Synergistic Engagement of an Ineffective Endogenous Anti-Tumor Immune Response and Induction of IFN-\(\gamma\) and Fas-Ligand-Dependent Tumor Eradication by Combined Administration of IL-18 and IL-2

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*J Immunol* 2002; 169:4467-4474; doi: 10.4049/jimmunol.169.8.4467

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IFN-γ is a critical component of the endogenous and many cytokine-induced antitumor immune responses. In this study we have shown that the combination of IL-18 and IL-2 (IL-18/IL-2) synergistically enhances IFN-γ production both in vitro and in vivo, and synergizes in vivo to induce complete durable regression of well-established 3LL tumors in >80% of treated mice. We have observed a nascent, but ineffective, host immune response against 3LL that depends on endogenous IFN-γ and IL-12 production and the Fas/Fas ligand (Fas-L) pathway. The combined administration of IL-18/IL-2 engages this endogenous response to induce tumor regression via a mechanism that is independent of NK and NKT cells or IL-12, but is critically dependent on CD8+ T cells, IFN-γ, and the Fas/Fas-L pathway. These studies demonstrate the importance of IFN-γ as well as the Fas/Fas-L pathway in both endogenous and cytokine-driven antitumor immune responses engaged by IL-18/IL-2 and provide preclinical impetus for clinical investigation of this potent anti-tumor combination. The Journal of Immunology, 2002, 169: 4467–4474.

Interleukin-18 is a potent immunoregulatory cytokine that was initially described as an IFN-γ-inducing factor (1, 2). IL-18 enhances T and/or NK cell cytokine production, proliferation, and cytotytic activity (3, 4) as well as the expression of Fas ligand (Fas-L), and Fas-L- or perforin-mediated cytotoxicity (5–7). Tumor cells engineered to produce IL-18 are less tumorigenic (8–10), and systemic administration of IL-18 protein has demonstrated considerable therapeutic activity in several murine tumor models (11–13). We have reported previously that IL-12, another potent IFN-γ-inducing immunoregulatory cytokine, synergizes with IL-2 in the treatment of mice bearing established primary and/or metastatic tumors (14). IL-2 has a wide range of immunoregulatory and/or anti-tumor effects, and is currently approved by the Food and Drug Administration for the treatment of patients with metastatic renal cell carcinoma and melanoma. Therefore, approaches that enhance the biological effects of IL-2 also may potentiate the therapeutic benefits of IL-2-based approaches to cancer treatment. IL-2 and IL-18 can synergistically enhance various aspects of immune function, including IFN-γ production, proliferation, and cytolytic activity by T and/or NK cells (15–18), and IL-18 enhances the expression of the IL-2Rα-chain on murine T cells (17). More recently, Son et al. (19) have demonstrated that IL-18 and IL-2 synergistically enhance proliferation, IFN-γ production, and cytotoxicity by human NK cells in vitro and suggested that this combination may warrant investigation as an anti-tumor regimen in vivo. The induction of endogenous IFN-γ production by IL-18/IL-2 may be particularly important given the critical role IFN-γ plays in the development of adaptive anti-tumor immune responses (20). As suggested previously (19), these in vitro observations collectively suggest that IL-18 and IL-2 may also interact favorably in the induction of an anti-tumor immune response. The present studies have now confirmed in vivo for the first time that systemic administration of IL-18 and intermittent IL-2 synergistically enhances the complete regression of well-established primary tumor by a mechanism that depends on CD8+ T cells, Fas/Fas-L, and the production of endogenous IFN-γ. In addition, these results suggest that IL-18/IL-2 may successfully amplify a detectable, but ineffectual, endogenous adaptive anti-tumor response to induce the regression of established tumors.

Materials and Methods

Mice and tumor cells

C57BL/6 mice were obtained from the Animal Production area of the National Cancer Institute. GKO/C57BL/6 (GKO) IFN-γ knock out mice, immunodeficient C57BL/6-J-SCID (SCID) mice, as well as B6 MLF/Fas (LPR) and B6C3H/FasLo/J (GLD) mice that express mutated, nonfunctional Fas and Fas-L genes, respectively, were derived from our breeding colonies. Breeding pairs of C57BL/6-IL-12 p40 knockout (p40−/−) mice were provided by Dr. J. Magram (Hoffmann-La Roche, Nutley, NJ) and maintained in our colony. Mice were maintained in a dedicated pathogen-free environment and generally used between 8 and 10 wk of age. The transplantable mouse Lewis lung carcinoma (3LL) cell line or dominant-negative Fas-transfected 3LL (3LL-DN) was used in all experiments as indicated. Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 86-23).

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Received for publication January 22, 2002. Accepted for publication August 14, 2002.

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3Abbreviation used in this paper: Fas-L, Fas ligand.
Generation of dominant-negative Fas 3LL transfectants

For construction of recombinant Fas expression vectors, the 1039-bp full-length Fas and 610-bp dominant-negative Fas that does not contain the intracellular signaling domain were amplified by RT-PCR from 3LL total RNA using the forward primer Fas-5A (GGCTTGGTCCATTGGCTGCA GAC) and the reverse primer Fas-3A (CTCTCCCTCTTCTAGCTGCT GACT or Fas-3B (CTACACGACCTTCTCCGTTGA), respectively. PCR-amplified products were ligated into the Unidirectional Eukaryotic TA Cloning Expression Vector (Invitrogen, San Diego, CA). The integrity of the insert was confirmed by sequencing. The expression vectors coding full-length Fas or dominant-negative Fas were transfected into 3LL tumor cells using Lipofectamine (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Briefly, 3LL cells (1 x 10^6) were plated in a six-well plate, complete medium and incubated at 37°C in a 5% CO2 incubator until the cells were 50–80% confluent. The cells were transfected with 2 μg recombinant expression vector DNA and 5 μg Lipofectamine for 6 h, passaged, and cultured continuously in the presence of 800 μg/ml genetricin from days 3–14. Genetricin-resistant clones were selected by limiting dilution, and flow cytometry was used to select clones that exhibited high levels of Fas expression. 3LL cells transfected with control vector alone were also selected under high concentrations of genetricin (1000 μg/ml) and used as a negative control.

Reagents

Recombinant murine IL-18 was purchased from PeproTech (Rocky Hill, NJ). For in vivo administration, aliquots of stock IL-18 were diluted with HBBS containing 0.1% (v/v) sterile-filtered C57BL/6 mouse serum. Recombinant human IL-2 was provided by Chiron (Emeryville, CA). After recirculation with sterile water, IL-2 was diluted with HBBS containing 0.1% C57BL/6 mouse serum. Recombinant mouse IFN-γ (sp. act., 47 x 10^4 U/mg) was provided by Genentech (San Francisco, CA). Recombinant murine TNF-α (sp. act., 1.2 x 10^5 U/mg), anti-murine Fas (Jo2) Ab, and hamster IgG isotype control were purchased from BD PharMingen (San Diego, CA). Monoclonal mouse anti-mouse CD8 (19/178) and rat anti-mouse CD4 (GK 1.5), derived from hybridoma supernatants, were used to identify the cell population. The cells were fixed in a 1/2 dilution of anti-NK1.1 ascites was injected i.p. on days 4 and 9 after the injection of 3LL tumor, and the effects of this regimen were analyzed 6 days after the last injection of Ab.

Cytokine production

Single-cell suspensions of C57BL/6 mouse splenocytes in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% FBS (Intergen, Purchase, NY) and additives including 1% sodium pyruvate, 1% l-glutamine, 1% penicillin/streptomycin, and 1% nonessential amino acids (Bio-Whittaker) were aliquoted (1 x 10^6 cells/well) in 24-well, flat-bottom plates (Costar, Cambridge, MA) in complete medium containing IL-18 (10 ng/ml), IL-2 (600 IU/well), IL-12 (200 IU), and IL-10 (250 ng/ml) in RPMI supplemented with 10% FBS (Intergen), 1% antibiotic/antimycotic, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1% sodium azide (Sigma-Aldrich). Cells were incubated for 48 h, and supernatants were assayed for IFN-γ by ELISA (R&D Systems, Minneapolis, MN). To investigate the impact of recombinant IL-18 and IL-2 on IFN-γ production in vivo, tumor-bearing mice were treated as outlined below. Serum samples obtained via cardiac puncture from mice euthanized as indicated below were also assayed for IFN-γ by ELISA.

Flow cytometry

The expression of Fas on 3LL tumor cells was analyzed using flow cytometry. Cells (5 x 10^6) were stained with PE-labeled anti-mouse Fas Ab (Jo2) or PE-labeled hamster IgG isotype control Ab at 4°C for 20 min. After washing, cells were analyzed on a FACScan (BD Biosciences, Mountain View, CA) flow cytometer using CellQuest software. The depletion of NK and NKT cell subsets in vivo was monitored using anti-mouse NK1.1 (PK136) and anti-mouse IgG2a to detect any cells that may have been missed by possible binding of residual NK1.1 Ab via flow cytometric analysis of single-cell leukocyte suspensions prepared from the liver and spleen. C57BL/6 mice were euthanized by cervical dislocation. Single-cell splenocyte suspensions were prepared by mechanical disruption and lysis to remove mature RBC using ACK lysis buffer (Quality Biological, Gaithersburg, MD). The resultant cell pellet was suspended in staining buffer (HBBS with 0.1% BSA, 0.1% sodium azide, 0.1% sodium citrate and 1% Tween-20). Cells were incubated with a combination of the following antibodies: CD4, CD8, Fas-1, and Fas-2. Cells were then washed and permeabilized with BD fixation and permeabilization buffer (BD Biosciences, San Jose, CA). Labeled cells were washed twice in cold HBSS, lysed as described above to remove RBC, and suspended in staining buffer for flow cytometric analysis as described above.

Cytotoxicity assay

Tumor target cells (1 x 10^6) were labeled with 111indium-oxine as previously described (21) and incubated in a volume of 200 μl complete medium/well in 96-well, flat-bottom plates with various concentrations of anti-Fas Ab (Jo2) in the presence of P815 (1 x 10^5 cells) to promote Ab cross-linking. After 18 h of incubation, supernatants were harvested and counted in a gamma spectrophotometer (model 1480; Wallac, Gaithersburg, MD). Specific killing (percent cytotoxicity) was calculated as [(experimental release – spontaneous release)/(maximal release – spontaneous release)] x 100. All samples conditions were run in triplicate.

Tumor models and in vivo treatment

Cohorts of 10 mice/group were used in the present studies unless otherwise noted. Mice received s.c. mid-flank injections of 10^6 3LL tumor cells and formed well-established tumors before the initiation of therapy. To investigate the impact of combined administration of IL-18 and IL-2 on the production of IFN-γ in vivo in a tumor-bearing host, IL-18 (2.5 μg) or vehicle alone was administered i.p. twice daily on days 8–11 and 14–17, and IL-2 (300,000 IU) or vehicle alone was delivered i.p. once daily on days 8, 10, 14, and 17 after tumor implantation. On day 18 cohorts of mice from the respective treatment groups (six mice per group) were euthanized, and serum samples were obtained from individual mice via cardiac puncture and assayed for IFN-γ as noted above.

To investigate the anti-tumor activity of combined systemic administration of IL-18 and IL-2, IL-18 (2.5 μg) or vehicle alone was administered i.p. twice daily on days 8–12, 15–19, and 22–26, and IL-2 (300,000 IU) or vehicle alone was delivered i.p. once daily on days 8, 10, 12, 15, 17, 19, 22, and 25 after tumor implantation. In a subsequent experiment IL-18 (2.5 μg) or vehicle alone was administered twice daily as described above on days 8–12 and 15–19, and IL-2 (300,000 IU) or vehicle alone was delivered once daily on days 8, 10, 12, 15, 18, and 22 after tumor implantation. The results of these experiments were pooled for analysis of the therapeutic activity of IL-18 with or without IL-2.

To define the role of an intact immune system and, more specifically, IFN-γ and IL-12 in the anti-tumor activity of IL-18/IL-2, we compared the therapeutic efficacy of this regimen in tumor-bearing wild-type, GKO, KO, and SCID mice. IL-18/IL-2 was also investigated by comparing the therapeutic efficacy of this regimen in tumor-bearing wild-type, LPR, and GLD mice treated as described above with three cycles of IL-18 and IL-2 on IFN-γ production in vivo, tumor-bearing mice were treated as outlined below. Serum samples obtained via cardiac puncture from mice euthanized as indicated below were also assayed for IFN-γ by ELISA.

Flow cytometry

The expression of Fas on 3LL tumor cells was analyzed using flow cytometry. Cells (5 x 10^6) were stained with PE-labeled anti-mouse Fas Ab (Jo2) or PE-labeled hamster IgG isotype control Ab at 4°C for 20 min. After washing, cells were analyzed on a FACScan (BD Biosciences, Mountain View, CA) flow cytometer using CellQuest software. The depletion of NK and NKT cell subsets in vivo was monitored using anti-mouse NK1.1 (PK136) and anti-mouse IgG2a to detect any cells that may have been masked by possible binding of residual NK1.1 Ab via flow cytometric analysis of single-cell leukocyte suspensions prepared from the liver and spleen. C57BL/6 mice were euthanized by cervical dislocation. Single-cell splenocyte suspensions were prepared by mechanical disruption and lysis to remove mature RBC using ACK lysis buffer (Quality Biological, Gaithersburg, MD). The resultant cell pellet was suspended in staining buffer (HBBS with 0.1% BSA, 0.1% sodium azide, 0.1% sodium citrate and 1% Tween-20). Cells were incubated with a combination of the following antibodies: CD4, CD8, Fas-1, and Fas-2. Cells were then washed and permeabilized with BD fixation and permeabilization buffer (BD Biosciences, San Jose, CA). Labeled cells were washed twice in cold HBSS, lysed as described above to remove RBC, and suspended in staining buffer for flow cytometric analysis as described above. Liver leukocyte suspensions were prepared via portal vein perfusion with HBBS, followed by mechanical disruption. The resulting single-cell suspension was collected by centrifugation at 800 x g for 10 min at 4°C and washed twice with cold HBBS, and the resulting pellet was resuspended in 40% Percoll (Amersham Pharmacia Biotech, Piscataway, NJ) in RPMI (BioWhittaker, Walkersville, MA). This single-cell suspen-
Fisher’s exact test. Interpretations regarding survival and tumor regression outcomes were in complete interpretative agreement. For simplicity we reported only $p$ values obtained from Fisher’s exact tests. All $p$ values were two-tailed and were considered significant at $p < 0.05$.

**Results**

**Synergistic enhancement of IFN-γ production by murine splenocytes treated in vitro with IL-18 with or without IL-2**

We initially investigated the impact of IL-18 and IL-2 on IFN-γ production by murine splenocytes. We found that IL-18 and IL-2 synergistically enhanced IFN-γ production, with splenocytes treated with IL-18 and IL-2 producing dramatically more IFN-γ (1820 pg/ml) than those treated with IL-18 (101 pg/ml), IL-2 (5 pg/ml), or medium (none detected) alone (Fig. 1A).

**Systemic administration of IL-18 and IL-2 synergistically enhances the complete regression of established 3LL tumors**

In light of their ability to synergistically up-regulate the production of IFN-γ, a cytokine known to be central to the development of adaptive anti-tumor immune responses (20), we speculated that IL-18 and IL-2 might also synergize for the induction of an anti-tumor immune response against a well-established primary tumor. In fact, twice-daily administration of IL-18 in combination with intermittent administration of IL-2 synergistically induced the complete regression of well-established 3LL tumors (Fig. 2). Sixteen of 20 mice (80%) treated with IL-18 plus IL-2 achieved curative responses compared with 5 of 20 mice (25%) treated with either IL-18 or IL-2 alone and 1 of 20 control mice (5%) treated with vehicle alone (Fig. 2; $p = 0.001$, IL-18 plus IL-2 vs IL-18 or IL-2 alone). An additional mouse in the IL-18 plus IL-2 group had undergone complete tumor regression, but was excluded from consideration after dying secondary to a traumatic injection. Notably, we also have found that 50% of mice cured of their original tumor with IL-18/IL-2 are resistant to rechallenge with viable 3LL cells compared with 0% of rechallenge control mice, demonstrating that immunologic memory responses are generated in these mice as the result of an initial adaptive immune response.

**Roles of T/B cells, IFN-γ, and IL-12 in the anti-tumor immune response induced by systemic administration of IL-18/IL-2**

The potential mechanisms mediating the potent anti-tumor activity of IL-18/IL-2, including the role of an intact immune system, and
the Th1 cytokines IFN-γ and IL-12 were subsequently investigated. Tumors were significantly larger on day 23 after implantation in vehicle-treated SCID (p = 0.0001) and GKO (p = 0.0002) mice and to a lesser degree in p40⁻/⁻ (p = 0.0015) mice compared with wild-type controls (Fig. 3A). Median tumor volumes among these groups were as follows: wild-type control, 150 mm³; p40⁻/⁻ control, 2197 mm³; GKO control, 7822 mm³; and SCID control, 6804 mm³ (Fig. 3A). Thus, a significant, but incomplete, endogenous immune response against 3LL occurs in vivo, and this response is critically dependent on endogenous IFN-γ and to a lesser degree on IL-12 production. An appreciable incidence of complete durable tumor regression was induced only in wild-type mice treated with IL-18/IL-2 (6 of 10 mice; 60%) or p40⁻/⁻ mice treated with IL-18/IL-2 (4 of 10 mice; 40%; Fig. 3B). No complete responses were induced by IL-18/IL-2 in either GKO or SCID mice. Thus, the ability of IL-18/IL-2 to induce complete responses in wild-type mice was significantly (p = 0.011) greater than its effect in GKO or SCID mice, but not in p40⁻/⁻ mice (p = 0.656). Collectively, these findings demonstrate that the production of both IFN-γ and IL-12 is an important component of the endogenous host immune response against an established 3LL tumor, but that only IFN-γ is critical for the anti-tumor response engaged by systemic administration of IL-18/IL-2.

Roles of T, NK, and NKT lymphocytes in the anti-tumor activity of IL-18/IL-2

The roles of specific T cell subsets in the anti-tumor activity of IL-18/IL-2 were defined in 3LL-bearing mice concurrently depleted of CD4⁺ vs CD8⁺ T lymphocytes (Fig. 4). Complete durable tumor regression was only induced by IL-18/IL-2 in nondepleted (8 of 10, 80%) or CD4-depleted (10 of 10, 100%) mice, while mice depleted of CD8⁺ T cells were completely refractory (0 of 10, 0%) to the anti-tumor effects of IL-18/IL-2. Thus, concurrent depletion of CD8⁺, but not CD4⁺, T cells ablates the anti-tumor activity of IL-18/IL-2 vs established 3LL. In subsequent studies we investigated the role of NK vs NKT cells as mediators of the anti-tumor activity of IL-18/IL-2 vs 3LL. Although administration of NK1.1 markedly depleted both NK (NK1.1⁺ CD3⁻; Fig. 5A) and NKT (NK1.1⁺ CD3⁺; Fig. 5B) cells in the liver and NK cells in the spleen (not shown), concurrent depletion of NK and/or NKT cells had no impact on the therapeutic efficacy of IL-18/IL-2 (Fig. 6). One surviving control mouse and another mouse in the group treated with IL-18/IL-2 and anti-NK1.1 had large progressive tumors at the last follow-up. All other surviving mice in the respective groups were tumor free. Complete durable tumor regression was induced in 12 of 15 (80%) mice treated with IL-18/IL-2 vs eight of 11 (73%) mice treated with IL-18/IL-2 and concurrently depleted of NK and NKT cells via administration of anti-NK1.1 Ab and zero of 10 (0%) control mice treated with vehicle alone.

Role of the Fas/Fas-L pathway in tumor regression induced by systemic administration of IL-18/IL-2

In light of the role of CD8⁺ T cells, we speculated that the Fas/Fas-L pathway might contribute to the anti-tumor activity of IL-18/IL-2. Notably, the tumor volumes were significantly larger on day 21 postimplantation among LPR (p = 0.0003) and GLD (p =

**FIGURE 3.** The role of an intact immune system, IFN-γ, and IL-12 in the anti-tumor activity of IL-18/IL-2. Cohorts of 9–11 C57BL/6 wild-type, GKO, p40⁻/⁻, or SCID mice/group received s.c. implants of 10⁶ 3LL tumor cells. Seven days after tumor implantation, mice in the respective groups were treated with IL-18 and IL-2 or vehicle as described in Materials and Methods. A, The estimated tumor volumes on day 23 post tumor implantation in the respective wild-type, GKO, p40⁻/⁻, and SCID control mice treated with vehicle alone. ○, Values for individual mice; ■, median values for the respective groups. B, Survival of mice in the respective strains after treatment with IL-18 with or without IL-2 or vehicle alone. Mice surviving at the last follow-up were tumor free unless otherwise noted.

**FIGURE 4.** The roles of T cell subsets in the anti-tumor activity of IL-18/IL-2. Cohorts of 10 C57BL/6 mice/group received s.c. implants of 10⁶ 3LL tumor cells and were treated with three cycles of IL-18 and IL-2 or vehicle alone as described in Materials and Methods. Anti-CD4 (GK 1.5) or anti-CD8 (19/178) mAbs were administered as described in Materials and Methods to deplete CD4⁺ or CD8⁺ T lymphocyte subsets, respectively. Mice surviving at the last follow-up were tumor free unless otherwise noted.
0.0002) mice treated with vehicle alone than in wild-type controls (Fig. 7A). Median tumor volumes among these groups were as follows: wild-type controls, 322 mm³; GLD controls, 5372.5 mm³; and LPR controls, 5994 mm³. Among wild-type, LPR, or GLD mice treated with IL-18/IL-2 or vehicle alone (Fig. 7B), an appreciable incidence of complete durable tumor regression was induced only in wild-type mice (9 of 10, 90%). The effectiveness of IL-18/IL-2 was significantly greater in wild-type C57BL/6 mice than in LPR (p < 0.001) or GLD (p < 0.005) mice. One GLD mouse treated with IL-18/IL-2 was excluded from analysis after it became sick and had to be euthanized on day 45. These findings demonstrate a critical role for the Fas/Fas-L system in both the endogenous immune response against 3LL and the anti-tumor effects induced by IL-18/IL-2.

Role of tumor vs host Fas responsiveness in the anti-tumor activity of IL-18/IL-2

Having demonstrated that an intact Fas/Fas-L pathway was an essential component of both the endogenous immune response against established 3LL and the anti-tumor mechanisms engaged by administration of IL-18/IL-2, we subsequently investigated the relative roles of tumor vs host cell Fas responsiveness in mediating...
the efficacy of this combination. Notably, although in vitro treatment of 3LL with IFN-\(\gamma\) with or without TNF-\(\alpha\) potently up-regulates the expression of Fas on the surface of 3LL as assessed by flow cytometry (Fig. 8), this does not translate to enhanced sensitivity to Fas-mediated killing upon subsequent exposure to Jo2 Ab (data not shown). Nonetheless, 3LL tumor cells engineered to overexpress cell surface Fas are exquisitely sensitive to Jo2, demonstrating that these cells possess the intracellular signaling mechanisms necessary to undergo Fas-mediated killing (J. K. Lee, T. C. Back, and R. H. Wilt trout, manuscript in preparation). To definitively address the role of tumor vs host cell Fas responsiveness in the anti-tumor activity of IL-18/IL-2, we established 3LL tumor cells (3LL-DN) engineered to overexpress a dominant-negative Fas molecule lacking the intracellular signaling domain. These 3LL-DN tumor cells overexpress cell surface Fas as detected by flow cytometry, but are completely resistant to Fas-mediated killing in response to in vitro treatment with Jo2 (data not shown). Although IL-18/IL-2 induces complete regression of tumors established with either wild-type 3LL (10 of 15, 67%) or dominant-negative 3LL (9 of 10, 90%) established in wild-type mice, it only rarely induces complete regression of either 3LL (1 of 9, 11%) or 3LL-DN (1 of 10, 10%) tumors established in LPR mice with dysfunctional Fas (Fig. 9; wild-type host/wild-type 3LL tumor vs wild-type host/dominant-negative 3LL tumor, \(p = 0.3449\); wild-type host/wild-type 3LL tumor vs LPR host/wild-type 3LL tumor, \(p = 0.0131\) or LPR host/dominant-negative 3LL tumor, \(p = 0.0119\); wild-type host/dominant-negative 3LL tumor vs LPR host/ wild-type 3LL tumor, \(p = 0.0011\) or LPR host/dominant-negative 3LL tumor, \(p = 0.0011\)). These studies demonstrate that although an intact endogenous Fas/Fas-L pathway is a critical component of the anti-tumor mechanisms induced by IL-18/IL-2 host but not tumor, cell Fas responsiveness is a necessary component of these mechanisms.

**Discussion**

Although IL-2 has been approved for use in the management of patients with advanced melanoma or renal cell carcinoma, its overall utility has been limited by the occurrence of significant toxicities with some high dose regimens. Further, IL-2 and other single cytokines, such as IL-12, have demonstrated only modest clinical benefits to date. However, there is considerable evidence that the immunoregulatory effects of IL-2 can be potentiated by IFN-\(\gamma\)-inducing cytokines, such as IL-12. IL-12 and IL-2 synergistically enhance various aspects of T and/or NK cell function, including proliferation, IFN-\(\gamma\) production, and cytolytic activity in vitro (22).

**FIGURE 8.** Cell surface Fas expression on 3LL cells treated in vitro with IFN-\(\gamma\) with or without TNF-\(\alpha\). 3LL cells were incubated at 37°C for 12 h with medium alone, IFN-\(\gamma\) (100 U/ml), TNF-\(\alpha\) (100 U/ml), or a combination of IFN-\(\gamma\) (100 U/ml) plus TNF-\(\alpha\) (100 U/ml). The expression of Fas was analyzed using flow cytometry. The dotted lines, solid lines, and shaded curves represent immunostaining with the isotype control Ab, anti-Fas Ab (Jo2) on cells in medium, and anti-Fas Ab (Jo2) on cells treated with cytokine, respectively.

Further, we have previously reported (14, 23) that systemic administration of IL-12/pulse IL-2 induces rapid and complete regression of even well-established primary and/or metastatic tumor in up to 88–100% of treated mice compared with 20% or fewer complete responses among mice treated with either single agent alone, and does so via mechanisms that are dependent on the induction of endogenous IFN-\(\gamma\)-production.

IL-18 is another potent IFN-\(\gamma\)-inducing cytokine (1, 2) with substantial preclinical anti-tumor activity (8–13). Tumor cells engineered to produce IL-18 are significantly less tumorigenic in vivo (8–10), and systemic administration of IL-18 protein has demonstrated therapeutic activity in several murine tumor models (11–13). Although combined systemic administration of IL-18/IL-12 protein is prohibitively toxic in murine tumor models (12), synergistic anti-tumor effects are achieved with systemic delivery of IL-12 in conjunction with IL-18 gene therapy (8), or systemic IL-18 in combination with IL-12 gene therapy (24), with appreciable reductions in toxicity. Concurrent vaccination with tumor cells engineered to overexpress IL-12 and IL-18 synergistically inhibits the establishment of wild-type tumor cells, and induces the regression of some early established tumors implanted at a distant site (25). Further, combined peritumoral injection of IL-18/IL-1\(\beta\)-converting enzyme/IL-12 cDNA has been reported to enhance the complete regression of established tumors (26). We have shown here that IL-18 and IL-2 synergistically enhance the production of IFN-\(\gamma\) by murine splenocytes, and a previous report suggests that IL-18 and IL-2 may do so more effectively than the combination of IL-12 and IL-2 (15). Several other studies also have demonstrated that IL-18 may markedly potentiate the effects of IL-2 on several aspects of T and/or NK cell function in vitro (15–18), suggesting that in combination these cytokines also may demonstrate synergistic anti-tumor activity. In a model of well-established primary tumor, where IL-18 or IL-2 alone has only modest therapeutic activity, we have now demonstrated that IL-18 and IL-2 synergize to induce complete and durable tumor regression in >80% of treated mice, and that immunologic memory responses are generated in many of these mice cured of their tumors with IL-18/IL-2. It is notable that this regimen consisting of 5-day cycles of IL-18 administered in conjunction with pulsatile intermittent dosing of IL-2 is not only therapeutically effective, but well tolerated as well.

**FIGURE 9.** Role of tumor vs host Fas responsiveness in the anti-tumor activity of IL-18/IL-2. Cohorts of 10 C57BL/6 wild-type or LPR mice/group received s.c. implants of 10\(^6\) wild-type 3LL or dominant-negative (DN) Fas 3LL-DN tumor cells. Mice were treated with three cycles of IL-18 and IL-2 or vehicle alone as described in Materials and Methods. The survival of mice in the respective strains after treatment with IL-18 with or without IL-2 or vehicle alone is shown. Mice surviving at the last follow-up were tumor free unless otherwise noted.
This contrasts quite markedly with the severe pulmonary toxicity observed in mice treated chronically with continuous cycles of both IL-18 and IL-2 for 10 days without interruption (27). In conjunction with our previous report regarding the potent anti-tumor activity of IL-12/pulse IL-2 and the severe toxicity of chronically administered IL-12 and IL-2 compared with IL-12/pulse IL-2 (14, 23), these observations suggest that careful evaluation of specific schedules and dosing regimens will probably be required to optimize the tolerance and therapeutic potential of new combination cytokine regimens as they are translated into the clinical setting.

The present studies also demonstrate that there is an active, but ultimately ineffective, endogenous immune response against 3LL, and that this response is critically dependent on IFN-γ and IL-12 production. Although others have reported that the anti-tumor activity of IL-18 gene therapy is IL-12 independent (24) and IFN-γ dependent (9, 25, 26) in several murine tumor models, systemic administration of IL-18 protein alone induces tumor regression via mechanisms independent of both endogenous IL-12 and IFN-γ production (12). We have shown here that combined systemic administration of IL-18 and IL-2 engages and amplifies this endogenous immune response against 3LL and induces tumor regression via mechanisms that are dependent on IFN-γ, but not IL-12 production. Thus, the current findings demonstrate that the anti-tumor mechanisms engaged by IL-18/IL-2 may contrast with those induced by IL-18 alone, and that although IL-12 is an important component of the endogenous immune response against 3LL, it does not appear to be central to the anti-tumor activity of IL-18/IL-2.

Both NK and T cell subsets have been shown to play a role in the anti-tumor activity of IL-18 alone in various murine tumor models. Several IL-18-based gene therapy approaches induce tumor regression via mechanisms dependent on CD8+ and/or CD4+ T cells (9, 26), while peritumoral injection of adenosine-overexpressing IL-18 induces tumor regression via mechanisms that are dependent on NK, but not IL-12 production. Thus, the current findings demonstrate that the anti-tumor mechanisms engaged by IL-18/IL-2 may contrast with those induced by IL-18 alone, and that although IL-12 is an important component of the endogenous immune response against 3LL, it does not appear to be central to the anti-tumor activity of IL-18/IL-2.

Because CD8+ T cells are vital for the anti-tumor activity of IL-18/IL-2, we speculated that the Fas/Fas-L pathway might play a role in mediating the tumor regression induced by this combination. IL-18 alone can activate cytolytic CD8+ T cells (16) and Fas-L mediated cytolytic activity by T and/or NK cells in vitro (5–7). Our results revealed that the Fas/Fas-L pathway is also an important component of the endogenous immune response against established 3LL as well as an essential mediator of the anti-tumor activity of IL-18/IL-2. Nonetheless, IL-18/IL-2 administration modestly prolongs the survival of 3LL-bearing LPR or GLD mice compared with mice treated with vehicle alone, suggesting that although the ability of IL-18/IL-2 to induce complete tumor regression is critically dependent on Fas and Fas-L mechanisms that can at least delay tumor growth independent of Fas/Fas-L can be engaged by IL-18/IL-2. In contrast, administration of IL-18 protein alone limits tumor establishment via mechanisms that are dependent on Fas-L, but not Fas (11), and administration of IL-12 protein efficiently induces 3LL tumor regression via mechanisms that are independent of Fas/Fas-L (J. M. Wigginton and R. H. Wiktrout, unpublished observations). Collectively, these findings demonstrate that the Fas/Fas-L pathway is an important component of both the endogenous and cytokine-induced immune responses against 3LL and show that the spectrum of anti-tumor mechanisms induced by IL-18/IL-2 differs from those mechanisms that have been attributed to the therapeutic effects of treatment with IL-18 or IL-12 alone. We have shown previously that combined administration of IL-12/pulse IL-2 inhibits tumor neovascularization and induces vascular endothelial injury and complete tumor regression via mechanisms that are critically dependent on the presence of an intact Fas/Fas-L pathway (23). Consistent with these observations, our studies using wild-type vs LPR (with dysfunctional host Fas) mice bearing established wild-type 3LL vs 3LL-DN (engineered to overexpress dominant-negative, nonfunctional Fas) tumors demonstrate that although tumor cell Fas responsiveness is not an essential component of the anti-tumor mechanisms engaged by IL-18/IL-2, host Fas responsiveness is essential for complete tumor regression induced by IL-18/IL-2. Notably, investigators recently have demonstrated a critical role for the Fas/Fas-L pathway in mediating the potent anti-angiogenic activity of thrombospondin-1 in vivo as well (28). Collectively, these observations and an increasing body of literature evidence suggest that the Fas/Fas-L pathway may play a critical role in a range of endogenous mechanisms governing tumor neovascularization as well as the spectrum of anti-tumor mechanisms induced by potent immunoregulatory cytokines such as IL-12 and IL-18. The specific impact of IL-18/IL-2 on endothelial and other host cell targets as well as the overall process of tumor neovascularization remains to be defined and is a focus of active investigation in our laboratory.

As has been observed during the evolution of combination chemotherapy strategies, optimal therapeutic enhancement of host anti-tumor mechanisms with biologics will most likely be achieved with rationally designed approaches combining various agents with complementary mechanisms of action. These may include cytokines administered in combination or in conjunction with other biologics, such as targeted inhibitors of tumor neovascularization or intracellular signal transduction. Such approaches offer the prospect for more potent anti-tumor activity with reduced toxicity compared with approaches using high doses of various single agents alone. The present studies describe an approach using IL-2 administered in conjunction with IL-18, a potent IFN-γ-inducing cytokine (1, 2), which results in profound anti-tumor activity that depends on CD8+ T cells, IFN-γ, and the Fas/Fas-L pathway. These results demonstrate the engagement of a novel therapeutic mechanism and suggest that clinical investigation of the immunoregulatory and anti-tumor activities of combined administration of IL-18 and IL-2 may be warranted.

Acknowledgments

We gratefully acknowledge the generous support of these studies by the Children’s Cancer Foundation. We also thank Dr. Kristin Komschlies for supplying the anti-CD4, anti-CD8, and anti-NK1.1 depletion Abs, and Joyce Vincent and Susan Charbonneau for editorial assistance and manuscript preparation.

References

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