NK Cells and Polymorphonuclear Neutrophils Are Both Critical for IL-2-Induced Pulmonary Vascular Leak Syndrome

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NK Cells and Polymorphonuclear Neutrophils Are Both Critical for IL-2-Induced Pulmonary Vascular Leak Syndrome

Eric Assier, Valérie Jullien, Jean Lefort, Jean-Louis Moreau, James P. Di Santo, B. Boris Vargaftig, Jose R. Lapa e Silva, and Jacques Thèze

The mechanism of IL-2-induced vascular leak syndrome (VLS) is still poorly understood. Cells of both innate and adaptive immune systems have been implicated, but no definitive conclusions have been reached concerning their respective roles. In this study we report a new mouse model of IL-2-induced pulmonary VLS used to obtain a detailed analysis of the early events (sequestration of polymorphonuclear neutrophils and bronchoconstriction) and late events (modifications in the cell and protein content of bronchoalveolar lavages, followed by edema) that characterize this lung injury. This model and knockout animals are used to reconsider the importance of the different leukocyte lineages in early and late events. Recombinase-activating gene 2−/− mice are used to demonstrate that adaptive lymphocytes, including NK T cells, are not required for pulmonary VLS induction. By contrast, results obtained with newly described recombinase-activating gene 2−/−/IL-15−−/− mice indicate that NK cells play a key role in both early and late events. In parallel, polymorphonuclear neutrophil depletion is used to evaluate the contributions made by these cells to the late alterations occurring in the lung. Furthermore, when used in combination with inhibition of NO synthase, granulocyte depletion was completely effective in protecting mice from the late events of IL-2-induced pulmonary VLS. Together our results indicate that both NK and PMN cells play a central role in the late events of IL-2-induced VLS. The Journal of Immunology, 2004, 172: 7661–7668.

Human rIL-2 (1, 2), used alone or in combination with the adoptive transfer of lymphokine-activated killer cells (LAK),3 has been shown to result in tumor regression in 25–30% of patients with metastatic melanoma or renal carcinoma (3, 4). Intermittent IL-2 therapy is also used in HIV-infected patients in combination with highly active antiretroviral therapy and restores sustained, protective levels of CD4+ T lymphocytes (5–9). However, the use of IL-2 is restricted by its dose-dependent toxicity, which manifests particularly as acute respiratory failure and hemodynamic instability (hypotension). Both these disorders are associated with vascular leak syndrome (VLS), which is characterized by increased vascular permeability and decreased microcirculatory perfusion, leading to interstitial edema and multiple organ failure within 2–24 h of IL-2 administration (10, 11). Human lungs are particularly susceptible to this complication, and the results of animal studies using human IL-2 are remarkably similar to those reported in humans, although organ sensitivity may vary with animal species (12, 13). At present there is no specific therapy for VLS. However, at the experimental level, it has been shown that inhibitors of NO synthase (NOS) are able to improve IL-2-induced VLS (14–16).

By contrast, results obtained with newly described recombinase-activating gene 2−/−/IL-15−−/− mice indicate that NK cells play a key role in both early and late events. In parallel, polymorphonuclear neutrophil depletion is used to evaluate the contributions made by these cells to the late alterations occurring in the lung. Furthermore, when used in combination with inhibition of NO synthase, granulocyte depletion was completely effective in protecting mice from the late events of IL-2-induced pulmonary VLS. Together our results indicate that both NK and PMN cells play a central role in the late events of IL-2-induced VLS. The Journal of Immunology, 2004, 172: 7661–7668.

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model suitable for studying IL-2-induced acute and chronic lung toxicity. We also investigated whether IL-2-induced VLS correlates with functional modifications in the airways. Using KO animals, we were able to study directly the involvement of the different lymphocyte subsets in VLS development. We demonstrated that B, T, and NK T lymphocytes are not required for pulmonary VLS. By contrast, our results clearly indicate that NK cells are involved in both the early and late phases of the syndrome. Furthermore, after granulocyte depletion, we showed that PMN are mainly associated with the development of the late response. Together, our data indicate that pulmonary VLS in the mouse is dependent on cells of the innate branch of the immune system.

Materials and Methods

Induction of VLS in mice

Mice were injected i.v. with 10 μg of IL-2 (180,000 IU Aldeleukin; Chiron, Amsterdam, The Netherlands). Control mice were injected with NaCl (0.9%). Acute responses were evaluated 2 and 24 h after the first injection. Late responses were measured after four daily i.v. injections, and the different biological parameters were analyzed at the times indicated below.

Six- to 9-wk-old male mice were used. C57BL/6 and recombinase-activating gene 2 (RAG2)/−/− mice were purchased from Centre National de la Recherche Scientifique-Centre de Distribution, Typage et Archivage Animal (Orlés, France). RAG2+/−/IL-15−/− (31) were bred at the Pasteur Institute with standard laboratory feed and water ad libitum.

Evaluation of bronchopulmonary hyper-reactivity

Airway resistance was measured by barometric plethysmography using unrestrained conscious mice placed in a plethysmographic chamber (Boxux Electronics, Sharon, CT). Respiratory parameters in each animal were measured in response to the aerosolization of 100 mM methacholine in sterile 0.9% NaCl for 20 s. Responsiveness to methacholine was determined 24 h after the last NaCl/IL-2 i.v. injection. Resistance was expressed as enhanced pause (32). The means of three results per minute for 10 min were calculated. The results were presented as cumulated areas under the curve (AUC), giving a quantitative representation of the results obtained over the 10 min of the test.

Myeloperoxidase (MPO) activity of lung

PMN accumulation in the lung was measured by MPO activity, a method commonly used to quantify this parameter in the lung (33, 34). We also confirmed by histopathology that MPO activity followed PMN adherence to vascular endothelium. This quantitative method was used to evaluate the total number of neutrophils sequestered in the lungs.

After bronchoalveolar lavage, lungs were perfused with 5 ml of Isoton (Beckman-Diagnostics, Krefeld, Germany) injected into the right ventricle, then were stored at −20°C pending MPO activity measurements. Briefly, lung tissues were homogenized using a Potter homogenizer (Potter-Elvejem glass homogenizer; Thomas Instruments, Philadelphia, PA) in 1 ml of PBS at room temperature. Homogenates were centrifuged at 10,000 × g for 10 min at 4°C. Cell pellets were suspended in 1 ml of PBS-hexadecyl trimethyl ammonium bromide (0.5%) and 1 mM EDTA before Potter lysis for 10 min at 4°C. After the supernatant was diluted in 950 μl of 1% sodium azide (Sigma-Aldrich), 100 μl of 0.5% O-dianisidine dihydrochloride (Sigma-Aldrich, Saint Quentin Fallavier, France), and 100 μl of 0.05% H2O2 (Sigma-Aldrich). After 15 min at 37°C, the reactions were stopped with 100 μl of 1% sodium azide (Sigma-Aldrich). Absorbance was determined on a spectrophotometer at 460 nm.

Analysis of bronchoalveolar lavage fluid (BALF) content

Alterations in the cell and protein contents of the BALF were studied using conventional procedures. Briefly, mice were deeply anesthetized with 0.5 ml of urethane (1.5 g/kg; Sigma-Aldrich) injected i.p. The trachea was cannulated, and the airways were rinsed five times with 0.5 ml of PBS. The fluid was withdrawn and stored on ice. Total cell counts were evaluated in 400-μl samples diluted in 20 ml of Isoton (Beckman Diagnostics) plus Zap-Oglobin (Beckman Coulter, Villepinte, France) to lyse the cells. Total nuclei were counted using an electronic counter (Coulter Electronics, Luton, U.K.). Cytosine preparations of the BALF were stained with Diff-Quick (Dade Behring, Marburg, Germany). A differential count of 200 cells was performed using standard morphological criteria.

The remaining BALF was centrifuged, and the supernatant was collected and stored at −20°C for total protein measurements. Bradford stain was added to 50 μl of the supernatant diluted in 950 μl of H2O plus 250 μl of Bradford solution (Bio-Rad, Ivry sur Seine, France), and absorbance was measured at 595 nm (UVIKON 860 spectrophotometer; Kontron Instruments, Zurich, Switzerland). Protein concentrations were calculated from a standard OVA curve.

Granulocyte depletion

Vinblastine sulfate (Velbe; Lilly, Saint Cloud, France) or rat RB6-8c5 mAbs were used to deplete granulocytes in vivo. Rat RB6-8c5 mAbs were purified from ascitic fluid by ammonium sulfate precipitation and dialysis against PBS. Rat control IgG was obtained from Sigma-Aldrich.

Granulocyte depletion by rat RB6-8c5 mAbs was achieved as previously described (35). In this study mAbs (50 μg, i.v.) were administered 16 h before the first IL-2/NaCl injection, followed by a second injection (25 μg of mAbs i.p.) between the second and third IL-2/NaCl injections. Mice injected with the same dose of rat IgG were used as controls. Vinblastine sulfate (5 mg/kg) was injected i.v. 3 days before the first IL-2/NaCl injection. An additional 1.25 mg/kg i.v. was administered between the second and third IL-2/NaCl injections. PMN depletion was confirmed by blood counts on slides stained with Diff-Quick.

The effects of granulocyte depletion and NOS inhibition were also evaluated. In this study mice were pretreated with N-nitro-L-arginine methyl ester (L-NAME; Sigma-Aldrich; 12.5 mg/kg s.c.) and returned to baseline after 24 h (0.06 ± 0.02). A similar pattern of analog of arginine and produces nonselective inhibition of NOS isoenzymes.

Edema evaluation

Lung edema was evaluated from the ratio between the weights of wet and dry tissues. Lung weight was immediately after collection were desiccated for at least 3 days at 50°C, and their weights were recorded after three constant values. The data are presented as wet/dry weight ratios for quantification of the water influx.

Data analysis

Two or more separate experiments were performed for each test using groups of five or more animals. Data, expressed as mean ± SEM, were tested by one-way ANOVA. Statistical analyses were performed using SPSS 6.1 software (SPSS, Chicago, IL).

Results

Kinetics of IL-2-induced VLS in C57BL/6 mice

The pathophysiological effects on the lung of repeated IL-2 administrations were assessed (Fig. 1) by selecting several parameters to monitor the early and late responses. As shown in Fig. 1a, the intensity of the bronchoconstrictive response to aerosolized methacholine was significantly enhanced 2 h after the first i.v. injection (AUC, 13.22 ± 2.16 after IL-2 vs 5.82 ± 0.52 after saline). However, this potentiation did not persist when IL-2 was injected daily from days 2 through 4.

Increased PMN influx into the lungs was also noted after each IL-2 injection. PMN sequestration was evaluated by assessing lung MPO activity. This assay has been shown to be reliable and able to quantify PMN accumulation in the lung (33, 34). Preliminary results showed that lung sequestration of PMN commenced 1 h after a single IL-2 injection. This peaked at 2 h and decreased after 3 h (data not shown). Fig. 1b clearly illustrates that MPO activity was increased 2 h after the first IL-2 injection (0.59 ± 0.03) and returned to baseline after 24 h (0.06 ± 0.02). A similar pattern was observed after each IL-2 injection.

By contrast, alterations in the content of BALF and increases in the wet/dry lung weight ratios were observed progressively over time. PMN counts were significantly higher 24 h after the third IL-2 injection (10.439 ± 2.982 vs 200 ± 20 PMN/ml in the saline controls). This was observed in the absence of any significant variation in total cell numbers (Fig. 1, d and e). The increase in BALF protein content became significant 24 h after the third IL-2 injection (16.30 ± 1.3 vs 6.66 ± 0.60 μg/ml in the controls; Fig. 1e).
Finally, the increase in the wet/dry lung weight ratio, expressing edema, also became significant 2 h after the third IL-2 injection (3.90 ± 0.30) and remained augmented after the fourth injection (Fig. 1f).

The persistence of lung injury was monitored after mice were treated with four IL-2 i.v. injections. The animals were sacrificed on day 4, 8, 12, 16, or 20. Fig. 2 shows the results on days 4, 12, and 20. Lung MPO activity, PMN infiltration, and BALF protein content returned to baseline 20 days after the first IL-2 injection. By contrast, the wet/dry lung weight ratio increased significantly over the same period (4.09 ± 0.28 on day 4 vs 4.80 ± 0.06 on day 20), suggesting that this parameter may become IL-2-independent under certain experimental conditions.

Collectively, these experimental conditions may therefore be considered appropriate to study the early and late events of pulmonary VLS. Early events (bronchoconstriction or PMN sequestration) could be analyzed as early as 2 h after an IL-2 injection. Late events, measured by changes in BALF (neutrophil counts and protein concentration), were easily measurable after four IL-2 injections.

**FIGURE 1.** Early and late responses after i.v. injections of IL-2. C57BL/6 mice received daily tail vein injections of 0.9% NaCl or 10 μg of IL-2 (180,000 IU) in a final volume of 0.1 ml. Results were obtained 2 or 24 h after each NaCl/IL-2 injection. a, AUC were calculated from plots obtained on the plethysmograph (Buxco Electronics) after methacholine aerosolization (100 mM, 20 s). Mice were exposed to methacholine 2 h after NaCl or IL-2 injection. b, MPO activity in lung homogenates was determined by spectrophotometry at 460 nm. c, Total cell counts in BALF. BALF was obtained after tracheal cannulation of urethane-anesthetized mice; 2.3 ml of BALF was obtained by successive lavages with PBS. d, BALF neutrophils were identified by staining cytospin preparations with Diff-Quick. e, BALF protein content was measured by Bradford staining. f, Wet/dry lung weight ratios were obtained by comparing lungs weighed immediately after dissection with lungs dried at 50°C for at least 3 days. In this experiment each group of mice comprises seven animals. Bars represent the mean ± SEM. *, p < 0.05 compared with saline controls.

Finally, the increase in the wet/dry lung weight ratio, expressing edema, also became significant 2 h after the third IL-2 injection (3.90 ± 0.30) and remained augmented after the fourth injection (Fig. 1f).
Effect of the absence of mature B, T, and NK T lymphocytes or NK cells on IL-2-induced VLS

Tests were conducted to determine whether the administration of IL-2 induced pulmonary VLS in RAG2\textsuperscript{-/-} mice, which lack mature B and T lymphocytes as well as NKT cells. Early events were comparable in both C57BL/6 and RAG2\textsuperscript{-/-} mice, as assessed by MPO activity (0.313 ± 0.04 vs 0.380 ± 0.02; Fig. 3a). Late events, measured by alterations in BALF PMN content (12,995 ± 1,100 vs 14,356 ± 1,700/ml) and protein concentration on day 4 (22.2 ± 0.7 vs 16.1 ± 0.4 μg/ml) were also very similar in the control and RAG2\textsuperscript{-/-} mice (Fig. 3b). The background responses in C57BL/6 and RAG2\textsuperscript{-/-} mice were not significantly different (p > 0.3). Therefore, our results clearly demonstrate that mature B, T, and NK T lymphocytes are not required for IL-2-induced pulmonary VLS.

RAG2\textsuperscript{-/-}/IL-15\textsuperscript{-/-} mice, which lack mature lymphocytes (T, B, and NK cells), were then used to determine whether NK cells are involved in the induction of pulmonary VLS. In these animals, hyper-reactivity to methacholine decreased significantly after a single IL-2 injection (decrease, 2.10 ± 1.22 vs 7.51 ± 2.23; Fig. 3c). Similarly, the sequestration of PMN was reduced, as assessed by MPO (Fig. 3d). As expected, BALF cell counts, BALF neutrophil counts, and protein concentration were not significantly different between the C57BL/6 and RAG2\textsuperscript{-/-}/IL-15\textsuperscript{-/-} mice 2 h after an IL-2 injection.

The late responses of C57BL/6 and RAG2\textsuperscript{-/-}/IL-15\textsuperscript{-/-} mice to repeated IL-2 administrations were then evaluated. VLS late events were substantially diminished in the absence of NK cells; PMN recruitment to BALF was greatly reduced after four IL-2 injections (reduction, 419 ± 173 vs 10,800 ± 379 PMN/ml; Fig. 3e). Protein leakage was also lower on day 4 in RAG2\textsuperscript{-/-}/IL-15\textsuperscript{-/-} mice than in C57BL/6 mice (reduction, 1.47 ± 0.58 vs 9.23 ± 0.3). Therefore, our results clearly demonstrate that mature B, T, and NK T lymphocytes are not required for IL-2-induced pulmonary VLS.

![FIGURE 3](image-url). Comparison of IL-2-induced VLS in C57BL/6 and RAG2\textsuperscript{-/-} mice. Mice received daily tail vein injections for four days with 0.9% NaCl or 10 μg of IL-2 and were sacrificed 2 h after the last injection. Background responses of C57BL/6 and RAG2\textsuperscript{-/-} mice did not differ significantly (p > 0.3). a, MPO activity of lung homogenates; b, BALF total cell counts; c, BALF neutrophil counts identified by staining cytospin preparations with Diff-Quick; d, BALF protein content measured by Bradford staining. In this experiment five RAG2\textsuperscript{-/-} mice and six C57BL/6 animals were used per group. Bars represent the mean ± SEM. *, p < 0.05 compared with saline controls. Background values and IL-2 responses were not significantly different between the C57BL/6 and RAG2\textsuperscript{-/-} mice.

![FIGURE 4](image-url). Comparison of VLS induced by a single IL-2 injection in C57BL/6 and RAG2\textsuperscript{-/-}/IL-15\textsuperscript{-/-} mice. Analysis of the early response after a single injection with 0.9% NaCl or 10 μg of IL-2 (180,000 IU). Mice were sacrificed 2 h postinjection. Results shown correspond to the difference between the IL-2 and NaCl values. The background response of C57BL/6 mice is given in Fig. 3. Background responses of RAG2\textsuperscript{-/-}/IL-15\textsuperscript{-/-} mice were comparable and did not differ significantly (p > 0.3). a, AUC as in Fig. 1; b, MPO activity of lung homogenates; c–e, cell and protein contents of BALF. In this experiment five RAG2\textsuperscript{-/-}/IL-15\textsuperscript{-/-} mice and six C57BL/6 mice were used per group. Bars represent the mean ± SEM. *, p < 0.05 compared with C57BL/6 mice.
Comparison of VLS induced by four IL-2 injections in C57BL/6 and RAG2−/−/IL-15−/− mice. Analysis of the late response after four injections of 0.9% NaCl or 10 μg of IL-2 (180,000 IU). Mice were sacrificed 2 h after the last injection. Results shown correspond to the difference between the IL-2 and NaCl values. The background responses of sacrificed 2 h after the last injection. Results shown correspond to the difference between the IL-2 and NaCl values. The background responses of C57BL/6 and RAG2−/−/IL-15−/− mice did not differ significantly (p > 0.3). a, AUC as in Fig. 1; b, MPO activity of lung homogenates; c–e, cell and protein contents of BALF. In this experiment five RAG2−/−/IL-15−/− mice and six C57BL/6 mice were used per group. Bars represent the mean IL-2-specific saline background response ± SEM. *, p < 0.05 compared with C57BL/6 mice.

Effect of PMN depletion on IL-2-induced VLS

The participation of PMN in VLS was investigated using C57BL/6 mice depleted with either RB6-8c5 mAbs or vinblastine (Fig. 5). Complete depletion was confirmed by blood smears. Bronchopulmonary reactivity did not differ significantly after a single IL-2 injection in depleted mice vs saline controls (data not shown). As expected, MPO activity and BALF PMN content were decreased on day 4 (Fig. 6, a and b). More significantly, protein leakage on day 4 was greatly diminished after four IL-2 injections. Inhibition with vinblastine was 55%, and that with RB6-8c5 was 34% (Fig. 6c). These results clearly indicate that PMN are important for the late events of IL-2-induced VLS.

As PMN depletion alone was only partially effective, the test was repeated in the presence of the NOS inhibitor, L-NAME. NO is known to be involved in VLS, and L-NAME is the only entity able to clearly reduce VLS (14–16). The effects of L-NAME, either alone or in combination with granulocyte depletion, are illustrated in Table I. L-NAME alone had limited effects on PMN mobilization into the BALF (25.9% inhibition on day 4; Table I), but resulted in 60.3% inhibition of protein leakage on day 4. This increased to become almost complete (83% inhibition on day 4) when the drug was combined with PMN depletion (p < 0.05). These results indicate that NO production is also implicated in our model of IL-2-induced pulmonary VLS, and that VLS can be entirely prevented when granulocyte depletion is combined with L-NAME treatment.

Discussion

The therapeutic usefulness of IL-2 in cancer and highly active antiretroviral therapy-treated HIV patients can only be enhanced by gaining a more in-depth understanding of VLS, a severe systemic side effect induced by this cytokine. To further examine the pathogenicity of this syndrome, we describe a mouse model that can be used to follow a number of relevant biological parameters...
and identify cell players. This model allowed us to focus our analysis on the early and late events that follow single or multiple injections of IL-2. Early events, such as bronchoconstriction, are intense, but transitory. This is suggestive of desensitization, although the mechanism involved remains to be clarified. By contrast, lung sequestration of PMN occurs rapidly after each IL-2 injection, independently of previous injections. Surprisingly, therefore, PMN sequestration did not lead to an increase in methacholine sensitivity in our model. Late events observed only after four IL-2 injections were mainly measured by changes in BALF. The total number of cells in BALF remained stable, whereas neutrophil counts increased significantly, as did protein concentrations. This was manifest as lung edema. This injury was critical and lasted for a considerable period (3 wk) without any further IL-2 injections. As the mouse lung is very sensitive to VLS, we believe that our model will be of considerable use in the study of IL-2-induced VLS.

RAG2−/− strain mice developed lung VLS comparable to their congenic C57BL/6 strain in terms of early and late events and the intensity of the responses measured. The possibility that endothelial cells from RAG2−/− mice are resistant to VLS or that this strain develops compensatory mechanisms is unlikely. Therefore, our results clearly establish that T lymphocytes are not involved in VLS induction and for the first time extend the results to B and NK cells. Previous studies showed that depletion was induced by RB6–8c5 Abs. Previous studies remained inconclusive in this matter. The observation that depletion of asialoganglioside-GM1-positive cells reduced IL-2-induced VLS did not exclude the participation of CD8 lymphocytes (25, 26). Similarly, IL-2 toxicity was shown to be reduced after mice were treated with mAb against NK 1.1 Ag (24). However, this last study could not exclude a role for NK T cells, whereas in our case the comparison of the results obtained with C57Bl/6 and RAG2−/− mice clearly indicates that NK cells are central in the late stage. When NK cells are involved, cell-cell contacts may be required for EC damage, as LAK and NK cell supernatants are unable to induce permeabilization of endothelial cell monolayers in vitro, whereas the same cells are lysed in coculture (11). KO animals were used to demonstrate the role played by NK cells, whereas previously published reports remained inconclusive in this matter. The observation that depletion of asialoganglioside-GM1-positive cells reduced IL-2-induced VLS did not exclude the participation of CD8 lymphocytes (25, 26).

The results obtained with the newly described RAG2−/−/IL-15−/− animals (31) suggest that NK cells are central in IL-2-induced VLS. The absence of NK 1.1+ cells has been verified in the blood, lymph nodes, and spleen of these animals. An additional direct or indirect role for IL-15 cannot be excluded in our studies. However, there is no published evidence that in vivo IL-15 controls the activation of another cell lineage or affects the physiology of endothelial cells. Adoptive transfer of purified NK cells would complement our data. However, these experiments are impossible to perform because NK cells do not survive in IL-15−/− animals (31, 36). We observed that early events were only partially inhibited in the RAG2−/−/IL-15−/− animals. This suggests that at a time when PMN do not play any significant role despite their intense sequestration in the lung, other mechanisms of EC injury are involved. Direct interaction of IL-2 with EC may participate directly in the early stages. The absence of VLS in RAG2−/−/IL-15−/− animals after four IL-2 injections indicates that NK cells are central in the late stages. When NK cells are involved, cell-cell contacts may be required for EC damage, as LAK and NK cell supernatants are unable to induce permeabilization of endothelial cell monolayers in vitro, whereas the same cells are lysed in coculture. KO animals were used to demonstrate the role played by NK cells, whereas previously published reports remained inconclusive in this matter. The observation that depletion of asialoganglioside-GM1-positive cells reduced IL-2-induced VLS did not exclude the participation of CD8 lymphocytes (25, 26).

The role played by PMN in IL-2-induced VLS was also studied. Despite the intense sequestration of PMN that followed an IL-2 injection, the depletion of these cells did not affect early VLS events. By contrast, PMN depletion affected the VLS response induced after four IL-2 injections; protein leakage was inhibited by 53% after vinblastine-induced granulocyte depletion and by 34% when depletion was induced by RB6–8c5 Abs. Previous studies have shown that PMN respond to IL-2 within a few hours, and mild neutropenia has been reported in clinical trials after IL-2 treatment (37). This neutropenia may be explained by increased

<table>
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<th>BALF neutrophils (10³/ml)</th>
<th>D1a</th>
<th>D4b</th>
<th>% Inhibition †</th>
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<td>Ig/NaCl</td>
<td>0.60 ± 0.01</td>
<td>1.44 ± 0.20</td>
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<th>% Inhibition †</th>
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<td>8.57 ± 0.34</td>
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</table>

† Mice were pretreated 24 h before IL-2 with l-Name and/or RB6–8c5, then sacrificed 2 h after a single IL-2 i.v. injection.  
† Mice were pretreated 24 h before the first IL-2 injection with l-Name and/or RB6–8c5, then sacrificed 2 h after the fourth IL-2 i.v. injection.  
† Percent inhibition was calculated vs (Ig/IL-2 – Ig/NaCl) values. Data are expressed as the means ± SEM.  
† p < 0.05 vs saline controls.  
† p < 0.05 vs NaCl/IL-2.
rolling and adherence of PMN to endothelial cells after IL-2 administration, as noted in our histological study (data not shown). Furthermore, PMN may cause indirect endothelial injury through the release of vasoactive mediators such as thromboxane B2 or direct damage by elastase, lysosomal enzymes, and the generation of oxygen free radicals (19). This is supported by the observation that administration of the hydroxyl-radical scavenger, dimethylthiourea, reduces IL-2-induced VLS in sheep (38).

IL-2 has been shown to induce NOS, and NO is an important component involved in the pathogenesis of VLS (39). IL-2 administration also results in elevated titters of endogenous NO that may mediate the hemodynamic abnormalities observed after prolonged IL-2 administration (40, 41). NO synthase inhibitors attenuate IL-2-induced edema and restore the structural integrity of the lungs, spleen, and kidney (14–16). In our study we show that granulocyte depletion by RB6-8c5 Abs is very effective in the presence of a non NOS inhibitor obtained with pk136 mAbs (data not shown).

Francesco Colucci for helpful discussions during the preparation of the manuscript.

A substantial reduction in PMN recruitment into the lung in response to a single IL-2 injection was observed using RAG2−/−/IL-15−/− mice and was confirmed during chronic treatment. These results raise the possibility that either NK lymphocytes or IL-15 are implicated in IL-2-induced PMN mobilization and VLS. However, it seems unlikely that PMN recruitment is dependent on NK lymphocytes after a single IL-2 injection. Studies using LPS from Gram-negative bacteria have not supported the hypothesis that PMN mobilization is controlled by NK cells (42). By contrast, NK influx into the lung may depend on PMN, which produce IL-12 in response to IL-2 stimulation. By inducing NK migration and their interaction with endothelial cells, IL-12 regulates crucial parameters of NK cell recruitment in tissues (43). Furthermore, the depletion of NK1.1+ cells by the pk136 mAb after LPS inhalation does not prevent the recruitment of PMN into airspaces (44). Similarly, preliminary results obtained with RAG2−/−/mouse Abs show that PMN mobilization in response to IL-2 is unaffected by the NK depletion obtained with pk136 mAbs (data not shown).

Thus, it is tempting to speculate that IL-15 may be implicated in PMN recruitment during IL-2-induced VLS in mice, because human PMN express functional IL-15Rs (45), and their proinflammatory and antimicrobial functions are known to be activated by IL-15.

This study shows that cells of the innate immune system are critical in IL-2-induced pulmonary VLS, whereas lymphocytes involved in adaptive immunity are not implicated. This fundamental idea is important in terms of understanding the mechanisms of IL-2 toxicity and in the design of IL-2-derived molecules with an increased therapeutic index (46–48).

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