Chapter 4: Evidence for the Involvement of Fas Ligand and Perforin in the Induction of Vascular Leak Syndrome

Abstract

Endothelial cell injury resulting in vascular leak syndrome (VLS) is one of the most widely noted phenomenons in a variety of clinical diseases, however, the underlying reason for which remains unclear. In the current study we used interleukin-2 (IL-2) induced VLS as a model to investigate the role of cytolytic lymphocytes in the direct cytotoxicity of endothelial cells. Administration of IL-2 (75,000 units/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 marked decrease in IL-2 induced VLS in all three organs tested. Also, FasL-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 histopathological studies in these mice demonstrated significant and often greater perivascular infiltration of lymphocytes when compared to the IL-2 treated wild-type mice. Ultrastructural studies of the lungs demonstrated significant damage to the endothelial cells in IL-2 administered wild-type mice, and decreased damage in perforin-KO mice. IL-2 administration caused upregulation 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 FasL may actively participate in endothelial cell injury and induction of VLS in a variety of organs.

Introduction

At sites of chronic inflammation as seen in a variety of infections, autoimmune diseases, graft-versus host disease (GVHD) and during treatment of cancer patients with high doses of interleukin-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 (LCM) 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 (Doherty et al., 1990). In multiple sclerosis, damage to the blood brain barrier has been known to occur, following injury to the endothelial cells by cytotoxic T cells (Tsukada et al., 1993). Also, in autoimmune disease models involving vasculitides, the lesions have been associated with infiltration of lymphocytes and macrophages at the vascular wall structure (Moyer et al., 1983). Such types of vasculitis have been described in human and murine autoimmune diseases (McCluskey et al., 1983; Hewicker and Trautwein, 1987). Similarly, in arteriosclerosis, endothelial cell damage and inflammatory cell activation have been shown to contribute to the further development of the cardiovascular disease (Fox et al., 1993; van der Wal et al., 1992).

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 (Bechard et al., 1989; Vial and Descotes ,1995). Such toxicity results from increased capillary leak, also known as vascular leak syndrome (VLS). A number of cytokines used as hematopoietic growth factors are also known to trigger VLS (Vial et al.,1995). 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 (Damle et al.

1987; Damle and Doyle, 1989). In contrast, some types of endothelial cell damage may also result from participation of neutrophils and complement components (Ward et al., 1996). The fact that cytotoxic T lymphocytes 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* (Hammond-Mckibben et al., 1995).

In the current study we used VLS as a model to study the endothelial cell damage seen following 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 (Kagi et al., 1994). The results demonstrated that the VLS was markedly decreased in all organs tested in perforin-KO mice and 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-6 weeks 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-knockout (KO) mice were bred in our animal facilities. The perforin-KO mice were kindly provided by WK Clark (Univ. of California, LA) (Walsch et al., 1994). It should be noted that *gld* and *lpr* mice used at 4-6 weeks of age did not exhibit any lymphoproliferative disease.

Cell Lines: YAC1, a NK-sensitive Moloney virus-induced lymphoma and TME-3H3, an SV40 transformed endothelial cell line were maintained *in vitro* by serial passages, as described (Hammond-McKibben et al., 1995, Seth et al., 1991).

Antibodies: Monoclonal MEL-14 (lymphocyte homing receptor; rat IgG) and anti-LFA-1 (M17/4; rat IgG) were grown *in vitro* as described elsewhere (Rosenstein et al., 1986). The FITC-CD3, PE-CD44, PE-CD8, FITC-CD4 and Jo2 (anti-Fas) mAbs were purchased from Pharmingen (San Diego, CA). FITC-conjugated F(abí)$_2$ fragment goat anti-syrian hamster IgG was purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA). ^{125}I-BSA was purchased from ICN (Costa Mesa, CA).

Interleukins: Recombinant IL-2 (rIL-2) was kindly provided by Hoffman LaRoche (Nutley, NJ) and by Dr. C. Reynolds, NIH, Bethesda, MD.

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 antibodies for 30 minutes on ice followed by washing three times. LFA-1 and MEL-14 expression was detected by staining the cells with unlabelled primary antibodies against these markers

for 30 minutes on ice followed by washing three times. After washing, FITC-conjugated secondary antibody (Ab) was added to detect the presence of LFA-1 and Mel-14. The secondary Ab consisted of FITC-conjugated anti-rat IgG $F(abí)_2$ (Cappel; Durham, North Carolina). 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 minutes on ice followed by washing three times. After washing, FITC-conjugated $F(abí)_2$ fragment 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 two times and analyzed flow cytometrically. CD3, CD4, CD44 and CD8 expression was detected by staining the cells with fluorescein labeled antibody against these markers 30 minutes on ice followed by washing three times. Nonspecific staining was blocked by incubation of cells with 0.5% normal mouse serum for 30 minutes prior to staining with labeled antibody. Next, 10,000 cells were analyzed by a flow cytometer (Epics V, Model 752; Miami, FL).

Quantitation of Vascular Leak Syndrome: Vascular leak was studied by measuring the extravasation of ^{125}I-BSA into various organs as described previously (Li et al., 1994). Groups of 5 mice were injected intraperitoneally with 75,000 rIL-2 or phosphate buffered saline (PBS) as a control, thrice a day for 3 days. On day 4, they received one injection in the morning and two hours later, were injected intravenously with 0.5µCi ^{125}I-BSA in 0.5 ml PBS. After two hours, the mice were bled to death under anesthesia, and the heart was perfused with heparin in PBS as described previously (Li et al., 1994). 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 percent

increase in the extravasation when compared to the PBS-treated controls and was calculated as:

$$\frac{(\text{cpm in the organ of IL-2 treated mice} - \text{cpm in the organ of PBS treated controls})}{(\text{cpm in the organ of PBS treated control})} \times 100$$

Each mouse was individually analyzed for vascular leak and the data from 5 mice were expressed as mean±SEM percent increase in VLS in IL-2 treated mice when compared to the PBS treated controls.

Cytotoxicity: The ability of splenic T cells to lyse various tumor targets was tested using ^{51}Cr-release assays described previously (Hammond-McKibben et al., 1995). Briefly, $5x10^6$ target cells (YAC-1 or TME-3H3) were labeled with $^{51}NaCrO_4$ by incubating at 37°C for 1 hour. Varying ratios of effector:target cells in triplicate were added in 96-well round-bottom plates (Falcon 3910, Becton Dickinson, Lincoln Park, NJ) and incubated for 4 hours at 37°C. Spontaneous release was measured by incubating the ^{51}Cr-labeled targets alone, and total release was determined by incubating the labeled target cells with 0.1M SDS. The supernatants were harvested after 4 hours and radioactivity was measured with a gamma counter (TmAnalytic, Elk Grove Village, IL)

Histology: For histopathological studies, groups of 5 separate mice were injected with IL-2 or PBS as described earlier 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 range for each group. Three samples were used for lung and ten samples for the liver.

Electron microscopy studies: Tissue samples were fixed in 5% Glutaldehyde/ 4.4% Formaldehyde/ 2.75% Picric Acid in 0.05M Sodium Cacodylate buffer, pH 7.4. Samples were washed in a sodium cacodylate buffer. Samples were post-fixed in osmium tetroxide and embedded in Polybed 812 resin (Polysciences, Warrington, PA) and studied with an electron microscope.

Statistical analysis: The VLS data in different strains of mice was compared using ANOVA and $p<0.05$ were considered to be 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 5 mice received 75,000 units of IL-2 three times daily for 3 days and once on day 4. On the last day, the mice were injected with ^{125}I-BSA and VLS was studied by determining the extravasation of ^{125}I-BSA in the lungs, liver and spleen. The data on VLS seen in each organ was expressed as mean percent increase in radioactive counts per minute (cpm) in IL-2-treated groups when compared to their respective controls that received PBS alone as described in 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. Similar approach was used to calculate the degree of VLS in each of the 5 mice per group and the mean percent increase in VLS±S.E.M. was plotted (Fig. 4.1) for each organ and each strain of mouse.

Figure 4.1 depicts a representative experiment in which the wild-type (B/6+/+) mice exhibited significant VLS following IL-2 injection in all 3 organs tested when compared to 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 the wild-type mice, further corroborated these results. In the liver, the perforin-KO, *gld*

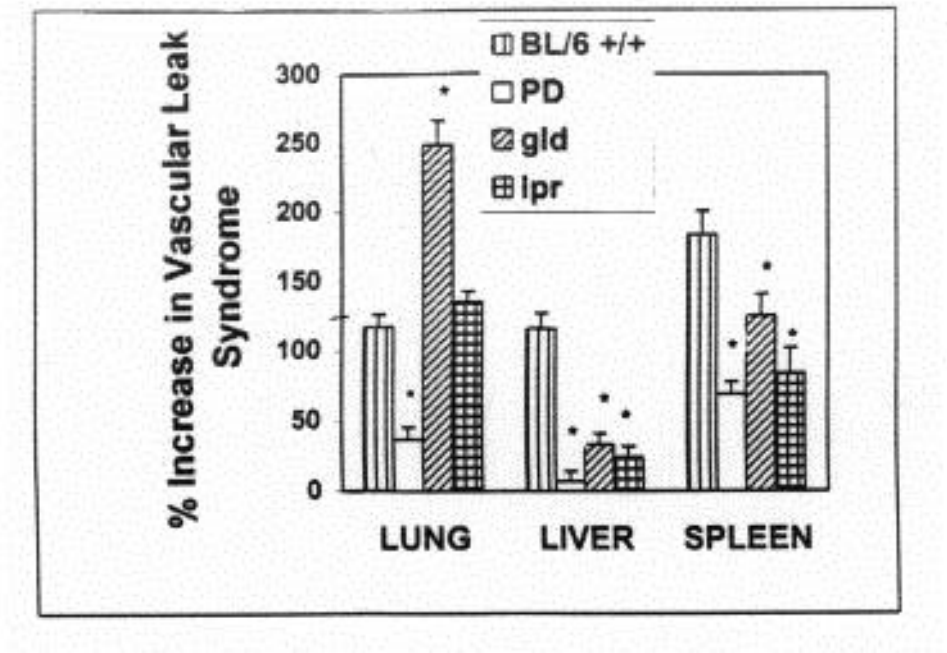

Figure 4.1: VLS induction in wild-type, perforin-KO, FasL-defective (*gld*) and Fas-deficient (*lpr*) mice: BL/6 wild-type (+/+), perforin-KO (PD), FasL-defective (*gld*) and Fas-deficient (*lpr*) mice received 75,000 units of IL-2 three times daily for 3 days and once on day 4. VLS was studied by determining the extravasation of ^{125}I-BSA in the lungs, liver and spleen. The vertical bars represent % increase in VLS± SEM, seen following IL-2 administration when compared to the PBS-treated controls, as described in methods. The counts for the lungs of +/+, perforin-KO, *gld* and *lpr* mice treated with PBS were 4,614±248, 6,604±147, 3,219±277 and 7,585±382 respectively. The counts for the liver of +/+, perforin-KO, *gld* and *lpr* mice treated with PBS were 17,120±1,628, 20,818±2,378, 18,140±2,773 and 21,616±656 respectively. The counts for the spleen of +/+, perforin-KO, *gld* and *lpr* mice treated with PBS were 1,455±22, 2,378±191, 1,700±217 and 1,874±335 respectively. The VLS data from perforin-KO, *gld* and *lpr* mice in each organ were compared to their respective data from BL/6 wild-type (+/+) mice and statistically significant differences were indicated with an asterisk. There was no statistically

significant difference in counts of specific organs of +/+, perforin-KO, *gld* or *lpr* treated with PBS.

and *lpr* mice exhibited a significant decrease in VLS.

These data suggested that both perforin and FasL played an important role in VLS induction in the liver. In the spleen, the perforin-KO, *gld* and *lpr* mice exhibited decreased levels of VLS when compared to the wild-type mice. This decrease was, however, less than that seen in the liver, using similar groups of mice. These experiments were repeated thrice with consistent results. The fact that perforin-KO mice exhibited decreased VLS in all 3 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 the lungs. This was further corroborated by the fact that VLS was decreased in the liver and spleen of *lpr* mice. 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 (Galandrini et al., 1993).

Histopathological studies on 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, there was perivascular infiltration of mononuclear cells, we carried out histopathological studies. To this end, wild-type, perforin-KO, *gld* and *lpr* mice were injected with PBS or IL-2 as described earlier. On day 4, the mice were euthanized and various organs fixed, processed and stained with hematoxylin and eosin. The representative studies on the lungs and liver are shown in Fig 4.2 and Fig 4.3 respectively.

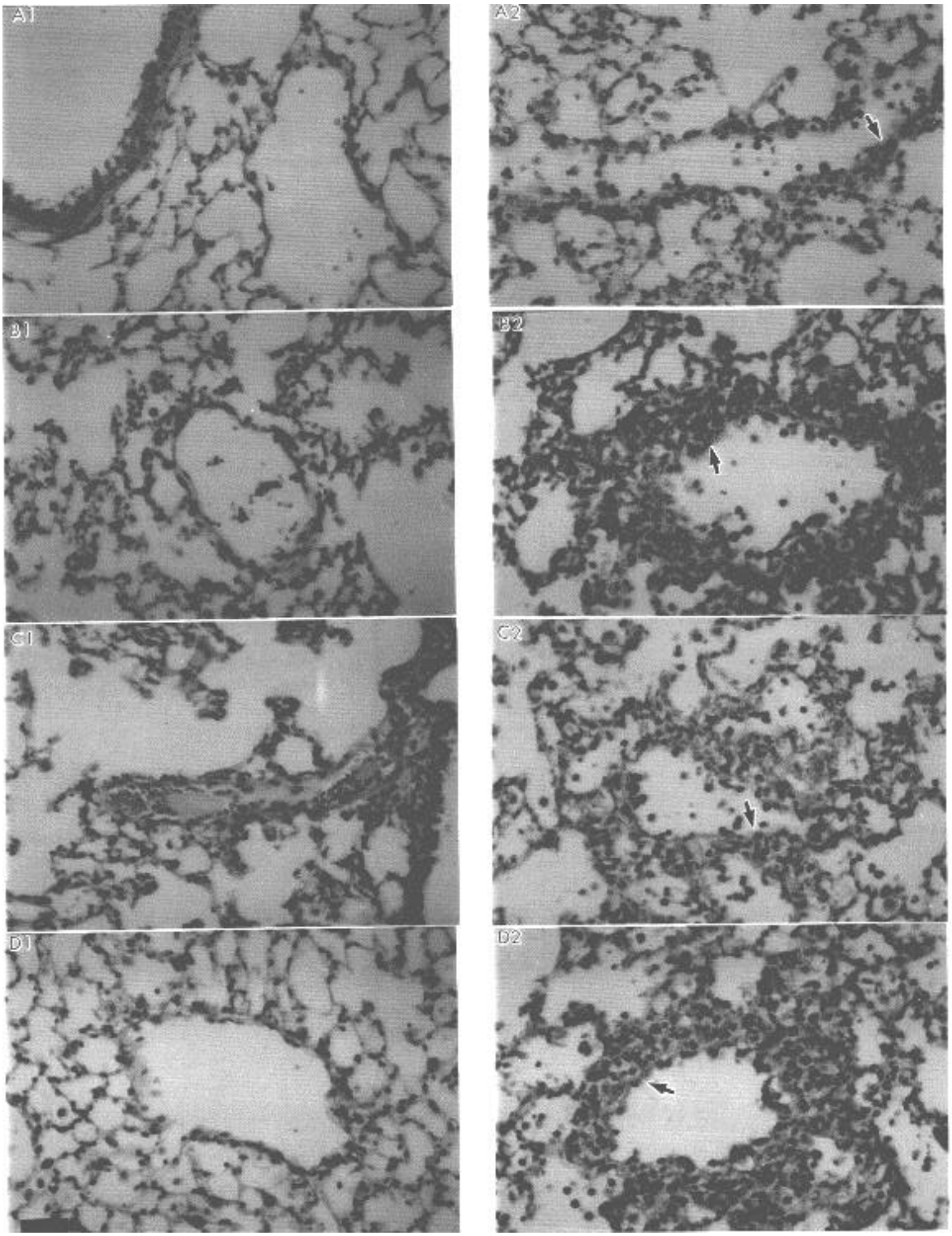
A1
A2
B1
B2
C1
C2
D1
D2

Figure 4.2: Histopathological studies on lung in mice: Lungs from wild-type (A1, A2), perforin-KO (B1, B2), *gld* (C1, C2) and *lpr* (D1, D2) treated with PBS (A1, B1, C1, D1) or IL-2 (A2, B2, C2, 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.

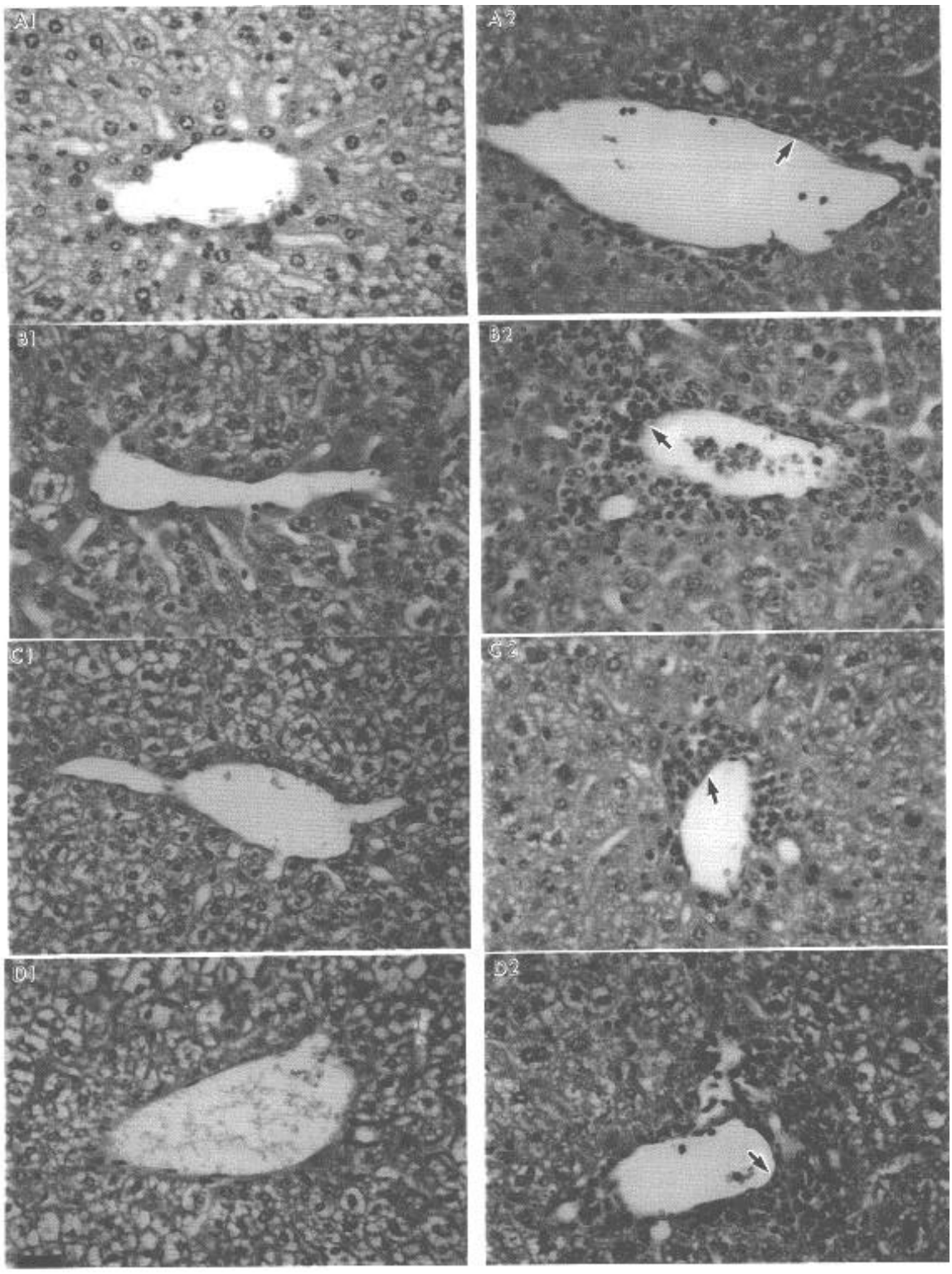
A1
A2
B1
B2
C1
C2
D1
D2

Figure 4.3: Histopathological studies on liver in mice: Livers from wild-type (A1, A2), perforin-KO (B1, B2), *gld* (C1, C2) and *lpr* (D1, D2) treated with PBS (A1, B1, C1, D1) or IL-2 (A2, B2, C2, 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.

In the lungs and liver, IL-2 treatment led to significant perivascular infiltration in all 4 strains of mice, consisting mainly of lymphocytes (Fig 4.2 and 4.3, panels A2, B2, C2 and D2) whereas, PBS-treated groups failed to exhibit significant lymphocytic infiltration (Fig 4.2 and 4.3, panels A1, B1, C1 and D1). The degree of infiltration was also measured by counting the number of lymphocytes infiltrating each vessel and averaging the range for each group (Table III). These results showed that IL-2 treated perforin-KO mice had the highest level of perivascular infiltration followed by *lpr*, *gld* and the wild-type mice (Fig 4.2 and 4.3, Table III). Although the reason why perforin KO, *gld* and *lpr* mice had higher levels of perivascular infiltrations was not clear, these data ruled out the possibility that the decreased VLS as seen in the lungs of perforin-KO mice and 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 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 lung were performed. As shown in Fig 4.4 (panel 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 4.4, panel A2). The perforin KO mice injected with IL-2 had the least amount of damage to the endothelial cells (Fig 4.4, panel B2). Many endothelial cells appeared to be morphologically normal as observed

Table III Perivascular Infiltrating Lymphocytes from Mice treated with IL-2[a]

	Strain			
Organ	+/+	Peforin-KO	*gld*	*lpr*
Lung[b]	3.17±0.12	5.95±0.38*	3.45±0.23	5.00±0.36*
Liver[c]	2.80±0.12	3.50±0.063*	2.98±0.25	3.12±0.19

[a] Mice were injected with IL-2 to induce VLS and the organs were processed for histopathological studies as described in Fig 4.2 and 4.3. The degree of perivascular infiltration was measured by counting the number of lymphocytes infiltrating each vessel.

[b,c]The number of lymphocytes infiltrating a venule. The data represent the Mean±S.E.M. 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 have been indicated with an asterisk.

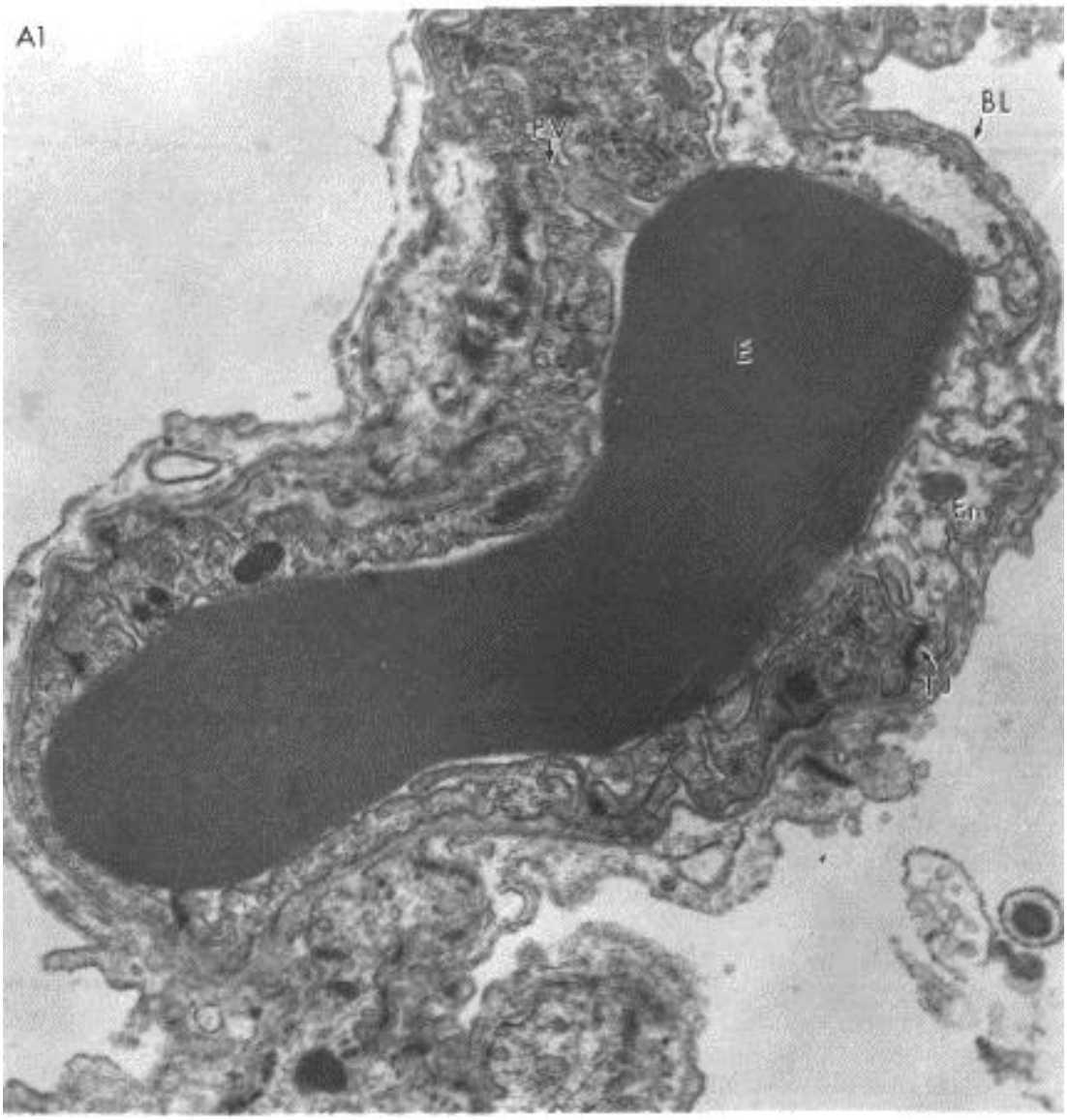

Figure 4.4 (A1): Ultrastructural studies on injury to endothelial cells during VLS
Lung samples from wild-type mice treated with PBS were fixed and studied with an electron microscope. A near cross-section of a capillary has an erythrocyte (E) in the central lumen surrounded by endothelial cells (En) 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 were structurally similar to PBS-treated wild-type mice and therefore not depicted. (Magnification = X16,000)

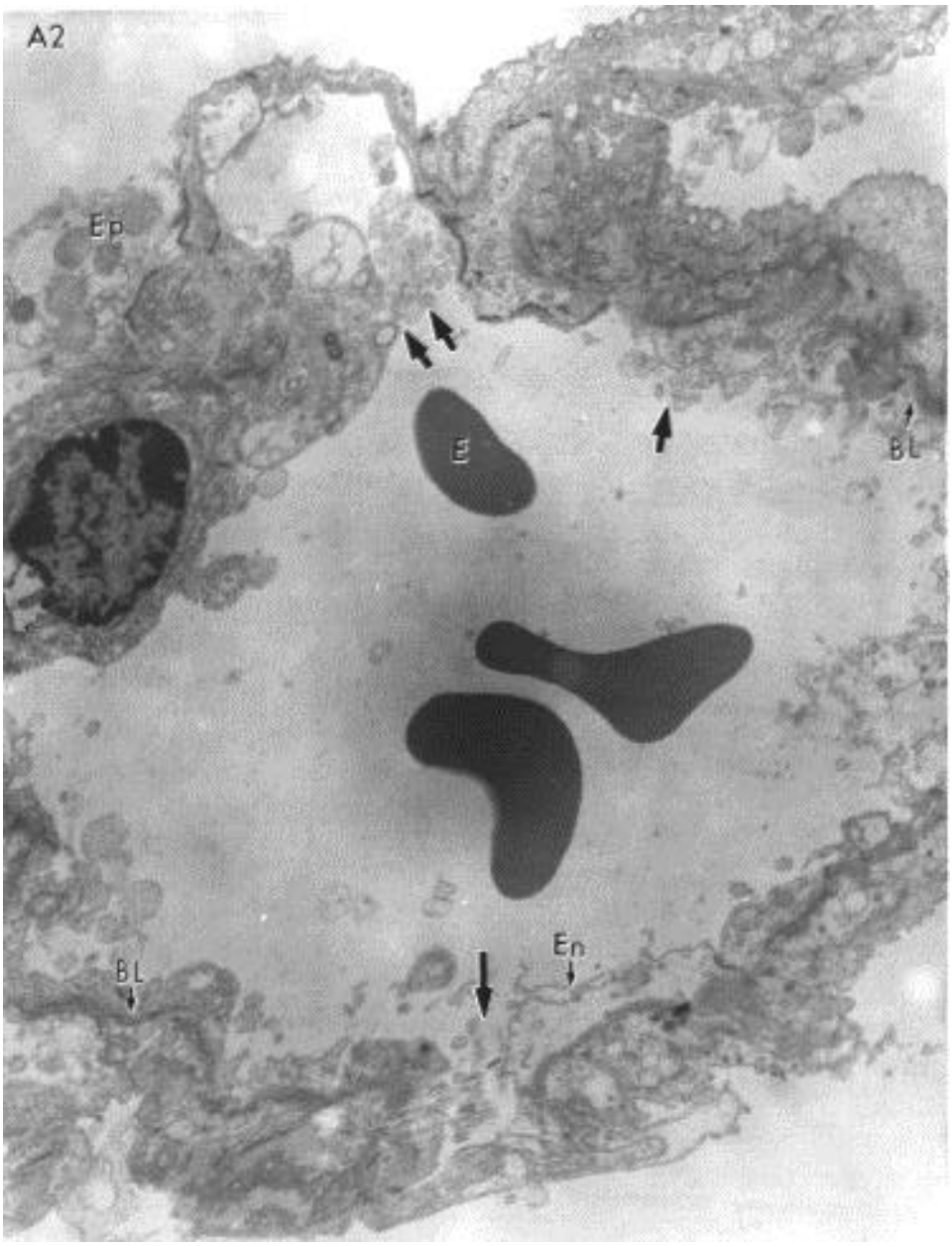

Figure 4.4 (A2): Ultrastructural studies on injury to endothelial cells during VLS
Lung samples from wild-type mice treated with IL-2 were fixed and studied with an electron microscope. Cross section of a capillary with erythrocytes (E) in the lumen. Almost complete destruction of the endothelial cell layer has occurred. Epithelial (Ep) cells of the alveolar air space on the other side of the basal lamina (BL) show extensive damage. (Magnification =X6,600)

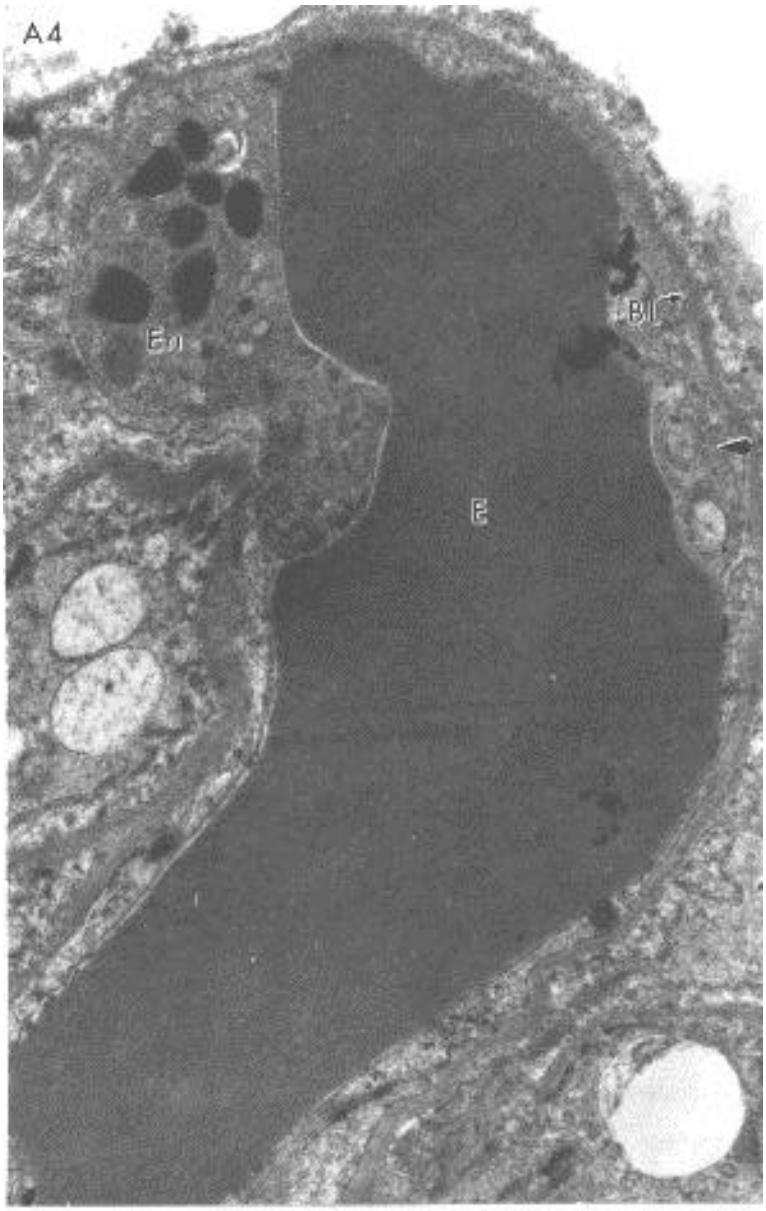

Figure 4.4 (B2): Ultrastructural studies on injury to endothelial cells during VLS
Lung samples from Perforin-KO mice treated with IL-2 were fixed and studied with an electron microscope. Erythrocytes (E) are moving through the lumen of the capillary. The endothelial cells are still pressed against the basal lamina (BL) but show indication of cellular deterioration. This is indicated by the formation of membranous circular structures (arrows) and apoptotic bodies formation 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, intact basal lamina and the lack of cellular debris and membrane fragments. (Magnification = X 20,400)

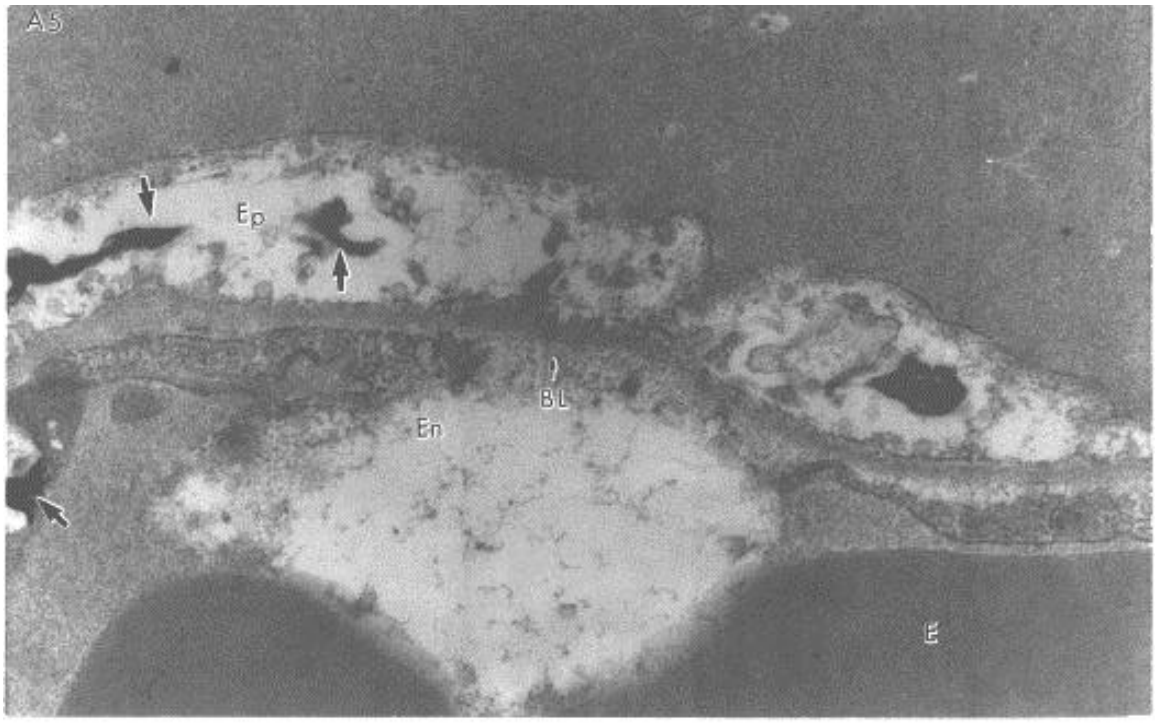

Figure 4.4 (B3): Ultrastructural studies on injury to endothelial cells during VLS
Lung samples from *lpr* mice treated with IL-2 were fixed and studied with an electron microscope. Cell damage to the endothelial and epithelial cells separated by the basal lamina is evident. Cell organization and structures have been lost leaving cell remnants. There are 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 = X 50,000)

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 4.4, panel 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 4.4, panel 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 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 Fig 4.5 indicated that perforin-KO (panel B) and *gld* (panel C) mice exhibited minimal spontaneous cytotoxicity against YAC-1 targets. However, after IL-2 administration, they exhibited significant increase in cytotoxicity, although such lytic activity was less than that seen in the wild-type mice (panel A). These data indicated that IL-2 upregulates 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

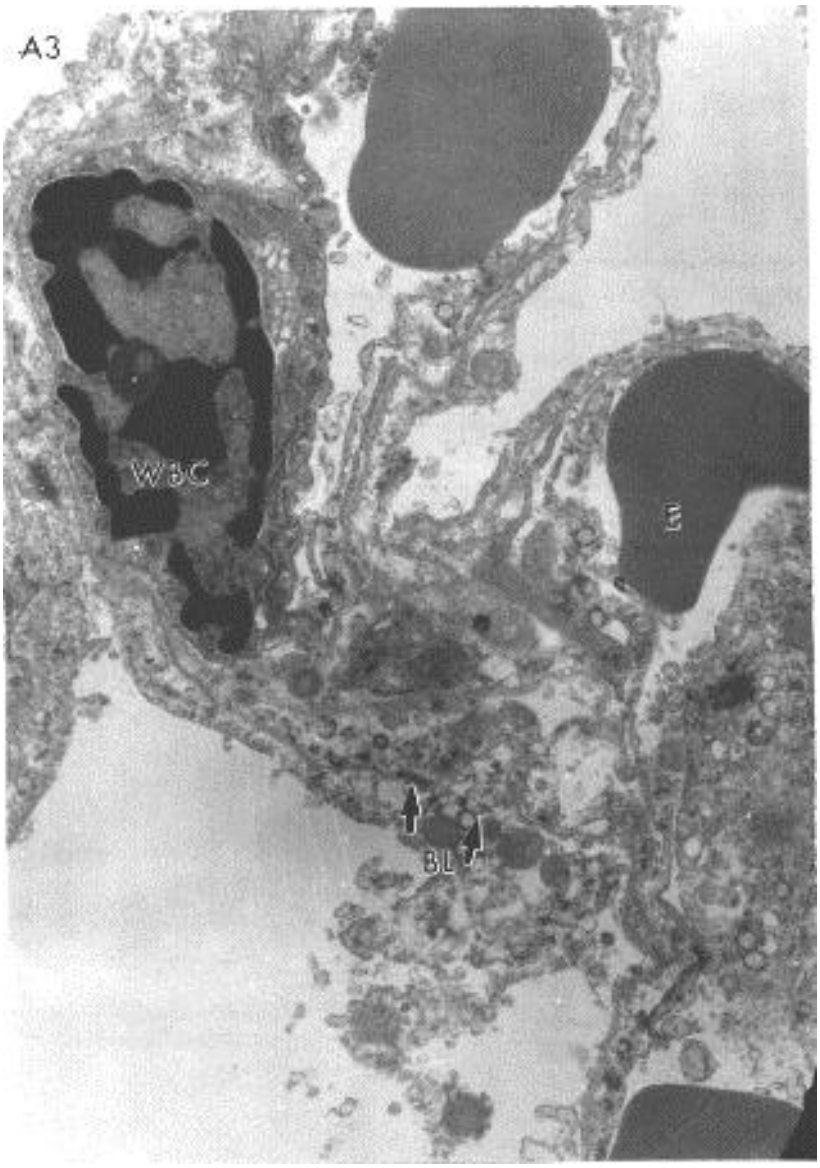

Figure 4.4 (B1): Ultrastructural studies on injury to endothelial cells during VLS Lung samples from*gld* mice treated with IL-2: Very extensive damage has occurred resulting in cell death within the blood vessel and outside the basal lamina (BL). Cellular cytoplasm of the endothelial cells and the epithelial 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 which are involved in the transport of fluids

(arrow) (Magnification = X12,600).

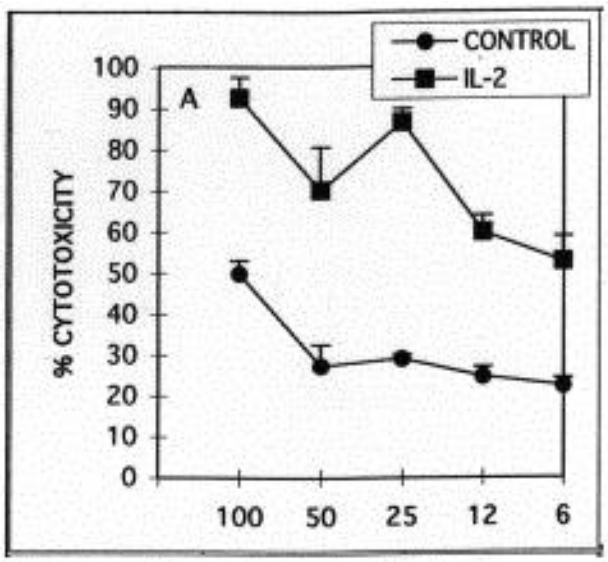

Figure 4.5A: LAK activity in mice undergoing VLS against YAC-1cell target: Wild-type mice were injected with IL-2 as described in Fig. 4.1. On day 4, spleen cells were passed over nylon wool to enrich T cells and tested for cytotoxicity against ^{51}Cr-labelled YAC-1 targets. The data indicate mean percent cytotoxicity triplicate cultures±S.E.M.

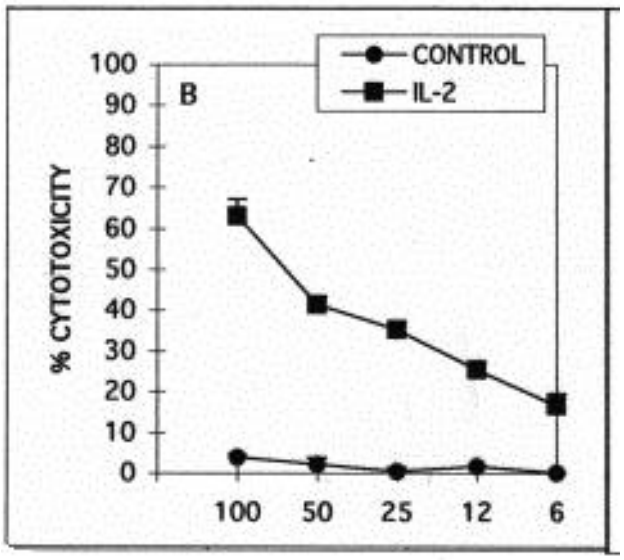

Figure 4.5B: LAK activity in mice undergoing VLS against YAC-1 cell target: Perforin-deficient mice were injected with IL-2 as described in Fig. 4.1. On day 4, spleen cells were passed over nylon wool to enrich T cells and tested for cytotoxicity against ^{51}Cr-labelled YAC-1. The data indicate mean percent cytotoxicity triplicate cultures±S.E.M.

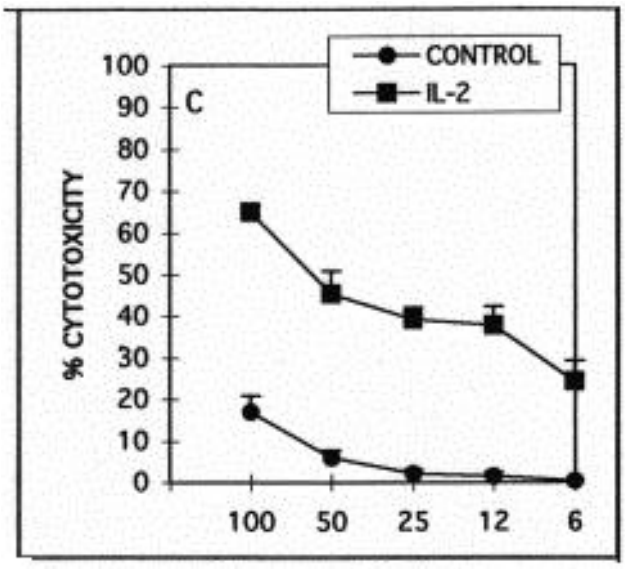

Figure 4.5C: LAK activity in mice undergoing VLS against YAC-1 cell target: *gld* mice were injected with IL-2 as described in Fig. 4.1. On day 4, spleen cells were passed over nylon wool to enrich T cells and tested for cytotoxicity against ^{51}Cr-labelled YAC-1 targets. The data indicate mean percent cytotoxicity triplicate cultures±S.E.M.

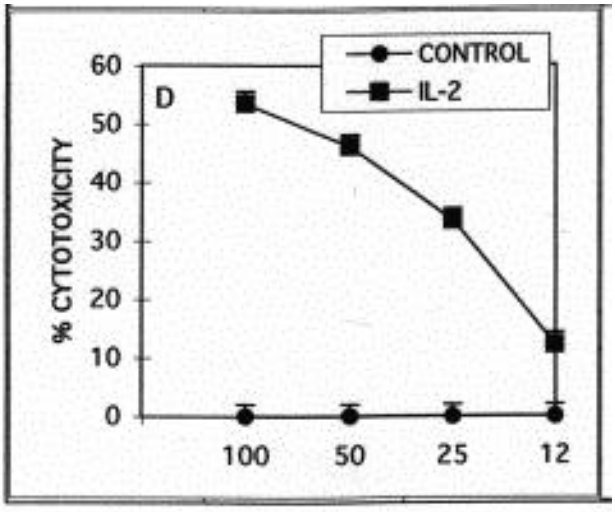

Figure 4.5D: LAK activity in mice undergoing VLS against endothelial cell targets: Wild-type mice were injected with IL-2 as described in Fig. 4.1. On day 4, spleen cells were passed over nylon wool to enrich T cells and tested for cytotoxicity against ^{51}Cr-labelled endothelial cell line, TME-3H3. The data indicate mean percent cytotoxicity triplicate cultures±S.E.M.

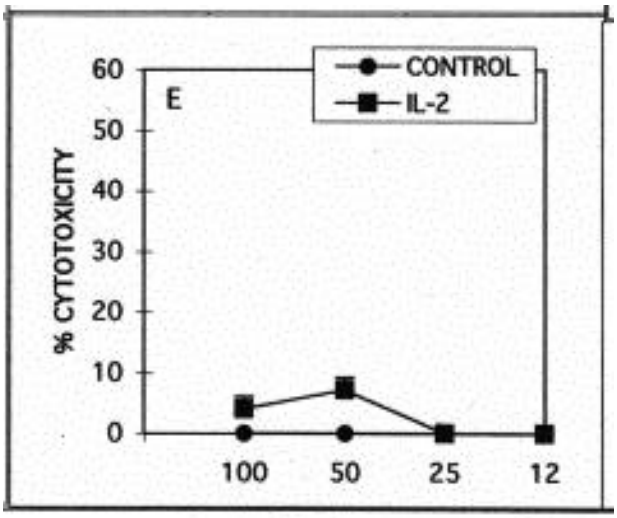

Figure 4.5E: LAK activity in mice undergoing VLS against endothelial cell targets: Perforin-KO mice were injected with IL-2 as described in Fig. 4.1. On day 4, spleen cells were passed over nylon wool to enrich T cells and tested for cytotoxicity against ^{51}Cr-labelled endothelial cell line, TME-3H3. The data indicate mean percent cytotoxicity triplicate cultures±S.E.M.

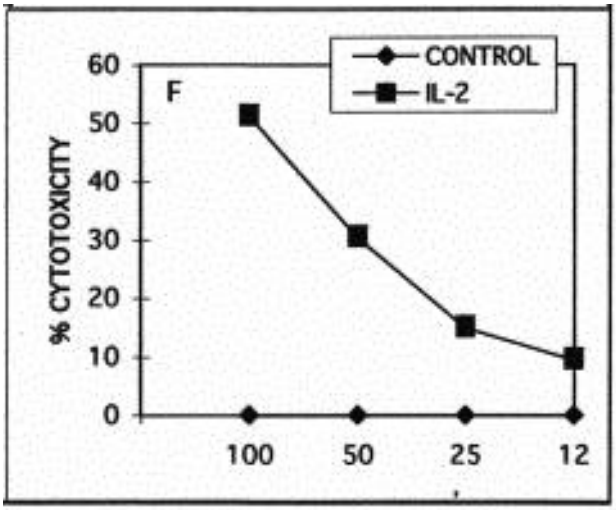

Figure 4.5F: LAK activity in mice undergoing VLS against YAC-1 and endothelial cell targets: *gld* mice were injected with IL-2 as described in Fig. 4.1. On day 4, spleen cells were passed over nylon wool to enrich T cells and tested for cytotoxicity against ^{51}Cr-labelled endothelial cell line, TME-3H3. The data indicate mean percent cytotoxicity triplicate cultures±S.E.M.

endothelial cell line, TME-3H3 (13). The data shown in Fig 4.5 indicated that wild-type (panel D), perforin-KO (panel E) and *gld* (panel F) mice failed to exhibit spontaneous cytotoxicity of endothelial cells. However, IL-2 induced LAK cells from wild-type mice mediated highest level of cytotoxicity followed by *gld* and perforin-KO mice. These data correlated well with the VLS results (Fig 4.1) in which perforin-KO mice were found to exhibit marked decrease in VLS and *gld* mice only 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 4.6). However, the endothelial cell line expressed lower levels of Fas and only 42% of the cells expressed Fas when compared to the thymocytes from wild-type mice which expressed higher levels. This may explain why endothelial cell lysis was more dependent on perforin rather than FasL as seen in Fig 4.5.

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 (Seth et al., 1991; Rosenstein et al., 1986; Tan et al., 1993; Rosenberg, 1985). 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 (Hammond-McKibben et al., 1995, Rosenstein et al., 1986 Galandrini et al., 1993). We therefore investigated whether IL-2 treatment would upregulate CD44 expression in T cells. To this effect, wild-type, perforin-KO, *gld* and *lpr* mice injected with IL-2 as described before, were sacrificed and purified T cells from the spleens were stained using mAbs against CD44 and the cells were analyzed flow cytometrically.

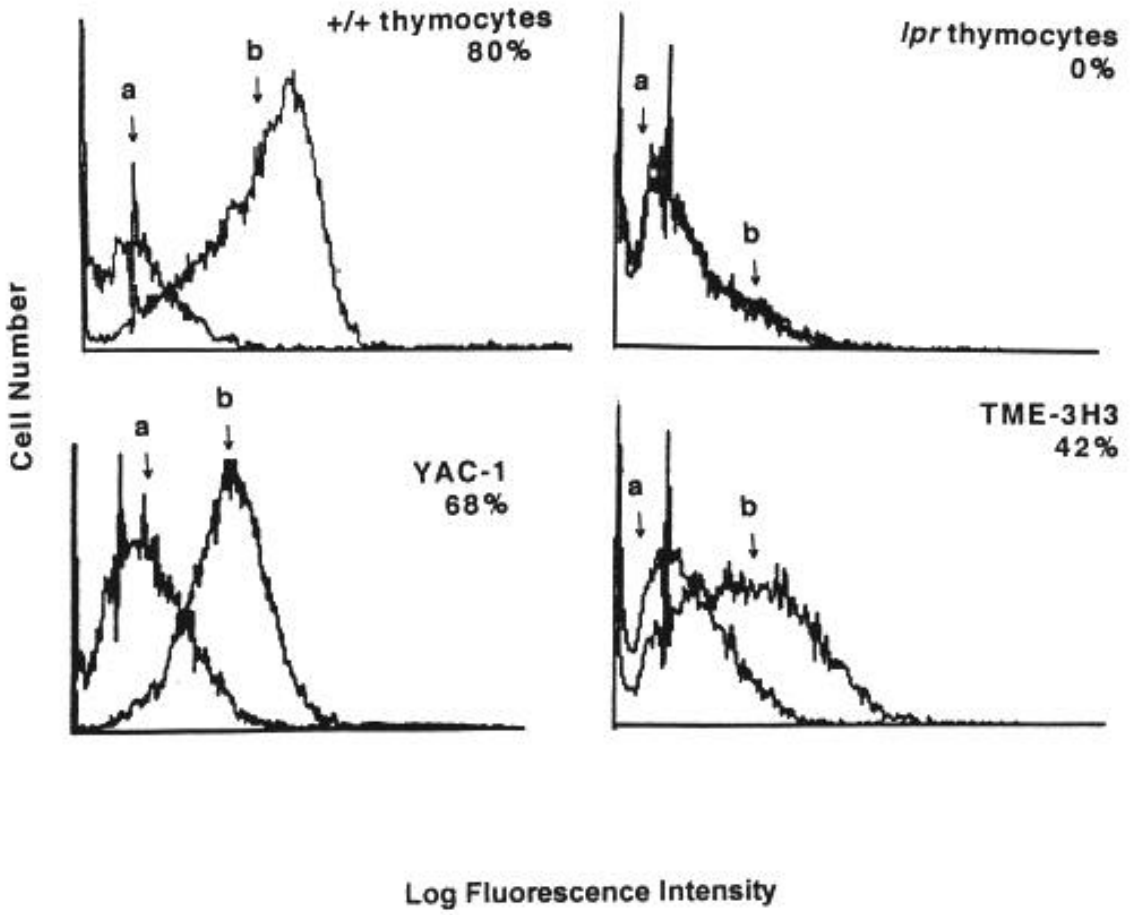

Figure 4.6: Fas expression in YAC-1 and endothelial cell line, TME-3H3. Cells were stained with Jo2 mAbs against Fas followed by FITC-conjugated $F(abí)_2$ fragment goat anti-syrian hamster IgG (b). Negative controls (a) consisted of cells stained with FITC-conjugated $F(abí)_2$ fragment goat anti-syrian hamster IgG alone. The cells were analyzed flow cytometrically.

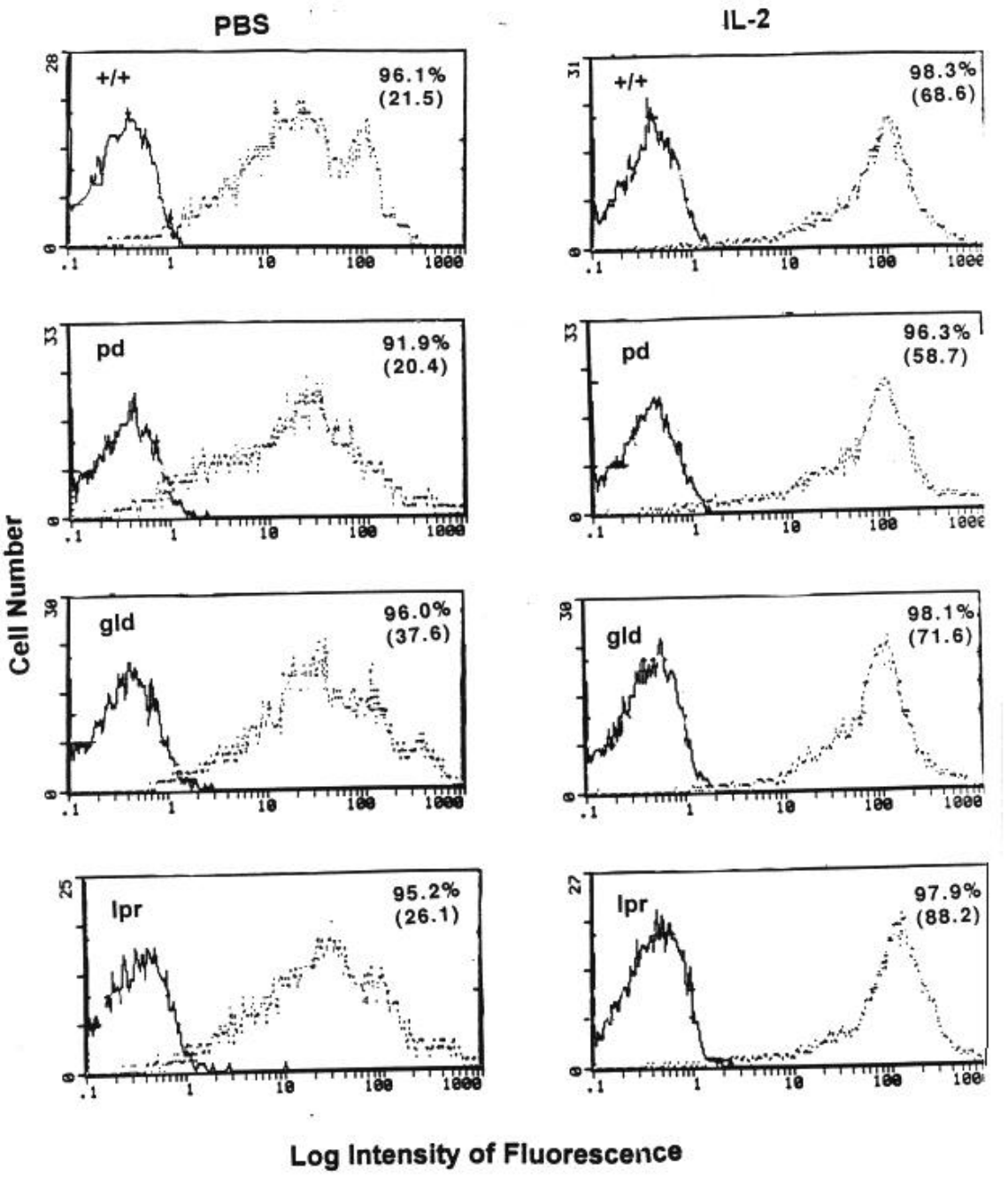

Figure 4.7: Effect of IL-2 administration on CD44 expression: Wild-type (+/+), perforin-KO (pd), *gld* and *lpr* mice injected with IL-2 as described in Fig. 4.1 were sacrificed and purified T cells from the spleens were stained using PE-conjugated mAbs against CD44 and the cells were analyzed flow

cytometrically. The solid histogram represents autofluorescence and the broken histogram represents cells stained with PE-antiCD44. The percent positive cells expressing CD44 and the mean intensity of fluorescence as shown in parenthesis have been depicted in each histogram.

The data shown in Figure 4.7 indicated that in IL-2 administered mice, there was significant upregulation in CD44 expression on T cells in all groups of mice tested when compared to the controls. This was evident from the increase in mean intensity of fluorescence on T cells following IL-2 administration. It should be noted that IL-2 treatment upregulated the expression of LFA-1 and L-selectin and downregulated 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 effect of IL-2 treatment on $CD4^+$ T and $CD8^+$ T cells in the periphery and to compare the level 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 IV 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.

Table IV Expression of CD4 and CD8 in the Spleen after IL-2 treatment[a]

	PBS		IL-2	
Strain	**CD4**	**CD8**	**CD4**	**CD8**
Wild-type	28.6±1.3	14.2±3.7	20.2±0.09*	24.2±1.1*
PD	21.2±0.18	17.6±0.82	16.87±0.62*	22.4±0.05*
GLD	17.1±1.2	15.8±0.62	12.78±0.93*	24.8±0.82*
LPR	20.7±0.81	19.45±1.9	14.15±0.43*	36.15±1.9*

[a] Mice were injected with PBS or IL-2 as described in Fig. 4.1. The spleen cells were stained for CD4 and CD8 markers and cells were analyzed flow cytometrically. The data are depicted as mean percent positive cells±S.E.M. obtained from 4-5 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 ($p<0.05$) were indicated with an asterisk.

Discussion

Administration of IL-2, although promising to treat certain types of cancer, triggers severe side effects in patients who develop a capillary leak syndrome resulting in anasarca and multi-organ system dysfunction (Lotze et al., 1986; Maling et al., 1973). 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 upregulates 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 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 density of homing molecules, including CD44, following IL-2 treatment. These data together suggested that IL-2 upregulates expression of CD44 thereby facilitating the migration of LAK cells to various organs. Furthermore, IL-2 also upregulates 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 (Li et al., 1994). 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 leak in acute inflammatory lesions are triggered by mediators of immediate hypersensitivity such as histamine, serotonin, and bradykinin (Willoughby, 1973). 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 (Li et al., 1994). Lymphocytes are also known to produce a variety of mediators that increase the vascular permeability (Sobel and Lagrue, 1980; Damle and Doyle, 1987). However, the current study demonstrates that IL-2 induced vascular leak may result from the involvement of cytolytic effector molecules, perforin and FasL.

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 (Damle et al., 1987). Also, the toxicity associated with IL-2 therapy has been shown to decrease after depletion of NK cells *in vivo* (Quinn et al., 1995). 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 (Hammond-McKibben et al., 1995). Also, 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 (Hammond-McKibben et al., 1995). Moreover, methotrexate was found to attenuate pulmonary vascular leak by preventing the proliferation of NK/LAK cells and also by inhibiting leukocyte binding to endothelium (Ohkobo et al., 1991). Similarly, dextran sulfate which blocks leukocyte-endothelial adhesion attenuates IL-2 induced VLS (Dubinett et al., 1994). 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 to the same extent as 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 of the 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 (Quinn et al., 1990; Thijs et al., 1990). In addition, the VLS may also result from complement activation (Rabinovici et al, 1994; Harlan et al., 1981).

In the current investigation the histopathological 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 (Martin et al., 1984; Diener et al., 1985; Anderson et al., 1985). The fact that IL-2 treated perforin-KO, *gld* and *lpr* mice demonstrated significant perivascular infiltration of lymphocytes, in fact greater than 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 upregulation 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 cytolytic effector molecules rather than lymphocyte migration and homing. Our data are consistent with previous histologic studies which showed lymphoid cell infiltration in lung, liver, kidney and heart following IL-2 therapy (Fujita et al., 1994;). 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 4-6 weeks of age during which time, they have similar proportions of T cells, as shown in our earlier studies on the thymus (Kakkanaiah et al., 1990). 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 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 (Hammond-McKibben et al., 1995; Rosenstein et al., 1986; Galandrini et al., 1993; Tan et al., 1993; Rosenberg et al., 1985; Sconocchia et al., 1994). 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 fragments or soluble hyaluronate (unpublished data). 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 the endothelial cell injury.