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Correction for “Improved IL-2 immunotherapy by selective stimulation of IL-2 receptors on lymphocytes and endothelial cells,” by Carsten Krieg, Sven Létourneau, Giuseppe Pantaleo, and Onur Boyman, which appeared in issue 26, June 29, 2010, of Proc Natl Acad Sci USA (107:11906–11911; first published June 14, 2010; 10.1073/pnas.1002569107).

The authors note that Fig. 2 appeared incorrectly. The corrected figure and its corresponding legend appear below. This error does not affect the conclusions of the article.

Fig. 2. Efficient control of tumor growth by IL-2/mAbCD122 complexes. (A and B) WT mice were injected s.c. with $10^6$ B16F10 melanoma cells, followed by daily injections (indicated by gray shaded area) of PBS (Control), IL-2, IL-2/mAbCD122 complexes, or IL-2/mAbCD25 complexes. Numbers in parentheses refer to the amount of IL-2 injected. Animals were treated for 5 d starting the day after tumor inoculation (A) or for 4 d starting 6 d after tumor inoculation (B). (C) WT mice were injected i.v. with $3 \times 10^5$ B16F10 melanoma cells, followed by treatment on day 4 after injection using either PBS (Control), 200,000 IU IL-2 (IL-2), 5,000 IU IL-2/mAbCD122 complexes or 5,000 IU IL-2/mAbCD25 complexes for 5 d. Photographs of lungs are shown on day 16 after tumor inoculation. Dashed lines indicate the day maximal tumor load was reached in control mice. Data are representative of three independent experiments.

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Improved IL-2 immunotherapy by selective stimulation of IL-2 receptors on lymphocytes and endothelial cells

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IL-2 immunotherapy is an attractive treatment option for certain metastatic cancers. However, administration of IL-2 to patients can lead, by ill-defined mechanisms, to toxic adverse effects including severe pulmonary edema. Here, we show that IL-2–induced pulmonary edema is caused by direct interaction of IL-2 with functional IL-2 receptors (IL-2R) on lung endothelial cells in vivo. Treatment of mice with high-dose IL-2 led to efficient expansion of effector immune cells expressing high levels of IL-2Rβγ, including CD8+ T cells and natural killer cells, which resulted in a considerable antitumor response against s.c. and pulmonary B16 melanoma nodules. However, high-dose IL-2 treatment also affected immune cell lineage marker-negative CD31+ pulmonary endothelial cells via binding to functional αβγ IL-2Rs, expressed at low to intermediate levels on these cells, thus causing pulmonary edema. Notably, IL-2–mediated pulmonary edema was abrogated by a blocking antibody to IL-2Rα (CD25), genetic disruption of CD25, or the use of IL-2Rβγ–directed IL-2/anti-IL-2R antibody complexes, thereby interfering with IL-2 binding to IL-2Rαβγ pulmonary endothelial cells. Moreover, IL-2/anti-IL-2R antibody complexes led to vigorous activation of IL-2Rγt effector immune cells, which generated a dramatic antitumor response. Thus, IL-2/anti-IL-2R antibody complexes might improve current strategies of IL-2–based tumor immunotherapy.

IL-2 exerts both stimulatory and regulatory functions in the immune system and is, along with other members of the common γ chain (γc) cytokine family, central to immune homeostasis (1, 2). IL-2 mediates its action by binding to IL-2 receptors (IL-2R), consisting of either trimeric receptors made of IL-2Rα (CD25), IL-2Rβγ (CD122), and IL-2Rγ (γc, CD132) chains or dimeric βγ IL-2Rs (1, 3). Both IL-2R variants are able to transmit signal upon IL-2 binding. However, trimeric αγγ IL-2Rs have a roughly 100–1000 times higher affinity for IL-2 than dimeric βγ IL-2Rs (3), implicating that CD25 confers high-affinity binding of IL-2 to its receptor but is not crucial for signal transduction. Trimeric IL-2RαRs are found on activated T cells and CD4+ forkhead box P3 (FoxP3)+ regulatory T cells (Treg), which are sensitive to IL-2 in vitro and in vivo (1, 4). Conversely, antigen-experienced (memory) CD8+, CD44high memory-phenotype (MP) CD8+, and natural killer (NK) cells are endowed with high levels of dimeric βγ IL-2Rs, and these cells respond vigorously to IL-2 in vitro and in vivo (5–7).

High-dose IL-2 is used for the treatment of patients with metastatic melanoma and metastatic renal cell carcinoma with a long-term impact on overall survival (8, 9). The major side effect of high-dose IL-2 immunotherapy is vascular leak syndrome (VLS), which leads to accumulation of intravascular fluid in organs such as lungs and liver with subsequent pulmonary edema and liver cell damage (10–13). There is no treatment of VLS other than withdrawal of IL-2. Low-dose IL-2 regimens have been tested in patients to avoid VLS, however, at the expense of suboptimal therapeutic results (10, 11, 14).

According to the literature, VLS is believed to be caused by the release of proinflammatory cytokines from IL-2–activated NK cells (11, 15–20). Instead, we show here that IL-2–induced pulmonary edema resulted from direct binding of IL-2 to lung endothelial cells, which expressed low to intermediate levels of functional αβγ IL-2Rs. Interaction of IL-2 with lung endothelial cells was abrogated by blocking anti-CD25 monoclonal antibody (mAb), in CD25-deficient host mice, or by the use of CD122–specific IL-2/anti-IL-2 mAb (IL-2/mAb) complexes, thus preventing VLS. Furthermore, IL-2/anti-IL-2 mAb complexes induced a potent antitumor response, leading to a dramatic reduction in tumor load.

**Results**

Comparison of Stimulatory Activities of IL-2 and IL-2/mAb Complexes.

We have described that the association of IL-2 with particular anti-IL-2 mAbs led to the formation of IL-2/mAb complexes, which markedly enhanced the in vivo activity of IL-2 (7). Moreover, depending on the anti-IL-2 mAb used, IL-2 was directed either preferentially to cells expressing high levels of IL-2Rβγ (CD122high) or, alternatively, cells carrying high levels of CD25 (CD25high) in addition to IL-2Rβγ (7). Accordingly, recombinant mouse IL-2 plus anti-mouse IL-2 mAb S4B6 formed CD122–specific IL-2/mAb (IL-2/mAbCD122) complexes, which stimulated CD122high immune cells, such as memory CD8+, MP CD8+, and NK cells and, to a lesser extent, also Tregs (7). Contrarily, recombinant mouse IL-2 plus anti-mouse IL-2 mAb JES6-1 resulted in CD25–specific IL-2/mAb (IL-2/mAbCD25) complexes, which were exclusively active on CD25high cells, including recently activated T cells and Tregs (7).

A similar selective pattern was seen with the use of recombinant human (rh) IL-2 plus particular anti-human (h) IL-2 mAbs. Thus, the association of rhIL-2 with the anti-hIL-2 mAb MBA602 generated IL-2/mAbCD122 complexes, which induced vigorous in vivo expansion of MP CD8+ and NK cells, as well as Tregs to a lesser degree (Fig. 1). Conversely, rhIL-2 plus anti-hIL-2 mAb 5344 formed typical IL-2/mAbCD25 complexes, inducing minimal proliferation of MP CD8+ and NK cells but instead robust expansion of CD25high FoxP3+ CD47 Tregs (Fig. 1). RhIL-2 plus either of these two anti-hIL-2 mAbs was used for generating IL-2/mAb complexes throughout this study.

To determine the difference in potency between IL-2 and IL-2/mAbCD122 complexes, we injected wild-type (WT) mice with titrated doses of IL-2 vs. IL-2/mAbCD122 complexes for 5 d and assessed proliferation of adoptively transferred carboxylfluorescein succinimidyl ester (CFSE)-labeled donor MP CD8+ cells and expansion of host MP CD8+ and NK cells (Fig. 1 and SI 1.4).

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Conflict of interest statement: O.B. is a shareholder of Nascent Biologics Inc.

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and B). The lowest dose of IL-2 showing some notable effect on donor MP CD8⁺ cells was 15,000 international units (IU), whereas the maximally tolerated dose of IL-2 was 200,000 IU, which led to proliferation of virtually all donor MP CD8⁺ cells and a 3- to 4-fold expansion of host MP CD8⁺ cells, NK cells, and Tregs. 5,000 IU IL-2/mAbCD122 complexes were comparable with 200,000 IU IL-2 alone in terms of proliferation of MP CD8⁺ and NK cells (Fig. 1), and IFN-γ production in MP CD8⁺ cells (Fig. S1C). The highest tolerated dose of IL-2/mAbCD122 complexes was 15,000 IU. Doses higher than 15,000 IU for IL-2/mAbCD122 complexes or superior to 200,000 IU for IL-2 given for 5 d were lethal for some animals. Collectively, these results indicate that a 40-fold lower dose of IL-2 in the form of IL-2/mAbCD122 complexes is as potent as 200,000 IU IL-2 alone in stimulating effector cells.

Efficient Control of Tumor Growth by IL-2/mAbCD122 Complexes. Because of its potent immune stimulatory capacity, high-dose IL-2 has been used in the treatment of metastatic melanoma (8, 11). Hence, after injection with syngeneic B16F10 melanoma cells, WT mice benefited from 200,000 IU (high-dose) IL-2 treatment by reducing their cutaneous tumor load (Fig. 2 A and B) or preventing the establishment of pulmonary tumor nodules (Fig. 2C and Fig. S2A). Notably, 5,000 IU IL-2/mAbCD122 were as efficient as 200,000 IU IL-2 in mediating tumor control (Fig. 2 A and C).

Strikingly, five daily injections of the maximally tolerated dose of 15,000 IU IL-2/mAbCD122 delayed tumor growth by 10 d, nearly doubling the time before tumors reached a size of 400 mm³ (Fig. 2A). As expected, IL-2–mediated tumor control depended on CD122⁺ T and NK cells (Fig. S2B). In contrast, use of IL-2/ mAbCD25 complexes at this dose of 15,000 IU led only to a minimal delay in tumor growth of 1–2 d (Fig. 2A). These observations also applied to mice that were treated later, i.e., starting on day 6, thus mimicking a therapeutic clinical setting (Fig. 2B). As expected, administration of either 5,000 IU IL-2 alone (Fig. 2 A and B) or anti-IL-2 mAb by itself did not exert any antitumor effect. In conclusion, IL-2/mAbCD122 Complexes are superior to high-dose IL-2 or IL-2/mAbCD25 complexes in generating efficient antitumor responses.

High-Dose IL-2 but Not Low-Dose IL-2/mAbCD122 Complexes Causes VLS. As mentioned previously, high-dose IL-2 treatment can cause VLS (10, 11), which affects lungs and liver leading to pulmonary edema, drop in blood oxygen saturation (SaO₂), liver cell damage, and subsequent elevation of liver enzymes in the serum such as aspartate aminotransferase (AST). Similar to previous reports (15, 21), WT mice injected with either titrated doses of IL-2 or 200,000 IU IL-2 for different lengths of time developed VLS when they received a cumulative IL-2 dose of >700,000–800,000 IU. In these mice, pulmonary weight drastically increased, which was accompanied by a sharp drop in SaO₂, and total liver weight and serum AST levels markedly increased (Fig. S3). Thus, five daily doses of 200,000 IU IL-2 produced robust VLS, which corresponds to ≈4 times the daily dose used in a clinical setting (10, 11) (Methods).

VLS is thought to be caused by activated NK cells. In accordance with the literature, ascending doses of IL-2 alone led to a notable expansion of NK and CD8⁺ cells, which was accompanied by a marked increase in pulmonary wet weight, significant histological changes of the lungs, and a drop in SaO₂ (Fig. 3 A–C). IL-2/mAbCD122 complexes dramatically boosted numbers of NK and CD8⁺ cells; however, pulmonary edema was very mild considering the vigorous immune stimulation (Fig. 3A). Hence,
sharp contrast, significant pulmonary edema was observed in immune-depleted mice after treatment with 200,000 IU IL-2 or 15,000 IU IL-2/mAbsCD25 complexes (Fig. 3D). Because immune cells are virtually absent in this model, these results suggest that nonimmune cells are involved in VLS.

**IL-2–Mediated Pulmonary Edema Depends on CD25+ Nonimmune Cells.** To identify which pulmonary cells IL-2 was acting on, we treated WT animals with 200,000 IU IL-2 for 5 d together with a panel of neutralizing mAbs to selectively deplete cell populations or block IL-2R subunits. As reported (15, 17), we found that IL-2–induced pulmonary edema was lowered discretely by targeting NK or T cells, notably CD8+ cells (Fig. 4A and Fig. S4). Interestingly, anti-CD25 mAb, and to a lesser extent anti-CD122 mAb, almost completely abolished IL-2–induced pulmonary edema (Fig. 4A).

With the use of above-mentioned immune-depleted mice, 200,000 IU IL-2 led to considerable pulmonary edema that, surprisingly, depended on CD25 as evidenced by a complete abrogation of pulmonary edema with coadministration of anti-CD25 mAb (Fig. 4B). Moreover, administration of 200,000 IU IL-2 to CD25−/− animals failed to induce pulmonary edema (Fig. 4C), despite the fact that NK and MP CD8+ cells expanded considerably. We generated mixed bone marrow (BM) chimeras by using WT or CD25−/− BM transferred to lethally irradiated WT or CD25−/− recipients, which were then left for 6 wk to allow for immune reconstitution before administration of 200,000 IU IL-2 for 5 d. WT → CD25−/− chimeras, expressing CD25 only on BM-derived cells, showed no significant IL-2–induced pulmonary edema.

**Fig. 3.** IL-2/mAbsCD25 complexes show an improved profile of immune stimulation to pulmonary edema. (A) Purified CFSE-labeled Thy1.1+ MP CD8+ cells were transferred to WT recipients, followed by daily injections of PBS, graded doses of IL-2, or graded doses of IL-2/mAbs complexes for 5 d. On day 6, mice were killed for determination of pulmonary wet weight (Left) and effector cell counts in host spleens (Right), including MP CD8+ (filled bars) and NK cells (open bars). Dashed lines indicate levels of pulmonary wet weight and total effector cell counts in mice receiving 200,000 IU IL-2. (B and C) WT mice were administered daily injections of PBS, 200,000 IU IL-2, 5,000 IU IL-2/mAbsCD25 complexes, or 5,000 IU IL-2/mAbsCD25 complexes for 5 d before staining of lungs with hematoxylin and eosin (B) or measurement of SaO2 (C). (D) Immune-depleted animals were generated by using RAG−/− mice that received sublethal irradiation (650 rad) and daily injections of depleting mAbs to Gr1 and NK1.1, followed by administration of PBS, 200,000 IU IL-2, 15,000 IU IL-2/mAbsCD25 complexes, or 15,000 IU IL-2/mAbsCD25 complexes before measurement of pulmonary wet weight on day 5. For B, representative regions are shown at a magnification of 40× (Upper) and 400× (Lower). Data are representative of three independent experiments.

Fig. 4.  IL-2–mediated pulmonary edema depends on CD25+ nonimmune cells. (A) WT mice received 5 daily injections of PBS or 200,000 IU IL-2 along with depleting mAbs to Thy1.2, NK1.1, CD25, or CD122. Pulmonary wet weight was determined on day 6. (B) Immune-depleted animals were generated as in Fig. 3D and received daily injections of PBS, 200,000 IU IL-2, or IL-2 plus anti-CD25 mAb. Pulmonary wet weight was determined on day 5. (C) CD25−/− mice or WT littermates received PBS or IL-2 as in A, followed by determination of pulmonary wet weight on day 6. (D) Mixed BM chimeras were generated with indicated combinations of WT or CD25−/− (KO) donor BM transferred to lethally irradiated WT or CD25−/− (KO) hosts and left for 6 wk, before treatment for 5 d with PBS or 200,000 IU IL-2. Pulmonary wet weight was calculated on day 6 by subtracting weights of PBS-treated from IL-2–treated animals. Data are representative of three independent experiments. ***P < 0.001; **P < 0.01; n.s., not significant.
edema, in contrast to WT→WT chimeras receiving IL-2, in which pulmonary edema was maximal (Fig. 4D). CD25<–/–→WT chimeras, expressing CD25 only on stromal cells, exhibited a considerable level of pulmonary edema upon IL-2 treatment, whereas CD25<–/–→CD25<+/– chimeras were resistant to IL-2–induced pulmonary edema (Fig. 4D). Collectively, these results demonstrate that CD25<–/– nonimmune cells are implicated in IL-2–induced pulmonary edema.

Lung Endothelial Cells Express Functional IL-2 Receptors. To identify which pulmonary nonimmune cells expressed CD25 and were responsible for pulmonary edema after IL-2 treatment, we stained lung cells with different immune cell lineage markers (Lin) along with CD25. A considerable fraction of lung cells from WT mice was Lin<–/– for markers of lymphoid cells (Ly5.2), B cells (B220), T cells (CD3), NK cells (NK1.1 and CD11b), granulocytes (Gr1), macrophages (CD11b and CD11c), and dendritic cells (CD11c). The majority of Lin<–/– lung cells expressed very high levels of CD31 and endoglin (also known as CD105), thus identifying them as endothelial cells (Fig. 5A Left). Notably, CD31<+ Lin<–/– lung cells expressed intermediate (int) surface levels of CD25 in comparison with Lin<+ immune cells, the majority of which exhibited low CD25 levels with a small subset expressing high CD25 levels (Fig. 5A Right). Moreover, CD122 and CD132 chains were also present on CD31<+ Lin<–/– lung cells, the expression of which was low to intermediate in comparison with immune cells (Fig. 5A). All three IL-2R chains were also evident on the mRNA level (Fig. 5B).

CD31<+ endothelial cells increased their CD25 mRNA levels upon IL-2 administration (Fig. 5C), suggesting IL-2–mediated signaling. Further proof for this hypothesis came from experiments showing a significant increase in levels of phosphorylated STAT5 (pSTAT5) in Lin<– CD31<+ cells in vivo upon treatment with 200,000 IU IL-2 (Fig. 6A). Moreover, purified Lin<– CD31<+ cells stimulated with IL-2 in vitro produced very high titers of nitrite (NO<2–), which has been reported to be elevated in VLS and toxic for endothelial cells (12, 22). In summary, IL-2 causes pulmonary edema by direct binding to lung endothelial cells expressing functional IL-2Rs, and this binding can be circumvented by the use of IL-2/mAbCD122 complexes.

Discussion

The literature suggests that IL-2–mediated pulmonary VLS is caused by the release of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, from IL-2–activated NK cells (10, 11). Using pulmonary edema as a fine measure of VLS, we observed in this study a minor but significant contribution of NK and CD8<+ T cells to IL-2–induced pulmonary edema, confirming previous findings. However, the major effect of IL-2 leading to pulmonary edema was not via activation of immune cells but the result of direct binding of IL-2 to functional IL-2Rs on lung endothelial cells.

IL-2 binding to IL-2Rαβγ<+ endothelial cells heavily depended on CD25. Conversely, CD122<high effector cells, including (MP) CD8<+ and NK cells, responded very efficiently to IL-2 via their dimeric βγ/IL-2Rs, present at very high levels on these cells. This situation led us to explore CD122-specific IL-2/mAbCD122 complexes, for which the anti-IL-2 mAb is thought to cover up the IL-2 binding epitope of IL-2, thus impeding interaction with CD25 (7, 11, 23). Accordingly, IL-2/mAbCD122 complexes caused efficient activation of IL-2Rαβγ<+ high effector cells, but not IL-2Rαβ<+ low/intermediate pulmonary endothelial cells. Therefore, 5,000 IU IL-2/mAbCD122 complexes were as potent as 200,000 IU IL-2 alone in activating CD122<high effector cells and generating antitumor immune responses. However, in contrast to IL-2 alone, IL-2/mAbCD122 complexes did not cause any notable toxicity at this dose. Moreover, with the use of 15,000 IU IL-2/mAbCD122 complexes administered for 5 d, we observed a dramatic antitumor response.

Anti-IL-2 mAbCD122 (such as anti-hIL-2 mAb MAB602 or anti-mouse IL-2 mAb S4B6) are thought to obscure the CD25-binding epitope of IL-2 (7, 11, 23). Thereby, anti-IL-2 mAbCD122 directs IL-2 to CD122<high cells, while interfering with CD25-dependent binding of IL-2 to IL-2Rαβγ<low/intermediate lung endothelial cells and, thus, preventing pulmonary VLS. Alternatively, reduction in toxicity by the use of IL-2/mAbCD122 complexes might be explained by interference of anti-IL-2 mAbCD122 with amino acids 22–58 of hIL-2, which have been shown to be responsible...
for IL-2-mediated VLS (12, 24). For anti-mouse IL-2 mAb S4B6, it is known that this mAb binds to amino acids 26–45 of mouse IL-2 (25), which overlaps with amino acids 22–31 of hIL-2 (26). It is therefore conceivable that anti-hIL-2 mAb MAB602, which was used in this study and exerts similar properties as S4B6 mAb, also interferes with a region within amino acids 22–58 of hIL-2.

Conversely, anti-IL-2 mAbCD25 (such as anti-hIL-2 mAb 5344 or anti-mouse IL-2 mAb JES6-1) are believed to cover up the CD122-binding epitope of IL-2 (7, 11, 23). In favor of this hypothesis, IL-2/mAbCD25 complexes behave similarly to hIL-2 mutein BAY50-4798, containing a single mutation at amino acid 58 of hIL-2, which interacts with the CD122-binding epitope of IL-2. Thus, both IL-2/mAbCD25 complexes and BAY50-4798 do not lead to significant expansion of NK cells but instead preferentially stimulate CD25+ cells, including recently activated T cells and Tregs (7, 23, 27, 28). Interestingly, high doses of IL-2/mAbCD25 complexes led in this study to pulmonary edema, and a clinical study demonstrated that patients receiving BAY50-4798 experienced a similar degree of IL-2–mediated VLS as IL-2, suggesting that activation of T cells or other immune or nonimmune cells might cause VLS (29). Notably, IL-2–mediated pulmonary edema was not an issue when Treg numbers were raised after a brief course of IL-2/mAbCD25 injections or with low doses of IL-2/mAbCD25 treatment. These latter approaches might be useful for the treatment of allergies, autoimmunity disease, and allograft rejection (11, 28, 30).

To our knowledge, this study is the first description of functional IL-2Rs on nonimmune cells, although others have observed expression of IL-2R subunits on nonimmune cell lines, such as human embryonic skin and lung fibroblast cell lines (31–33).

Why do endothelial and other nonimmune cells express IL-2Rs? For immune cells, CD25 is known to associate with β2 IL-2Rs to increase IL-2R affinity (34). Interestingly, CD25+ mice show increased serum IL-2 levels (35–37), which might result from a lack of IL-2–consuming Tregs in these mice or of CD25 molecules on immune or nonimmune cells, the suggestion being that CD25 serves as a sort of scavenger or decoy receptor for IL-2, thus regulating IL-2 levels in vivo (23, 33, 38). In this study, lung endothelial cells increased CD25 expression upon IL-2 treatment in vivo, maybe to reduce local IL-2 levels, thus limiting an overstimulation of the immune system in vital organs such as the lungs.

Methods

Mice. C57BL6 (referred to as WT, from Charles River), Thyl.1-congenic, RAG-1−/−, and CD25−/− (all on a C57BL6 background, from The Jackson Laboratory) were maintained under specific-pathogen-free conditions and used at 4–8 wk of age. Experiments were performed in accordance with the Swiss Federal Veterinary Office guidelines and approved by the Cantonal Veterinary Office.

Flow Cytometry. Cell suspensions of spleen were prepared according to standard protocols (39) and stained for analysis by flow cytometry using PBS containing 4% FCS and 2.5 mM EDTA. Fluorochrome-conjugated or biotinylated mAbs (from eBioscience unless otherwise stated) were used against CD3 (145-2C11), CD4 (RM4-5 or GK1.5), CD8a (S3-6.7, BD Biosciences), CD11b (M1/70, BD Biosciences), CD11c (N418), CD25 (PC61 or 7D4), CD31 (MEC13.3, BD Biosciences), CD44 (IM7, BD Biosciences), CD49b (DX5), CD105 (M71/18), CD122 (TM-β1), CD123 (AG3, BD Biosciences), B220 (RA3-68, Caltag Laboratories), Gr1 (RB6-8C5), NK1.1 (PK136), and Thy1.1 (HIS51). Intracellular cytokine staining for IFN-γ (XMG1.2, eBioscience) and FoxP3 (FJK-166) was performed as described (40). pS-STAT5 (pY694, BD Biosciences) staining was performed according to manufacturer’s instructions. Immune and nonimmune cell subsets were separated by using FITC-conjugated lineage markers for CD4, CD8, CD11b, CD11c, B220, Gr1, NK1.1, and CD62. Fc receptor binding was blocked by preincubating cells with anti-CD16/CD32 mAbs. At least 100,000 viable cells were acquired on a digital LSRII by using Cell Quest Diva software (BD Biosciences) and analyzed by using FlowJo software (TriStar).

Cytokines and mAbs. RhlL-2 and anti-hIL-2 mAb MAB602 (clone S355) were obtained from R&D Systems. Anti-hIL-2 mAb S344.111 was purchased from BD Biosciences. Alternatively, rhlL-2 was obtained from the National Cancer Institute’s Biological Resources Branch. As described (7), mice were injected intraperitoneally (i.p.) with IL-2 or IL-2/mAb complexes. For IL-2/mAb complexes, IL-2 and anti-IL-2 mAbs were mixed at a 2:1 molar ratio. Where indicated, starting 1 d before cytokine administration, mice were received daily i.p. injections of 100 μg of depleting mAbs to CD25 (PC61, Gr1 (RB6-8C5), NK1.1 (PK136), Thy1.2 (30H12), CD4 (GK1.5), and CD8 (YTS169), all from BioXcell.

IL-2–Induced Toxicity in Vivo. WT mice received daily i.p. injections of IL-2, either as titrated doses, ranging from 6,000 to 200,000 IU for 7 d or as daily injections of 200,000 IU IL-2 for different lengths of time (ranging from 1 to 7 d) in the experiments described in Fig. 53. For the experiments shown in Figs. 3 and 4 and Fig. S4, 200,000 IU IL-2 for 4–5 d was used to produce robust VLS. The regimen in mice of 200,000 IU IL-2 once daily corresponds to 58.8 μg IL-2/kg of body weight. This dose is 3.7 times higher compared with 720,000 IU IL-2 three times daily used in humans, which corresponds to 158.8 μg IL-2/kg body weight. This difference in dose is justified by the faster metabolism in mice (41). One day before sacrifice, SaO2 was determined by using a pediatric pulse oximeter (BCI 3302). For determination of pulmonary wet weight, lungs were weighed before and after lymphophiliation overnight at 58 °C under vacuum according to published protocols (15, 21). Pulmonary wet weight was calculated by subtracting initial pulmonary weight from the wet weight, which was blocked by preincubating cells with anti-CD16/CD32 mAbs. At least 2 × 106 purified cells were injected i.v. to mice.

Mixed BM Chimeras. BM cells from WT or CD25−/− mice were depleted for mature B, T, and MHC-II-expressing cells by negative selection (StemCell Technologies) using mAbs to Thy1.2 (30H12), NK1.1 (PK136), and MHC-II (M5/114.15.2). Subsequently, BM cells were injected i.v. to lethally irradiated (1,200 rad) WT or CD25−/− hosts.

Lung Histology. Lungs were fixed in 4% formalin, embedded in paraffin, sectioned at 6 mm, and stained with hematoxylin and eosin.

Preparation of Lung Cells. After perfusion of anesthetized mice with PBS, lungs were removed, cut into small pieces, and stirred at 37 °C for 30 min in PBS containing 1.3 mM EDTA. Subsequently, lung tissue was digested for 1 h at 37 °C in RPMI 1640 medium containing 150 U/ml collagenase-I (Sigma-Aldrich) and 1 mM MgCl2, 1 mM CaCl2, 2.5 mM L-glutamine, and 5% FCS. Cells were then mashed through a cell strainer and washed and resuspended in RPMI 1640 medium supplemented with 10% FCS. Immune cell lineage marker (Lin)− lung cells were identified by gating on Ly5.2− lung cells. For purified Lin− CD31+ lung cells, cells positive for CD31+ but negative for Ly5.2 or negative for CD3, B220, NK1.1, CD11b, CD11c, and Gr1 were sorted by using a BD Aria, with both sorting strategies yielding similar purities (≈97%) and results.

Tumor Model in Vivo. For the generation of s.c. tumors, either 106 (Fig. 2A and B) or 5 × 104 (Fig. 2D) B16F10 melanoma cells in 100 μL of RPMI 1640 medium were injected into the upper dermis in the back of mice. Treatment was started either on day 1 (Fig. 2A) or on day 6 (Fig. 2B) after tumor inoculation, the latter of which corresponds to 1 d after tumor nodules were clearly visible and palpable at a size of ≈25 mm3. To establish lung tumors, 3 × 105 B16F10 melanoma cells were injected i.v. Treatment was started 3 d after injection and administered for 5 d. On day 16 after injection, mice were killed and lungs fixed in Fekete’s solution (70% ethanol, 3.7% paraformaldehyde, 0.75 M glacial acetic acid) before counting of pulmonary micrometastases.

Quantitative Real-Time PCR. Total mRNA was isolated by using the RNeasy Micro/Micro kit (Qiagen), cDNA was obtained by using the Quantitect Reverse Transcription kit, and IL-2 mRNA expression was analyzed by using quantitative real-time PCR according to manufacturer’s instructions (Qiagen). Lin− CD31+ lung cells were purified from WT animals by using a BD Aria sorter, before mRNA isolation, cDNA amplification with the SuperSMArt CDNA

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Synthesis kit (Clontech), and subsequent processing for quantitative real-time PCR by using QuantiTect primers (both from Qiagen).

Nitrite (NO$_2^-$) Production. We measured NO$_2^-$ in culture supernatants by using the Griess assay, as reported (24). Briefly, 1–2 $\times$ 10$^5$ Lin-2CD31$^+$ cells per well were left untreated or stimulated with 10 IU/mL IL-2. Alternatively, 1–2 $\times$ 10$^5$ CD11b$^+$ cells per well, either untreated or stimulated with 200 U/mL IFN-γ plus 200 U/mL lipopolysaccharide (LPS), served as controls. After 16 h of culture in 96-well U-bottom plates, supernatants were collected and incubated for 10 min at room temperature with 1% sulfanilamide hydrochloride and 0.1% naphthylenediamine dihydrochloride in 2.5% phosphoric acid, before measuring optical density at 550 nm with a microplate reader (Versamax; Molecular Devices). The concentration of NO$_2^-$ was calculated from a NO$_2^-$ standard curve, which was linear between 6 and 100 μM NO$_2^-$.

Statistical Analysis. Results are expressed as mean ± SD. Differences between groups were examined for statistical significance by using one-way ANOVA with a Bonferroni’s post test.

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