences, Warrington, PA); and studied with an electron microscope.

Statistical analysis

The VLS data in different strains of mice were compared using analysis of variance, and p < 0.05 was considered statistically significant.

Results

VLS induction in wild-type, perforin-KO, FasL-defective (gld), and Fas-deficient (lpr) mice

To directly test whether VLS seen following injection of IL-2 is caused by cytolytic effector molecules such as perforin and FasL, we used perforin-KO and FasL-defective (gld) mice with B/6 background and compared the degree of VLS seen with IL-2-treated wild-type mice. To this end, groups of five mice received 75,000 U of IL-2 three times daily for 3 days and once on day 4. On the last day, the mice were injected with [125I]BSA, and VLS was studied by determining the extravasation of [125I]BSA in the lungs, liver, and spleen. The data on VLS seen in each organ were expressed as the mean percent increase in radioactive counts per minute in IL-2-treated groups compared with that in their respective controls that received PBS alone as described in Materials and Methods. For example in one experiment, the lungs of PBS-treated controls exhibited 4,614 mean cpm, while the lungs from IL-2-treated mice showed 10,012 mean cpm. Thus, the percent increase in vascular leak was considered to be 117. A similar approach was used to calculate the degree of VLS in each of the five mice per group, and the mean percent increase in VLS ± SEM was plotted (Fig. 1) for each organ and each strain of mouse.

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Figure 1 depicts a representative experiment in which the wild-type (B/6) mice exhibited significant VLS following IL-2 injection in all three organs tested compared with PBS-treated controls. Interestingly, the VLS induction varied in the perforin-KO and FasL mutant mice. In the lungs, VLS was markedly reduced in perforin-KO mice, whereas gld mice did not exhibit any decrease, but, in fact, demonstrated a significant increase in VLS. These data suggested that perforin, but not FasL, played a key role in VLS induction in the lungs. The fact that VLS in the lungs of Fas-deficient (lpr) mice was seen to the same extent as that in the wild-type mice further corroborated these results. In the liver, the perforin-KO, gld, and lpr mice exhibited a significant decrease in VLS. These data suggested that both perforin and FasL played important roles in VLS induction in the liver. In the spleen, the perforin-KO, gld, and lpr mice exhibited decreased levels of VLS compared with the wild-type mice. This decrease was, however, less than that seen in the liver, using similar groups of mice. These experiments were repeated three times with consistent results. The fact that perforin-KO mice exhibited decreased VLS in all three organs tested, indicated that perforin may constitute an important factor involved in the induction of VLS. In contrast, FasL-based lytic activity appeared to play a key role in the induction of VLS in liver and spleen but not lungs. This was further corroborated by the fact that VLS was decreased in the liver and spleen of lpr mice.
The counts for the liver of mice treated with PBS were 17,120 ± SEM

These findings supported our hypothesis that IL-2 may activate LAK cells, which mediate lysis of endothelial cells using perforin and FasL. The involvement of FasL also suggested that endothelial cells may express Fas, as previously shown in our laboratory (20).

**Histopathologic studies of organs exhibiting VLS**

It was possible that the reason why perforin-KO, gld, or lpr mice exhibited decreased VLS was because of the inability of cytolytic lymphocytes to migrate to the vascular tissues of various organs rather than the inability to mediate cytotoxicity of endothelial cells. To rule out this possibility and to confirm that in IL-2-treated mice perivascular infiltration was not clear, these data ruled out the possibility that the decreased VLS seen in the lungs of perforin-KO mice and the livers of perforin-KO, lpr, and gld mice resulted from decreased migration/homing and ability to cause perivascular infiltration. These data also suggested that the decreased VLS in the lung, liver, and spleen in perforin-KO mice and the decreased VLS in the liver and spleen of gld and lpr mice may have resulted from the inability of cytolytic lymphocytes to mediate lysis of endothelial cells due to deficiency of perforin, FasL, or Fas, respectively.

**Ultrastructural studies on injury to endothelial cells during VLS**

To further corroborate that IL-2-induced VLS resulted from actual damage to the endothelial cells, ultrastructural studies of the lungs were performed. As shown in Figure 4a, A1, the wild-type mice injected with PBS (control) displayed normal ultrastructural morphology of blood vessels. In contrast the wild-type mice injected with IL-2 had extensive to almost complete destruction of the endothelial cell layer of the blood capillaries as well as epithelial cell damage in the alveolar air spaces (Fig. 4a, A2). The perforin KO mice injected with IL-2 had the least amount of damage to the endothelial cells (Fig. 4b, B2). Many endothelial cells appeared to be morphologically normal, as observed in the controls. The IL-2-treated lpr mice displayed more damage in the blood vessels, with shrinkage of endothelial cells away from the basal lamina and extensive deterioration of the endothelial and epithelial cells, which are separated by the basal lamina (Fig. 4b, B3). The most severe destruction of endothelial cells and epithelial cells of the alveolar air spaces and breakage of the basal lamina occurred in the IL-2-treated gld mice. In the lungs of these mice, widespread areas of cellular debris could be observed (Fig. 4b, B1). In some places the basal lamina was broken, and cell debris extruded into the alveolar air spaces.

These data together indicated that IL-2 treatment caused significant damage to the endothelial cells in wild-type mice, thereby confirming that the VLS resulted from actual damage to the endothelial cells. Furthermore, when KO/mutant mice were screened, the endothelial cell damage correlated with the VLS data. It was interesting to note that perforin-KO mice exhibited the least damage to the endothelial cells in the lungs.

**LAK activity in mice undergoing VLS**

To test whether the lymphocytes from IL-2-treated mice would exhibit increased LAK activity and to investigate the nature of cytolytic effector molecules triggered by in vivo IL-2 administration, splenic T cells collected from mice undergoing VLS were tested for cytolytic activity against Fas+ YAC-1 tumor targets. The data shown in Figure 5 (upper panel) indicated that perforin-KO (B) and gld (C) mice exhibited minimal spontaneous cytotoxicity against YAC-1 targets.

**Table I.** *Perivascular infiltrating lymphocytes from mice treated with IL-2*<sup>a</sup>

<table>
<thead>
<tr>
<th>Organ</th>
<th>Strain</th>
<th>+/+</th>
<th>Perforin-KO</th>
<th>gld</th>
<th>lpr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.17 ± 0.12</td>
<td>5.95 ± 0.38*</td>
<td>3.45 ± 0.23</td>
<td>5.00 ± 0.36*</td>
<td></td>
</tr>
<tr>
<td>Liver&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.80 ± 0.12</td>
<td>3.50 ± 0.063*</td>
<td>2.98 ± 0.25</td>
<td>3.12 ± 0.19</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Mice were injected with IL-2 to induce VLS and the organs were processed for histopathological studies as described in Figures 2 and 3. The number of perivascular infiltration was measured by counting the number of lymphocytes infiltrating each vessel.

<sup>b</sup>The number of lymphocytes infiltrating a venule. The data represent the mean ± SEM obtained from 3 (for lungs) or 10 (for liver) samples per mouse (4 mice were analyzed for each organ). Data showing statistically significant differences (*p* < 0.05) when compared to the wild-type mice are indicated with an asterisk.
However, after IL-2 administration, they exhibited a significant increase in cytotoxicity, although such lytic activity was less than that seen in the wild-type mice (A). These data indicated that IL-2 up-regulates both FasL-based and perforin-based cytotoxicity, which may subsequently play a role in endothelial cell lysis. To test the ability of IL-2-induced LAK cells to kill endothelial cells, we used a...
well-characterized endothelial cell line, TME-3H3 (13). The data shown in Figure 5 (lower panels) indicated that wild-type (D), perforin-KO (E), and gld (F) mice failed to exhibit spontaneous cytotoxicity of endothelial cells. However, IL-2-induced LAK cells from wild-type mice mediated the highest level of cytotoxicity, followed by those from gld and perforin-KO mice. These data correlated well with

FIGURE 3. Histopathologic studies on liver in mice. Livers from wild-type (A1 and A2), perforin-KO (B1 and B2), gld (C1 and C2), and lpr (D1 and D2) mice treated with PBS (A1, B1, C1, and D1) or IL-2 (A2, B2, C2, and D2) were harvested and preserved in 10% formalin solution. Sections were stained with hematoxylin and eosin. Perivascular infiltration consisting mostly of lymphocytes is indicated by arrows. The 1-cm horizontal bar equals 35 μm.
The VLS results (Fig. 1) in which perforin-KO mice were found to exhibit marked decrease in VLS, and gld mice showed only a partial decrease in certain organs. It should be noted that YAC-1 and TME-3H3 cell lines expressed significant levels of Fas as determined by flow cytometry (Fig. 6). However, the endothelial cell line expressed lower levels of Fas, and only 42% of the cells expressed Fas compared with the thymocytes from wild-type mice, which expressed higher levels. This may explain why endothelial cell lysis was more dependent on perforin than FasL as shown in Figure 5 (lower panel).

**Effect of IL-2 administration on CD44 expression**

We and others have demonstrated that activation through CD44 can trigger lytic activity in CTL and NK/LAK cells (16, 17, 21, 22). Inasmuch as endothelial cells express the ligand for CD44, we have hypothesized that LAK cells expressing CD44 may spontaneously kill endothelial cells following interaction between LAK cells and endothelial cells (13, 17, 20). We therefore investigated whether IL-2 treatment would up-regulate CD44 expression in T cells. To this effect, wild-type, perforin-KO, gld, and lpr mice injected with IL-2 as described before and were sacrificed, and purified T cells from the spleens were stained using mAbs against CD44 and were analyzed flow cytometrically.

The data shown in Figure 7 indicated that in IL-2-treated mice, there was significant up-regulation in CD44 expression on T cells in all groups of mice tested compared with that in the controls. This was evident from the increase in the mean intensity of fluorescence on T cells following IL-2 administration. It should be

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**FIGURE 4.** a. Ultrastructural studies on injury to endothelial cells during VLS. Lung samples from wild-type mice treated with IL-2 (A2) or PBS (A1) were fixed and studied with an electron microscope. A1 shows wild-type mice treated with PBS. A near cross-section of a capillary has an erythrocyte (E) in the central lumen surrounded by endothelial cells (En) with their cytoplasm tightly appressed against the basal lamina (BL), which separates the endothelial cell from the flattened cytoplasmic portion of a lining epithelial cell. Note the numerous pinocytotic vesicles (PV) and a few tight junctions (TJ). It should be noted that the lung tissue from perforin-KO, gld, and lpr mice treated with PBS alone did not show any damage to the endothelial cells and was structurally similar to that in PBS-treated wild-type mice and therefore is not depicted. Magnification = ×16,000. A2 depicts wild-type mice treated with IL-2. A cross-section of a capillary with erythrocytes (E) in the lumen is shown. Almost complete destruction of the endothelial cell layer has occurred. Endothelial (Ep) cells of the alveolar air space on the other side of the basal lamina (BL) show extensive damage. Magnification = ×6,600. b. Ultrastructural studies on injury to endothelial cells during VLS. Lung samples from gld (B1), perforin-KO (B2), and lpr (B3) mice treated with IL-2 were fixed and studied with an electron microscope. B1 shows gld mice treated with IL-2. Very extensive damage has occurred, resulting in cell death within the blood vessel and outside the basal lamina (BL). The cellular cytoplasm of the endothelial cells and the epithelial cells of the alveolar sac are completely fragmented into cellular debris. In the lumen a white blood cell (WBC) is in the process of being fragmented. The basal lamina (BL) has been broken, with cellular debris spilling into the alveolar air space, including pinocytotic vesicles that are involved in the transport of fluids (arrow). Magnification = ×12,600. B2, shows perforin-KO mice treated with IL-2. Erythrocytes (E) are moving through the lumen of the capillary. The endothelial cells are still pressed against the basal lamina (BL), but show indications of cellular deterioration. This is indicated by the formation of membranous circular structures (arrows) and apoptotic bodies in the endothelial cells. However, this deterioration is much less than that seen in the wild-type mice treated with IL-2. This is based on the intact membranes of the cellular constituents, the intact basal lamina, and the lack of cellular debris and membrane fragments. Magnification = ×20,400. B3 shows lpr mice treated with IL-2. Cell damage to the endothelial and epithelial cells separated by the basal lamina is evident. Cell organization and structures have been lost, leaving cell remnants. There is condensed disorganized dark-staining cellular debris (large arrows) in the endothelial (En) and epithelial (Ep) cells. The cell blebbing and the loss of cell membranes are indicative of dying and dead cells. The basal lamina (BL) is losing its definition and has become diffuse between the endothelial and epithelial cells, which indicates severe leakage. Magnification = ×50,000.

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**FIGURE 5.** LAK activity in mice undergoing VLS against YAC-1 and endothelial cell targets. Wild-type (A and D), perforin-KO (B and E), and gld (C and F) mice were injected with IL-2 as described in Figure 1. On day 4, spleen cells were passed over nylon wool to enrich T cells and were tested for cytotoxicity against 51Cr-labeled YAC-1 targets (A–C) or the endothelial cell line, TME-3H3 (D–F). The data indicate the mean percent cytotoxicity of triplicate cultures ± SEM.
noted that IL-2 treatment up-regulated the expression of LFA-1 and L-selectin and down-regulated CD3 expression to a moderate extent in all strains of mice (data not shown).

**IL-2 treatment increases the percentage of CD8+ T cells and decreases the proportion of CD4+ T cells in the periphery**

To investigate the effects of IL-2 treatment on CD4+ T and CD8+ T cells in the periphery and to compare the levels of their induction in different groups of mice, the proportions of CD4+ and CD8+ T cells in the spleens were detected using flow cytometry. The data shown in Table II indicated that IL-2 treatment caused an increase in the percentage of CD8+ T cells and a consequent decrease in the percentage of CD4+ T cells in the periphery of wild-type, perforin-KO, gld, and lpr mice. These data indicated that IL-2 induced similar activation of CD8+ in all strains of mice tested and that the differences in cytotoxicity seen in various groups did not result from altered activation of T cell subsets.

**Discussion**

Administration of IL-2, although promising in the treatment of certain types of cancer, triggers severe side effects in patients who develop a capillary leak syndrome resulting in anasarca and multiorgan system dysfunction (23, 24). The exact mechanism of induction of VLS is not known. In the current study we tested the hypothesis that IL-2-induced VLS may result from the cytolytic activity of endothelial cells by LAK cells. Because perforin and FasL constitute two important cytolytic effector molecules involved in LAK cell-mediated cytotoxicity, we used perforin-KO and FasL-mutant (gld) mice to study the VLS. Our data indicated that IL-2 up-regulates perforin and FasL activity and that these molecules do participate in the induction of VLS. In wild-type mice exhibiting VLS, there was significant perivascular infiltration with lymphocytes, and endothelial cell damage was evident from ultrastructural studies. In perforin-KO mice, despite perivascular infiltration with lymphocytes, there was no significant endothelial cell damage, and VLS was markedly reduced in all organs tested. Similarly, the VLS was decreased in certain organs of gld and lpr mice, thereby suggesting that Fas-FasL interactions may also regulate VLS induction. Also, the IL-2-treated wild-type mice exhibited increased LAK cell activity against an endothelial cell line, whereas gld and particularly the perforin-KO mice exhibited a significant decrease in their ability to kill endothelial cells. Lymphocytes from all strains of mice tested expressed increased levels of CD8+ T cells and higher densities of homing molecules, including CD44, following IL-2 treatment. These data together suggested that IL-2 up-regulates the expression of CD44, thereby facilitating the migration of LAK cells to various organs. Furthermore, IL-2 also up-regulates perforin and FasL, and the interaction between LAK cells and endothelial cells may trigger their lysis, leading to extravasation of intravascular fluid.

Earlier studies have demonstrated that immunosuppression of mice by pretreatment with irradiation or injection of cyclophosphamide or cortisone markedly reduces or eliminates the development of IL-2-induced VLS (18). These data suggested that IL-2 does not act directly on the blood vessels to alter their permeability, but that it may do so indirectly by involving cells of the immune system. Vascular leaks in acute inflammatory lesions are triggered by mediators of immediate hypersensitivity, such as histamine, serotonin, and bradykinin (25). Although the IL-2-induced vascular leak resembles that triggered by the above-mentioned mediators, earlier studies have ruled out the involvement of vasoactive amines in IL-2-induced VLS (18). Lymphocytes are also known to produce a variety of mediators that increase the vascular permeability (26, 27). However, the current study demonstrates that IL-2-induced vascular leak may result from the involvement of cytolytic effector molecules, perforin and FasL.
Table II. Expression of CD4 and CD8 in the spleen after IL-2 treatment

<table>
<thead>
<tr>
<th>Strain</th>
<th>PBS</th>
<th>IL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4</td>
<td>CD8</td>
</tr>
<tr>
<td>Wild-type</td>
<td>28.6±1.3</td>
<td>14.2±3.7</td>
</tr>
<tr>
<td>PD</td>
<td>21.2±0.18</td>
<td>17.6±0.82</td>
</tr>
<tr>
<td>GLD</td>
<td>17.1±1.2</td>
<td>15.8±0.62</td>
</tr>
<tr>
<td>LPR</td>
<td>20.7±0.81</td>
<td>19.45±1.9</td>
</tr>
</tbody>
</table>

*Mice were injected with PBS or IL-2 as described in Figure 1. The spleen cells were stained for CD4 and CD8 markers and cells were analyzed by flow cytometrically. The data are depicted as mean percent positive cells ± SEM obtained from four to five mice. The CD4+ and CD8+ T cell percentages in IL-2-treated mice were compared with their respective PBS-treated controls, and those showing statistically significant differences (p < 0.05) are indicated with an asterisk.

Several studies have suggested that endothelial cells can be the targets of lymphocyte-mediated destruction. Damle et al. demonstrated that IL-2-activated LAK cells adhered and killed endothelial cells efficiently (10). Also, the toxicity associated with IL-2 therapy has been shown to decrease after depletion of NK cells in vivo (29). The role of CTL in VLS induction was also demonstrated in our earlier studies, in which it was noted that administration of a CTL clone plus IL-2 into irradiated syngeneic mice, but not the CTL clone or IL-2 alone, triggered VLS (13). Also, the IL-2-activated CTL clone could mediate efficient lysis of an endothelial cell line, but not a fibroblast cell line, in an MHC-unrestricted fashion (13). Moreover, methotrexate was found to attenuate pulmonary vascular leak by preventing the proliferation of NK/LAK cells and also by inhibiting leukocyte binding to endothelial (30). Similarly, dextran sulfate, which blocks leukocyte-endothelial adhesion, attenuates IL-2-induced VLS (31). Such studies together with the data presented in the current study suggest that IL-2-induced VLS may result from the direct cytotoxicity of endothelial cells by LAK cells.

In the current study it was observed that perforin-KO mice exhibited marked decrease in VLS in the lungs, whereas gld mice did not exhibit any decrease, but, in fact, exhibited a significant increase in VLS in the lungs. These data suggested that perforin played a key role in VLS induction in the lungs and that FasL was not critical. The fact that VLS in Fas-deficient (lpr) mice was of the same extent as that in the wild-type mice, further corroborated these results. In the current study it was not clear why the gld mice exhibited increased VLS in the lungs. This was seen in all three repeated experiments. It can be speculated that increased perivascular infiltration of lymphocytes as seen in the histopathology of lung tissue and/or increased susceptibility of lung endothelial cells to cytolytic activity may have triggered increased VLS in gld mice. Also, in gld mice, there was greater VLS in the lungs but less in the liver and spleen, thereby suggesting more damage to lung endothelium and less to the endothelium of spleen and liver. However, when LAK cells from gld mice were tested for cytotoxicity against the endothelial cell line in vitro, they exhibited similar levels of cytotoxicity as the wild-type mice. This may be because the endothelial cell line used was derived from the lymph node. Thus, it is possible based on our data that endothelial cells from various organs, following IL-2 treatment in vivo, may vary in their susceptibility to lysis by LAK cells. The fact that in the liver and the spleen, both the perforin-KO and gld mice had significantly diminished VLS suggested that both Fasl and perforin may play a significant role in VLS induction in these organs. These data also suggested that the endothelial cells from different organs may exhibit differential susceptibility to perforin and FasL-mediated cytotoxicity. The fact that lpr mice also exhibited decreased VLS in the liver and spleen suggested that the endothelial cells in these organs may express Fas. The endothelial cell line used in the current study expressed significant levels of Fas. Whether the endothelial cells in different organs express varying levels of Fas, thereby accounting for the differential susceptibility to Fasl-based cytotoxicity, remains to be determined. It should be noted that in the lungs, perforin, but not Fasl, played an important role in VLS induction. However even in perforin-KO mice the VLS was not completely abolished. This suggested the possibility that other cytotoxic molecules such as TNF may play a role in VLS induction. It should be noted that the role of TNF in VLS is controversial. Studies involving attempts to block TNF have demonstrated either beneficial or no effect on IL-2-induced toxicity (32, 33). In addition, the VLS may result from complement activation (34, 35).

In the current investigation the histopathologic studies indicated that following IL-2 administration, there was significant infiltration of lymphocytes, but not neutrophils, in the perivascular tissue. These studies ruled out the possible damage to endothelial cells caused by neutrophils as reported in other models (36–38). The fact that IL-2-treated perforin-KO, gld, and lpr mice demonstrated significant perivascular infiltration of lymphocytes, in fact greater than that in the IL-2-treated wild-type mice, ruled out the possibility that the decreased VLS seen in the KO/mutant mice was because of the inability of cytolytic lymphocytes to migrate to the vascular tissues of various organs. Also, the fact that all strains tested exhibited similar up-regulation of CD44, a molecule involved in lymphocyte homing, further suggested that decreased VLS in the KO/mutant mice was due to the deficiency of the cytolitic effector molecules rather than lymphocyte migration and homing. Our data are consistent with previous histologic studies that showed lymphoid cell infiltration in lung, liver, kidney, and heart following IL-2 therapy (39, 40). Also, the electron microscopic studies confirmed that IL-2 treatment in wild-type mice caused significant damage to the endothelial cells, and furthermore, the results on VLS as seen in various mutant/KO mice correlated well with the ultrastructural studies on endothelial cell damage. It should be noted that the lpr and gld mice used in the current study were 4 to 6 wk of age, during which time they have similar proportions of T cells, as shown in our earlier studies on the thymus (41). This, however, did not rule out the possibility that such differences could exist in the periphery, particularly after IL-2 treatment. In the current study it was noted that IL-2 treatment caused a similar increase in the percentage of CD8+ T cells and a decrease in CD4+ T cells in all groups of mice. These data ruled out the possibility that the differences in the cytotoxicity and VLS induction resulted from differential activation of CD4+ or CD8+ T cells in various groups of mice.

Although in the current study we used IL-2-induced VLS as a model to study LAK cell-endothelial cell interactions, there is growing evidence that similar endothelial cell injury may occur in a variety of disease models. Thus, the vascular leak seen at sites of chronic inflammation involving mononuclear cells may be triggered by the direct killing of endothelial cells by cytolytic lymphocytes involving perforin and Fasl-based pathways.

We and others have shown earlier that CTL, double-negative T cells and NK cells upon activation express high levels of CD44 and mediate efficient MHC-unrestricted TCR-independent lysis following ligation of CD44 (13, 17, 20–22, 42). We have also demonstrated that the lysis of endothelial cells ex vivo by cytolytic lymphocytes can be blocked by soluble CD44 fusion protein, anti-CD44 Fab, or soluble hyaluronate (unpublished observations). These data suggested that CD44-hyaluronate interactions may play an important role in the migration, homing, and lysis of endothelial...
cells. Further studies on the CD44 isoforms involved in lymphocyte adhesion and cytotoxicity of the endothelial cells should provide useful information on therapeutic intervention to prevent endothelial cell injury.

References


