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Role of Effector Cell-Derived IL-4, IL-5, and Perforin in Early and Late Stages of Type 2 CD8 Effector Cell-Mediated Tumor Rejection

Mark J. Dobrzanski, Joyce B. Reome, and Richard W. Dutton

Type 2 CD8 T cells (Tc2) secrete IL-4 and IL-5 and display perforin-dependent cytolysis in vitro. Using an OVA-transfected B16-melanoma model, we show that tumor-reactive Tc2 effector cells accumulated at the tumor site and induced tumor regression that enhanced survival in mice with pulmonary tumors. Transfer of perforin-deficient Tc2 cells generated from perforin gene knockout mice showed no differences in therapeutic efficiency when compared with wild-type Tc2 cells. In contrast, Tc2 cells derived from select cytokine gene-deficient mice showed that therapeutic effects were dependent on effector cell-derived IL-4 and IL-5 that led to a local elevation in lung-derived chemokine production and accumulation of activated host-derived CD8/CD44^high, CD4/CD44^high, and OVA-specific tetramer-positive CD8 cells in vivo. Host-derived T and non-T immune cells increased in the lung over time and correlated with an elevated production of type 1-related chemokines. Conversely, donor Tc2 cell numbers markedly diminished at later times, suggesting that prolonged therapeutic responses were due to host-derived mechanisms. Moreover, type 1 host responses were detectable with increased levels of IFN-γ production by lung-derived CD4 and CD8 T cells from surviving Tc2-treated mice. Transfer of Tc2 cells into IFN-γ-deficient tumor-bearing mice was markedly less effective than into wild-type mice, suggesting that host-derived IFN-γ-dependent mechanisms play a role in Tc2-mediated antitumor responses. The Journal of Immunology, 2001, 167: 424–434.

B oth type 1 (IFN-γ) and type 2 (IL-4, IL-5, and IL-10) cytokines have been demonstrated to be useful in various cancer therapies (1–5). In the latter, protection against tumor challenge was strongly associated with the presence of eosinophils and macrophages (1, 3, 6); however, the local recruitment and activation of T cells still appears to be critical for the generation and persistence of long-lasting systemic immunity (1, 7–10). Moreover, such therapeutic responses appeared to be highly dependent on chemokines that can preferentially recruit select T cell populations to the site of tumor growth (11). Studies from this laboratory and others have demonstrated that differential migration patterns by various effector T cell subpopulations are dependent, in part, on diverse chemokines and the expression of T cell subset-specific chemokine receptors (unpublished observations and Refs. 12–16). Thus, enhanced local chemokine production at the site of tumor growth may play a role in the regulation of tumor cell growth and metastases and involve the participation and orchestration of specific effector cell types and their soluble products.

As in the case with CD4 T cells, CD8 T lymphocytes can be further classified into distinct effector cell types based on their cytokine-secreting profiles after tumor Ag encounter (17–23). Type 2 CD8^+ T cells (Tc2)^3 preferentially secrete IL-4, IL-5, IL-10, and IL-13 and kill predominantly by the perforin pathway, whereas type 1 CD8 T cells predominantly secrete IFN-γ and kill by either perforin or Fas-mediated mechanisms (22, 24). Although Tc2 effector cell subpopulations have been identified in human peripheral blood and in patients with various clinical disorders (25–28), their role in tumor rejection and cancer immunotherapy remains relatively undefined.

In the current study, we investigated the therapeutic mechanisms involved in the effector phase of tumor rejection induced by adoptively transferred Tc2 effector cells in vivo. Using a previously described OVA Ag-expressing B16 melanoma lung metastasis model (17–19), we examined the antitumor mechanisms involved in tumor rejection by OVA Ag-specific Tc2 effector cells and the role(s) of effector cell-derived perforin, IL-4, and IL-5. At early time points after therapy, tumor-reactive Tc2 effector cells accumulated at the tumor site and induced tumor regression that subsequently enhanced survival in mice with established pulmonary tumors. On adoptive transfer of perforin-deficient Tc2 effector cells, generated from perforin gene knockout OT-I mice, we show no differences in therapeutic efficiency when compared with that of wild-type Tc2 effector cells. In contrast, Tc2 cells derived from select cytokine gene-deficient OT-I mice showed that therapeutic effects were dependent on perforin- and non-T immune cells significantly increased in the lung, which correlated with an elevated production of IP-10, RANTES, and MIP-1β and a continued reduction in tumor burden. Conversely, donor Tc2 effector cell numbers markedly diminished at corresponding times, suggesting that prolonged therapeutic

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1 Abbreviations used in this paper: Tc2, CD8^+ CTL-producing type 2 cytokines; B16-OVA, OVA-expressing B16 melanoma; OT-IL-5 mice, OVA TCR^+ IL-5 knockout mice; OT-IL-5 mice, OVA TCR^+ IL-5 knockout mice: OT-1PDKO mice, OVA TCR^+ perforin knockout mice; IP-10, IFN-inducible protein-10; MIP, macrophage-inflammatory protein; FAB, fluorescent Ab buffer.
responses were due, in part, to the presence of host-derived anti-tumor mechanisms. Moreover, local type 1-like immune responses were detectable with increased levels of IFN-γ production by lung-derived host CD4 and CD8 T cells from Tc2-treated tumor-bearing mice. Adaptive transfer of Tc2 effector cells into syngeneic IFN-γ-deficient tumor-bearing mice were less effective than into wild-type recipients. This suggest that recipient-derived IFN-γ plays a significant role in Tc2 cell-mediated antitumor responses and that Tc2 effector cell immunotherapy is predominantly mediated at later stages after therapy by type 1-like IFN-γ-dependent antitumor responses of the host.

Materials and Methods

Animals

Female C57BL/6 mice, 6 to 10 wk of age, were obtained from the Animal Breeding Facility at Trudeau Institute (Saranac, NY). The OT-I mouse strain (Thy-1.2), on a C57BL/6 background (H-2b), was originally obtained from Dr. M. Bevan (University of Washington, Seattle, WA). These mice express a transgenic TCR Vα2 and Vβ5 specific for the SIINFEKL peptide of OVA in the context of MHC class I, H2-Kb (29). Perforin−/−, IL-4−/−, IL-5−/−, IFN-γ−/−, and B6.PL/Thy-1.1 mice, on a B6 background, were purchased from The Jackson Laboratory (Bar Harbor, ME). Homozygous perforin−/− (OT-I.PKO), IL-4−/− (OT-I-4L4), IL-5−/− (OT-I-5L5), and IFN-γ−/− (OT-I-IFN-γ−/−) knockout mice, expressing the TCR Vα2 and Vβ5 transgenes, were generated by backcrossing OT-I mice onto specified syngeneic knockout mice (H-2b) for more than two generations. Animals were maintained and treated according to animal care committee guidelines of the National Institutes of Health and Trudeau Institute.

Tumor cells

The weakly immunogenic OVA-transfected B16 melanoma tumor cell line (B16-OVA) that is syngeneic to the C57BL/6 background was kindly provided by Drs. E. Lord and J. Frelinger (University of Rochester, Rochester, NY). EL4 and the derivative OVA-expressing EG7-OVA cell lines were obtained from the American Type Culture Collection (Manassas, VA).

Lung cell preparation

For preparation of single-cell suspensions from lung parenchyma, lungs were flushed in situ with HBSS via cannulation of the heart to remove residual intravascular blood pools. Minced lung tissues were incubated for 1 h at 37°C on a rocker platform, in 1.5 ml/lung RPMI 1640 supplemented with D-Nase I (50 U/ml; Sigma, St. Louis, MO), collagenase I type 4197 (250 U/ml; Sigma), and 5% FCS. After incubation, digested lung tissues were mechanically dispersed through stainless steel mesh screens in RPMI 1640–5% FCS. After three washes in RPMI 1640–5% FCS, lymphoid cells were resuspended in RPMI 1640 (Life Technologies, Gaithersburg, MD), supplemented with 2 mM pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, and 10% heat-inactivated FCS (Life Technologies) to attain a cell concentration of 1 × 10⁷ viable cells/ml.

Generation of OVA-specific CD8 effector T cells

To obtain effector cells to OVA peptide, single-cell suspensions from spleen and lymph nodes of OT-I mice were washed twice in HBSS and resuspended in RPMI 1640–10% FCS. CD8-enriched T cells were obtained by passing lymphoid cell suspensions through nylon wool columns and adjusting with anti-CD4 (RL172.4), anti-heat-stable Ag (31D), and anti-MHC class II (D3.137, M5114, CA4) mAbs and complement. Small resting CD8 T cells were harvested from Percoll gradients (Sigma) and resuspended to appropriate cell concentrations in culture medium. Naive CD8 cells were typically 90% pure as demonstrated by immunofluorescent Ab staining. APCs were enriched from spleens of normal C57BL/6 mice by anti-Thy-1.2 (H913.14 and F7D5), anti-CD4 (RL172.4), and anti-CD8 (3.155) mAbs and complement. T cell-depleted APCs were pulsed with OVA peptide (10 μM) for 30 min at 37°C and treated with mitomycin C (50 μg/ml; Sigma) for an additional 30 min at 37°C. For Tc2 effector cell generation, naive CD8 T cells from OT-I-transgenic mice (2 × 10⁶ cells/ml) were stimulated with mitomycin C-treated OVA peptide-pulsed APCs (6 × 10⁶ cells/ml) in the presence of IL-2 (20 U/ml; X6.5-II-2 supernatants, Ref. 30), and anti-IFN-γ mAb (20 μg/ml; XMG1.2). Effector cell cultures were incubated for 4 days with additional IL-2 (20 U/ml) added to the cultures on day 2 to promote CD8 cell expansion of Tc2 populations.

Adoptive immunotherapy model

Syngeneic B6 or B6.PL/Thy-1.1 mice were injected i.v. with 2 × 10⁶ B16-OVA melanoma cells to establish pulmonary metastases. Seven days after tumor challenge, mice were treated i.v. with various doses of Tc2 OVA-specific effector T cells and survival times monitored daily. Control groups received no treatment. At either early (5–12 days) or late (21–28 days) time points after Tc2 effector cell transfer, mice were sacrificed for enumeration of pulmonary metastatic nodules. Metastases on freshly isolated lungs appeared as discrete black pigmented foci that were easily distinguishable from normal lung tissue. The number of pulmonary metastases in treated and untreated control groups was counted in a blind manner. Metastatic foci too numerous to count were assigned an arbitrary value of >250. For survival studies, data are expressed as either the percent survival or the relative percent survival as a percent of mortality curves (31). The 95% confidence interval was determined by regression analysis (95% confidence intervals of values derived from the percent survival of tumor-bearing mice over time. The absolute value of the negative slope of the regression line is the mortality rate. Data are expressed as the ratio of the mortality rate for groups of effector cell-treated mice to corresponding groups of untreated mice × 100. The significance of the regression coefficients was determined by analysis of variance (31).

Flow cytometric analysis

Single-cell suspensions of processed murine lung were washed three times in a fluorescent Ab buffer (FAB) consisting of 1% BSA and 0.02% sodium azide in 0.1 M PBS, pH 7.2. Recipient immune cell populations were phenotyped by their expression of surface markers using direct immunofluorescence staining techniques. Lymphocytes (10⁶), pretreated with FcR block, were mixed with 100 μl FAB containing 1 μg/ml either fluorescein-conjugated anti-CD90.1 (Thy-1.1; BD Pharmingen; clone H155, 1; fluorescein-conjugated anti-CD90.2 (Thy-1.2; BD Pharmingen; clone 53-2.1), Cy-Chrome-conjugated anti-CD8 or anti-CD4 (BD Pharmingen), PE-conjugated CD44 (BD Pharmingen; clone IM7), PE-conjugated MAC-1 (CD11b), PE-conjugated CD19, or PE-conjugated NK1.1 mAbs and incubated for 20 min on ice. Stained cell preparations were then washed three times in FAB and analyzed by multicolor flow cytometry using a FACScan (BD Biosciences, San Jose, CA). For B6-OVA tumor cells, direct immunofluorescence staining with either fluorescein-conjugated class I H2-Kb, class I H2-Db, or class II Ld or PE-conjugated CD95, FasL, and CD44. Ten thousand cells were analyzed per sample with dead cells excluded on the basis of forward light scatter. Surface marker analysis was performed using CellQuest software (BD Biosciences), and the percent positive and absolute cell numbers were determined.

MHC tetrameric reagents and analysis

The construction of folded MHC class I-peptide complexes and their tetramerization have been described previously (32). Tetramers were generated by the Molecular Biology Core Facility at Trudeau Institute. The OVA tetramer in K‘/SIINFEKL epitope. No cross-reactivity was detected in studies using cell preparations from OT-I mice mixed with wild-type C57BL/6 or non-strain-related BALB/c mice. T cell-enriched populations from either spleen or lungs of either effector cell-treated or untreated tumor-bearing mice were incubated with FcR block for 10 min and stained with PE-conjugated tetrameric reagent for 1 h at 20°C, followed by staining with Tricolor anti-CD8 and FITC-Thy-1.1 or FITC-Thy-1.2 mAbs on ice for 20 min. Stained samples were washed and analyzed using a FACScan flow cytometer and CellQuest software.

Intracellular cytokine staining

Single-cell suspensions were obtained from lungs of effector cell-treated or untreated tumor-bearing mice as described above. Cells were stimulated with PMA (10−7 M) and ionomycin (1 μg/ml) for 4 h. Two hours before harvesting, brefeldin A (10 μg/ml) was added to cultures to retain cytoplasmic cytokines. Cells were pretreated with FcR block followed by either/for FITC-conjugated anti-CD90.1 (Thy-1.1; BD Pharmingen; clone HIS51), FITC-conjugated anti-CD90.2 (Thy-1.2; BD Pharmingen; clone 53-2.1), Cy-Chrome-conjugated anti-CD8, or anti-CD4 (BD Pharmingen). Subsequently, cells were fixed with 2% paraformaldehyde followed by intracellular staining with fluorescein isothiocyanate-saponin and 1% BSA in PBS and PE-conjugated IFN-γ, IL-4, or IL-10 (BD Pharmingen). Cells were washed and resuspended in 1% BSA-PBS solution and analyzed by flow cytometry. Data were analyzed using CellQuest software.
Cytokine/chemokine mRNA expression in lung tissue

Lungs were flushed in situ with HBSS via cannulation of the heart to remove residual intravascular blood pools. Total RNA from either cell cultures or whole lungs of effector cell-treated or untreated tumor-bearing mice were prepared by tissue homogenization in TRIzol reagent (Life Technologies). mRNA levels were quantitated using the RiboQuant Multiprobe Ribonuclease Protection Assay system (BD PharMingen) with the mCK-1, mCK-3, and mCK-5 cytokine/chemokine mRNA detection probe sets. Bands were detected using the Molecular Imager FX with the Quantity One Software analysis program (Bio-Rad Laboratories, Hercules, CA) and normalized against the L32 housekeeping gene as relative U.

Statistical analysis

For statistical analysis, the two-tailed Student t test or nonparametric Mann-Whitney rank sum test was used.

Results

Phenotypic characterization of in vitro-generated OVA-specific Tc2 effector T cells

CD8+ Tc2 effector T cells were generated in vitro from OVA-specific TCR-transgenic OT-1 mice as described in Materials and Methods. As shown in earlier in vitro studies (17), Tc2 effector cells demonstrated potent tumor Ag-specific cytolytic activity to OVA Ag-expressing tumor cell targets (EG7-OVA) that was predominantly mediated by the perforin pathway. Furthermore, Tc2 populations released considerable amounts of IL-5 (>20,000 U/ml/10^6 cells in 24 h) and IL-4 (>20 ng/ml/10^6 cells in 24 h), with low yet detectable levels of IFN-γ on restimulation with OVA-expressing tumor cells. Flow cytometric analysis showed that Tc2 effector cell populations expressed similar patterns of cell surface Ag markers that are characteristic of effector cell phenotype (17). Effector cell populations were generated from OVA-expressing B16 parent line showed no detectable therapeutic effect in survival times when compared with those of untreated tumor-bearing mice (Fig. 1B). Both cytokine gene-deficient effector cell populations were less effective in prolonging survival times than those of corresponding groups of tumor-bearing mice receiving similar doses of wild-type Tc2 effector cell therapy. All mice receiving no treatment succumbed to progressively growing tumors within 31 days post-tumor challenge (Fig. 1, A and B). Transfer of either wild-type or cytokine gene-modified Tc2 effector cells into mice challenged with the non-OVA Ag-expressing B16 parent line showed no detectable therapeutic effect in survival times when compared with those of untreated B16 tumor-bearing control mice (data not shown). This suggests that Tc2 effector cell-derived IL-4 and IL-5, but not perforin, play a significant and unique role in Tc2 effector cell-mediated tumor Ag-specific immunity and rejection.

To quantitatively assess the therapeutic efficacy of effector cell-derived IL-4 and IL-5 in Tc2 effector cell-mediated antitumor responses, titration studies of donor Tc2 effector cell populations were performed. As shown in Fig. 1C, groups of mice receiving wild-type Tc2 effector cell populations showed a marked decrease in the relative percent rate of mortality among groups of tumor-bearing mice when compared with that of mice receiving either IL-4- or IL-5-deficient Tc2 effector cells. Moreover, the absolute mortality rate (100%) among groups of tumor-bearing mice were delayed with administration of wild-type effector cells at numbers as low as 1.0 x 10^5. Whereas corresponding groups of tumor-bearing mice receiving similar doses of Tc2 effector cells deficient in either IL-4 or IL-5 showed no therapeutic effect (100% mortality) and only when a 5-fold higher effector cell number (5 x 10^5) did groups of mice start to show diminished rates of group mortality (75-90%) among tumor-bearing mice. Moreover, at higher doses of effector cell transfer, the relative death rates among IL-4-deficient Tc2-treated mice was lower than that of corresponding groups of IL-5-deficient Tc2-treated mice (Fig. 1C). These results suggest that, on a per cell basis, wild-type Tc2 effector cells are 5-fold more therapeutically effective than corresponding IL-4- or IL-5-deficient effector cells and that alternative mechanisms of action by effector cell-derived IL-4 and IL-5 may be involved in lowering the rates of mortality among tumor-bearing mice.

Exogenous IL-4 and IL-5 have no direct effects on B16-OVA tumor cell growth or phenotype in vitro

We have previously shown that exogenous cytokines, such as IFN-γ, can substantially inhibit B16-OVA tumor cell growth and increase tumor cell immunogenicity by up-regulating select tumor cell surface Ag or chemokine and “death” (proapoptotic) gene expression in vitro (19). In the current study, we assessed the direct effects of the Tc2-related cytokines, IL-4 and IL-5, on B16-OVA tumor cell surface phenotype. B16-OVA tumor cells were cultured...
in the presence of IL-4 or IL-5 and harvested at various intervals for flow cytometric analysis. Tumor cell treatment with either IL-4 (20 ng/ml) or IL-5 (20,000 U/ml) induced no change in the surface expression of either MHC class I Ag (K\textsuperscript{b} and D\textsuperscript{b}), MHC class II Ag (I-A\textsuperscript{b}), CD95, or CD44 when compared with that of corresponding untreated B16-OVA tumor cell populations (data not shown). Moreover, cell counts at various time intervals during a culture period of 36 h showed that neither IL-4 nor IL-5 affected tumor cell growth in vitro (data not shown). Concomitantly, IL-4- or IL-5-treated tumor cells showed no change in the levels of either chemokine or “death” gene expression when compared with that of untreated cultures, suggesting that neither IL-4 nor IL-5 had any detectable direct effect on B16-OVA tumor cell phenotype (data not shown).

Tc2 effector cell immunotherapy induces local accumulation of activated recipient CD8\textsuperscript{T} cells that are partially dependent on donor effector cell-derived IL-4 or IL-5

Therapeutic efficacy of class I-expressing tumors is largely dependent on the localization and persistence of tumor-reactive CD8\textsuperscript{T} T cells at the site of tumor growth. We next attempted to assess the local accumulation of activated recipient CD8\textsuperscript{T} cells by using Thy-1.1 congenic mice. Normal syngeneic B6.PL/Thy-1.1 recipient mice were injected i.v. with $2 \times 10^5$ B16-OVA tumor cells. Seven days later, when metastases were established, $2 \times 10^6$ OVA Ag-specific Tc2 effector cells, generated from either Thy-1.2 wild-type OT-I, IL-4-deficient OT-I/IL-4\textsuperscript{−/−}, or IL-5-deficient OT-I/IL-5\textsuperscript{−/−} knockout mice were systemically transferred into tumor-bearing mice as previously described. Recipient Thy-1.1/CD8 cell subpopulations from lungs of effector cell-treated mice at early (5–12 days) or late (21–28 days) time points following effector cell therapy were enumerated by multicolor analysis. Because up-regulated CD44 expression is indicative of T cell activation and Ag recognition (33), we assessed the numbers of recipient Thy-1.1/CD8\textsuperscript{T} cell subpopulations from lungs of effector cell-treated mice at these time points.

Because Tc2 effector cell therapy induced accumulation and localization of activated recipient CD8\textsuperscript{T} cells, we assessed the level of CD8\textsuperscript{T} cell accumulation and persistence at sites of tumor growth. As shown in Fig. 2, both the absolute cell numbers and percentages of recipient Thy-1.1/CD8\textsuperscript{T} CD44\textsuperscript{high} cells in the lungs of mice receiving wild-type Tc2 effector cell therapy were significantly ($p < 0.05$) greater at early time points after effector cell transfer (days 5–12) than those in groups of either untreated IL-4-deficient Tc2 or IL-5-deficient Tc2-treated mice. In contrast, Thy-1.1/CD8\textsuperscript{T} CD44\textsuperscript{high} T cell numbers from groups of mice receiving IL-4- or IL-5-deficient Tc2 effector cells were not significantly ($p > 0.05$) different from that of corresponding groups of untreated tumor-bearing control mice (Fig. 2). Similar results were obtained in corresponding groups of mice at later (21–28 days) times after effector cell transfer (Fig. 2). This suggested that accumulation and activation of recipient Thy-1.1/CD8\textsuperscript{T} cells were dependent, in part, on donor effector cell-derived IL-4 and/or IL-5. Similar results were obtained in three independent experiments.

Adoptively transferred Tc2 effector cells localize and facilitate accumulation of recipient OVA Ag-specific CD8\textsuperscript{T} cells at the site of tumor growth

Because Tc2 effector cell therapy induced accumulation and localization of activated recipient-derived CD8\textsuperscript{T} cells at the site of tumor growth, we assessed the local accumulation of tumor Ag-specific CD8\textsuperscript{T} cells in tumor-bearing mice at either early or late stages after Tc2 effector cell therapy. Using Thy-1.1 congenic mice, donor (Thy-1.2) and recipient (Thy-1.1) CD8\textsuperscript{T} T cell populations, from lungs of effector cell-treated mice at either early (5–12 days) or late (21–28 days) stages of effector cell-mediated therapy, were enumerated by OVA Ag-specific tetramer staining and multicolor flow cytometric analysis. As shown in Fig. 3, OVA Ag-specific donor Thy1.2/CD8\textsuperscript{T} Tc2 effector cells accumulated in lungs of tumor-bearing mice at early time points after effector cell transfer. Similar proportions were obtained in corresponding groups of mice receiving either IL-4-deficient or IL-5-deficient Tc2 effector cells, suggesting that effector cell-derived IL-4 or IL-5
was not necessary for donor cell accumulation at the site of tumor growth. However, in all instances, the percentages of donor Thy-1.2/CD8+ T cells markedly diminished to nearly undetectable levels over time (Fig. 3). In contrast, the percentages and frequencies of recipient OVA Ag-specific Thy-1.1/CD8+ T cells persisted at both early and late time points after effector cell transfer, suggesting that Tc2 effector cell therapy can induce recipient tumor Ag-specific CD8+ T cell populations at sites of established tumor growth. Although untreated tumor-bearing mice did contain low yet detectable levels of OVA tetramer-positive staining Thy-1.1/CD8+ T cells, recipient CD8+ T cell percentages and frequencies remained comparatively lower than that of corresponding cell populations in lungs of effector cell-treated mice at later time points after therapy. Concomitantly, the number of lung metastases in mice receiving effector cell therapy was substantially lower than that of untreated tumor-bearing control mice at both early and late time points after cell transfer (Fig. 3). This suggested that although tumor-reactive Tc2 effector cell numbers diminished over time in tumor-bearing mice, recipient tumor Ag-specific CD8+ T cells persisted at the site of tumor growth and potentially prolonged antitumor immunity and survival in effector cell-treated mice with established pulmonary malignancy.

Tc2 effector cell immunotherapy induces local accumulation of activated recipient CD4+ T cells that are dependent on donor effector cell-derived IL-4 or IL-5

Because CD4+ T cells have been shown to express cytokines that participate in the recruitment and maturation of other antitumor effector cells to the sites of tumor growth (8, 10), we evaluated the accumulation and activation of recipient CD4+ T cells at the site of tumor growth after Tc2 effector cell-mediated therapy. Recipient Thy-1.1/CD8+ T cell subpopulations from lungs of effector cell-treated mice at either early (5–12 days) or late (21–28 days) time points after effector cell transfer were enumerated by multicolor flow cytometric analysis. As shown in Fig. 4, the cell numbers and percentages of activated early stage recipient CD4+ T cell subpopulations, coexpressing elevated levels of CD44 surface Ag (Thy-1.1/CD4+/CD44high), were significantly (p < 0.05) greater in the lungs of mice receiving wild-type Tc2 effector cell therapy than that of either untreated or cytokine-deficient Tc2 effector cell-treated mice. Moreover, Thy-1.1/CD4+CD44high T cell numbers from groups of mice receiving IL-4- or IL-5-deficient Tc2 effector cells were not significantly (p > 0.05) different from that of corresponding groups of untreated tumor-bearing control mice (Fig. 4). Similar results were obtained in corresponding groups of mice at later (>21 days) times after effector cell transfer (Fig. 4). This suggested that accumulation and activation of recipient Thy-1.1/CD4+ T cells were dependent, in part, on donor effector cell-derived IL-4 and/or IL-5. Similar results were obtained in three independent experiments.

Tc2 effector cell immunotherapy induces accumulation of NK and myeloid cell populations that are partially dependent on donor effector cell-derived IL-4 and IL-5

Because nonspecific inflammatory responses have been shown to effectively delay tumor progression (34), we assessed the kinetics of myeloid and NK cell recruitment to sites of tumor cell growth after Tc2 effector cell-mediated therapy. Lung metastases were...
IL-5 cytokine-deficient Tc2 effector cell therapies were not significantly different from that of corresponding groups of untreated tumor-bearing control mice (Fig. 5A). Cell numbers of lung-derived myeloid/macrophage populations (MAC-1+) were significantly (p < 0.03) elevated at all time points tested in groups of tumor-bearing mice receiving wild-type Tc2 effector cell therapy (Fig. 5B). In contrast, the absolute cell numbers of such cell populations in mice receiving IL-4 or IL-5 cytokine-deficient Tc2 effector cells were nearly 5- to 7-fold lower for both groups at corresponding stages of effector cell therapy. Moreover, myeloid cell population numbers from groups of mice receiving either cytokine-deficient Tc2 effector cell therapy were not significantly (p < 0.60) different from that of corresponding groups of untreated tumor-bearing control mice (Fig. 5B). This suggests that Tc2 effector cell therapy can effectively induce accumulation of host-derived myeloid and NK cell populations that are dependent, in part, on effector cell-derived IL-5 and/or IL-4.

FIGURE 3. Identification of recipient OVA-specific CD8+ T cells induced by Tc2 effector cell immunotherapy in mice bearing established lung metastases. Tumor-bearing mice (Thy-1.1) were treated with Tc2 effector cells (Thy-1.2) generated from OT-I-IL-4, OT-I-IL-5, or wild-type OT-I mice as described in Fig. 2. Lungs were harvested from either untreated or effector cell-treated groups of mice at early or late time points after therapy, and single-cell suspensions were obtained. Cells were labeled with FITC-Thy-1.1 or Thy-1.2, Tricolor-CD8, and PE-OVA (SIINFELK) tetramer and assessed by multicolor flow cytometry. Numbers indicate the percentages of total recipient (Thy-1.1+) or donor (Thy-1.2+)-OVA tetramer-positive staining T cells per lung. Results are representative of three to four mice per group. KO, Knockout.

Induced in normal syngeneic C57BL/6 mice by systemic injection of \(2 \times 10^5\) B16-OVA tumor cells. Seven days later, Tc2 effector cells were adoptively transferred into tumor-bearing mice as previously described. Lung cell populations from effector cell-treated tumor-bearing mice at either early (5–12 days) or late (21–28 days) time points after therapy were enumerated by single-color flow cytometry. As shown in Fig. 5A, the absolute cell numbers of host NK1.1+ cells from lungs of wild-type Tc2 effector cells were nearly 3- to 4-fold greater than that of corresponding cell populations from mice either untreated or treated with either IL-4 or IL-5 cytokine-deficient Tc2 effector cells. Moreover, NK1.1+ cell numbers from groups of mice receiving either IL-4 or IL-5 cytokine-deficient Tc2 effector cell therapies were not significantly (p < 0.60) different from that of corresponding groups of untreated tumor-bearing control mice (Fig. 5A).

Cell numbers of lung-derived myeloid/macrophage populations (MAC-1+) were significantly (p < 0.03) elevated at all time points tested in groups of tumor-bearing mice receiving wild-type Tc2 effector cell therapy (Fig. 5B). In contrast, the absolute cell numbers of such cell populations in mice receiving IL-4 or IL-5 cytokine-deficient Tc2 effector cells were nearly 5- to 7-fold lower for both groups at corresponding stages of effector cell therapy. Moreover, myeloid cell population numbers from groups of mice receiving either cytokine-deficient Tc2 effector cell therapy were not significantly (p < 0.60) different from that of corresponding groups of untreated tumor-bearing control mice (Fig. 5B). This suggests that Tc2 effector cell therapy can effectively induce accumulation of host-derived myeloid and NK cell populations that are dependent, in part, on effector cell-derived IL-5 and/or IL-4.

Because Tc2 effector cell therapy induced accumulation of activated recipient immune cell populations in mice with established pulmonary malignancy, we investigated the expression of lung-derived chemokine genes in mice at either early or late stages of effector cell therapy. RNase protection assays were performed to assess chemokine gene expression among whole lungs of tumor-bearing mice either untreated or receiving wild-type Tc2 effector cell therapy. As shown in Fig. 6, the latter showed a consistent and preferential elevation in the type I-related chemotactants IP-10, MIP-1β, and MIP-1α at early time points after therapy when compared with that of the former. Moreover, expression of IP-10, MIP-1β, and MIP-1α was significantly (p < 0.05) lower at corresponding times in mice receiving either IL-4- or IL-5-deficient Tc2 effector cells. In contrast, expression of RANTES was similarly elevated in both untreated and all effector cell-treated mice at early time points, suggesting that early RANTES expression was not dependent on either Tc2 cells or their cytokines.

At later stages of therapy, wild-type Tc2 effector cells showed a significant elevation in IP-10, MIP-1β, and RANTES when compared with either untreated or IL-4 and IL-5 cytokine-deficient Tc2 effector cell treatments (Fig. 6). Moreover, in mice treated with IL-4 or IL-5 cytokine-deficient Tc2 effector cells, corresponding levels of late stage lung-derived RANTES, IP-10, and MIP-1β mRNA were not significantly different than that of untreated tumor-bearing control mice. MIP-1α was not detectable at later stages of therapy for either untreated or all groups of effector cell-treated tumor-bearing mice (Fig. 6). This suggests that Tc2 effector cells can differentially up-regulate and/or facilitate gene expression of select type-1-related chemokines at different stages of therapy at the site of tumor growth and that this expression is dependent, in part, on effector cell-derived IL-4 and IL-5.

Elevated levels of T cell-derived IFN-γ in lungs of late surviving mice treated with Tc2 effector cell therapy

Because Tc2 effector cell therapy induced heightened numbers of recipient T cells and selectively up-regulated gene expression of type 1-related chemokines at sites of tumor growth in mice with established malignancy, we investigated type 1 (IFN-γ) and type 2 (IL-4, IL-5, IL-10, and IL-13)-related T cell cytokine gene expression in surviving tumor-bearing mice treated with Tc2 effector cell therapy. Enriched T cells, from lungs of mice surviving beyond 45 days after tumor challenge, were restimulated for 5 h with plate-bound anti-CD3 and IFN-γ, IL-4, IL-5, IL-10, and IL-13 cytokine...
gene expression were assessed by RNase protection assays as described in Materials and Methods. As shown in Fig. 7A, T cells derived from lungs of Tc2 effector cell-treated tumor-bearing mice showed a nearly 3-fold elevation in the levels of IFN-γ after re-stimulation with anti-CD3 when compared with that of corresponding T cell populations from normal age-matched mice. IFN-γ gene expression levels from unstimulated control cultures of T cells derived from either effector cell-treated or normal mice were negligible (Fig. 7A). Concomitantly, expression of IL-4, IL-5, IL-10, and IL-13 was significantly (p < 0.05) lower among T cells derived from effector cell-treated mice and for the most part greater than that of unstimulated control cultures. Type 2-like cytokine gene profiles from either stimulated or unstimulated corresponding T cells from lungs of normal age-matched mice were negligible (Fig. 7A). Similar results were obtained from T cell populations of spleens from these same animals were nearly negligible. Collectively, this suggested that treatment with Tc2 effector cells elicit both systemic and local Th1 and Tc1 IFN-γ-producing T cell responses in mice with established pulmonary disease and that such responses may aid in prolonging survival in these mice.

Prolonged Tc2 effector cell-mediated therapy requires recipient-derived IFN-γ

The findings that treatment with Tc2 effector cells can elicit local IFN-γ-producing T cell responses in mice with established pulmonary disease and that IFN-γ has a profound effect on both B16 tumor cell growth and immunogenicity in vitro (19) suggest that IFN-γ may play a potential role in Tc2-mediated tumor rejection in vivo. Because we have previously shown that Tc2 effector cell-derived IFN-γ does not play a significant role in Tc2 effector cell-mediated therapy (17, 18), we extended our current studies to assess the role of recipient-derived IFN-γ in Tc2-mediated therapy. OVA-Ag-specific Tc2 effector cell populations were generated as treated mice between days 21–28 after therapy (late stage) were noticeably elevated by nearly 2- to 3-fold when compared with that of corresponding T cell populations in untreated tumor-bearing mice (data not shown). Levels of either intracellular IL-4 or IL-10 among corresponding T cells from these same animals were nearly negligible. Collectively, this suggested that treatment with Tc2 effector cells elicit both systemic and local Th1 and Tc1 IFN-γ-producing T cell responses in mice with established pulmonary disease and that such responses may aid in prolonging survival in these mice.
previously described and transferred into either wild-type or syngeneic IFN-γ−/− knockout C57BL/6 mice bearing 7-day-established B16-OVA lung tumors. As shown in Fig. 8, survival times among Tc2 effector cell-treated tumor-bearing wild-type recipients were significantly (p < 0.001) prolonged when compared with that of corresponding groups of untreated control mice. However, transfer of these same effector cell populations into tumor-bearing IFN-γ−/− knockout recipients showed no significant (p > 0.05) therapeutic effect when compared with corresponding groups of untreated control knockout mice (Fig. 8). Both groups of untreated tumor-bearing wild-type or IFN-γ−/− knockout mice similarly died within 32 days post-tumor challenge (Fig. 8). This suggested that effective Tc2 effector cell-mediated therapy was markedly dependent on host-derived IFN-γ.

Discussion

In the current study, we assessed the mechanisms involved in the effector phase of tumor rejection induced by adoptively transferred tumor-reactive Tc2 effector cells in mice bearing established pulmonary malignancy. Systemic transfer of OVA Ag-specific Tc2 effector cell populations resulted in the local accumulation of adoptively transferred cells at the tumor site that significantly reduced the number and growth of OVA-expressing B16 melanoma lung metastases and subsequently enhanced survival times in tumor-bearing mice. Tc2-mediated therapeutic effects were dependent, in part, on effector cell-derived IL-4 and IL-5 but not perforin. Moreover, effector cells not only induced elevated levels of lung-derived IP-10, MIP-1α, and MIP-1β chemokine message in vivo but also increased the local accumulation of activated host-derived CD8/CD44high, CD4/CD44high, and OVA Ag-specific tetramer-positive CD8 T cells at the tumor site. With time, the numbers of host-derived T and non-T immune cells significantly increased in the lung, which correlated with an elevated production of IP-10, RANTES, and MIP-1β and a continued reduction in tumor burden. Conversely, donor Tc2 effector cell numbers became markedly diminished at corresponding times, suggesting that prolonged therapeutic responses were due in part to the presence of host-derived antitumor mechanisms at later stages following therapy.

Effective tumor rejection by adoptively transferred cell populations may be dependent on several potential direct and indirect mechanisms. Effector T cell populations have been shown to directly eradicate tumor cells through cognate interactions that involve predominantly perforin-mediated lytic mechanisms (34). Interestingly, perforin derived from Tc2 effector cells had little or no effect on enhancing survival among tumor-bearing mice, whereas absence of effector cell-derived IL-4 or IL-5 severely impaired survival among tumor-bearing mice receiving Tc2 cell therapy, suggesting that both cytokines contributed to the effector phase of the immune response. Although neither IL-4 or IL-5 had any detectable direct effects on B16-OVA tumor cell growth, immunogenicity, or cytotoxicity in vitro, we found that such cytokines were, in part, necessary for the localization and accumulation of host-derived CD4 and CD8 lymphocytes and NK and myeloid cell populations at the tumor site. This was consistent with previous reports that tumors transplanted with either IL-4 or IL-5 inhibited in vivo tumor growth through a strong inflammatory effect associated with local recruitment of granulocytes and macrophages (1, 3, 6). These effects were partially attributed to the ability of both cytokines to rapidly induce infiltration of nonspecific inflammatory cells that resulted in increased vascular permeability and heightened leukocyte infiltration at the tumor site. In contrast, our data
show that Tc2 effector cell therapy not only elevated nonspecific tumor-reactive leukocytes but also enhanced the generation and persistence of tumor Ag-specific host-derived CD8 T cells among tumor-bearing mice. These observations emphasize the potentially critical role of Tc2 effector cells in two phases of the antitumor immune response: 1) priming of de novo Ag-specific host-derived CD8 T cells, as determined by tetramer-positive staining; and 2) amplification of the effector response through the recruitment of activated host-derived effector cell populations directed in part by local release of cytokines such as IL-4 and IL-5.

Although both IL-4 and IL-5 appear to have overlapping regulatory effects that induce similar physiological responses, we show in our tumor model that differences in the mortality rates exist among tumor-bearing mice receiving either IL-4- or IL-5-deficient Tc2 effector cell therapy. In the former, the rates of mortality were lower than those of the latter, which suggests that alternative mechanisms of action by Tc2-derived IL-4 and IL-5 may be involved. In other tumor models, it has been proposed that IL-4, but not IL-5, can function as growth/differentiation factors for CD8 T cells and enhance CTL responses during primary stimulation (35–38). Whereas IL-5 may act either independently or synergistically with other cytokines by enhancing development, activation, and tissue survival of eosinophils recruited to the site of tumor growth (2, 3, 37, 38). Aside from such potentially different mechanisms of action involving different cell populations, differences in therapeutic efficacy by cytokines may be further dependent on different spatial and temporal patterns of expression during the effector phase of Tc2-mediated antitumor responses (11, 39–41). Further studies to examine the potential interrelationship between these and other cytokines for the development of effective Tc2-mediated antitumor immune responses in our adoptive immunotherapy tumor model are currently under way.

FIGURE 7. Elevated levels of local T cell-derived IFN-γ in lungs of surviving mice treated with Tc2 effector cell immunotherapy. Mice were injected i.v. with $2 \times 10^5$ B16-OVA tumor cells. Seven days later, $2 \times 10^6$ OVA Ag-specific Tc2 effector cells were adoptively transferred into mice bearing established lung metastases. Lung cell suspensions from surviving tumor-bearing mice treated with Tc2 cell therapy 45 days earlier were obtained and restimulated with plate-bound anti-CD3 for 5 h at 37°C. Cultured T cells were harvested and total RNA was prepared as described in Materials and Methods. Type 1 (IFN-γ) and type 2 (IL-4, IL-5, IL-10, IL-13) cytokine mRNA were detected by RNase protection assays and normalized against the L32 housekeeping gene. Normal age-matched mice served as controls. Results are expressed as the mean ± SEM of three to five mice per group.

B. Tc2 Effector Cell Therapy (>45 Days Post Therapy)

Recipient CD8 (Thy 1.1/CD8)

Recipient CD4 (Thy 1.1/CD4)

FIGURE 8. Role of recipient-derived IFN-γ in Tc2 effector cell-mediated therapy of mice with established lung tumors. Wild-type or IFN-γ-deficient mice ($n = 6–10$ group) were injected i.v. with $2 \times 10^5$ B16-OVA tumor cells. Seven days later, $2 \times 10^6$ OVA Ag-specific Tc2 effector cells were adoptively transferred into mice bearing established lung metastases, and survival times were monitored. Corresponding groups of untreated tumor-bearing mice served as controls. Results are representative of two independent experiments.
Another explanation for differences in the mortality rates among IL-4- and IL-5-deficient Tc2 effector cells may be attributed to differences by either cytokine to influence the production and secretion of different chemokines from the tumor site. Others have shown that type 2 cytokines, such as IL-4, IL-5, IL-10, and IL-13, can influence the production and secretion of select chemokines and their receptors among various cell populations including mononuclear, epithelial, and endothelial cells that can regulate the influx of various immune cell populations at sites of inflammation (11, 42–47). Our data showed that Tc2 cells not only produced substantial amounts of various chemokines on restimulation in vitro but also selectively up-regulated local gene expression of IP-10, MIP-1α, MIP-1β, or RANTES at both early and late stages after therapy in vivo. Moreover, this effect appeared to be dependent, either directly or indirectly, on adoptively transferred Tc2 effector cell-derived IL-4 and IL-5. Conceivably, the differential and/or preferential induction and expression of such chemokines by either IL-4 or IL-5 secreted by Tc2 cells may provide a mechanism by which host-derived T cell and non-T cell populations can selectively be recruited to sites of tumor growth and subsequently enhance therapeutic effects.

Although some experimental tumor models have demonstrated either favorable or deleterious contributions by type 2 cytokines in tumor rejection (1, 2, 8, 10, 48, 49), multiple models have suggested a dominant role for type 1 T cell responses in promoting optimal tumor rejection (50–52). Previous studies from this laboratory and others have demonstrated that differential migration patterns by Tc1/Th1 (type 1) and Tc2/Th2 (type 2) effector cell populations are dependent, in part, on select chemokines and the expression of T cell subset-specific chemokine receptors (12–16). Because Tc2 effector cells not only induced recruitment and accumulation of activated recipient-derived CD4 and CD8 T cells at the site of tumor growth but also selectively up-regulated local gene expression levels of type 1-related chemotacticants (IP-10, MIP-1α, MIP-1β, or RANTES) in vivo, we investigated local T cell-derived cytokine response profiles in lungs of surviving mice at later stages after therapy. Our data showed elevated levels of host T cell-derived IFN-γ in lungs of mice treated with Tc2 effector cells when compared with those of corresponding type 2-like cytokines (IL-4, IL-5, IL-10, and IL-13). This suggested that treatment with Tc2 effector cells may in part elicit both systemic and local Th1 and Tc1 IFN-γ-producing T cell responses in tumor-bearing recipients that may aid in prolonging survival in these animals. To further address this, we assessed the role IFN-γ in Tc2 effector cell-mediated therapy by using tumor-bearing IFN-γ knockout recipient. Our results showed that effective Tc2 effector cell-mediated therapy was markedly dependent on host-derived IFN-γ at later stages after therapy and further supported a dominant role for type 1 T cell responses in promoting tumor rejection in mice with established malignancy.

Another interesting finding with therapeutic relevance to vaccine design was that Tc2 effector cell therapy induced the generation and persistence of OVA-specific host-derived CD8 T cells among B16-OVA-bearing mice. Although metastatic lesions in effector cell-treated mice were greatly reduced, residual disease, tumor outgrowth, and eventual mortality among these animals were still prevalent. Collectively, these observations suggest that Tc2 effector cells, which are relatively short-lived in vivo, initiate antitumor responses but are unable to successfully maintain and/or induce “complete” tumor immunity that competes with aggressive tumor growth. Multiple mechanisms of tumor escape have been proposed to explain the failure of such adoptive T cell immunotherapies and include global immunosuppression (53), tumor-specific anergy (54), insufficient T cell costimulation (55), apoptosis of tumor-specific effector cells (56), recruitment of other inhibitory cell types such as myeloid cells (57), and a barrier effect of the tumor stroma (58). Alternatively, it has been shown that diverse CTL repertoires that exhibit different clonal origins and TCR usage can be generated to a single immunodominant epitope after some chronic viral infections and in patients with melanoma (59–61). Subsequently, these T cell populations exhibited qualitatively different functional behaviors after Ag reencounter (61). Such functional heterogeneity among clonally expanded Ag-specific CD8 T cell populations may emerge during the immune response and possess high levels of functional heterogeneity with respect to tumor Ag–TCR signal recognition, activation, persistence, and effector function (62). This may influence the level of host-mediated effector cell therapeutic efficacy after adoptively transferred Tc2 effector cell disappearance in vivo. Moreover, such T cell heterogeneity may suggest that functionally and phenotypically different types of tumor-reactive T cells may play distinct roles in tumor immunity and thus affect host-mediated tumor protection and therapeutic efficacy. Studies are currently under way to determine whether such host-derived tetramer-positive staining T cells, recognizing OVA Ag, possess different clonal origins and/or TCR usage repertoires that ineffectively respond to cognate recognition of OVA-Ag-expressing tumor cells and thus render ineffective antitumor responses in vivo.

In summary, we speculate that Tc2 effector cell-mediated immunotherapy involves sequential events involving initial tumor-reactive Tc2 effector cell accumulation and localization to the tumor site. On appropriate Ag recognition, release of IL-4 and IL-5 induce, in part, selective expression of either donor- or host-derived chemokines that contribute to recruitment and perhaps activation of recipient CD8, CD4, myeloid, and NK cells to the site of tumor growth. At later stages after therapy, this process appears to be amplified in the presence of host-derived type 1-like tumor immunity that is markedly dependent on host-derived IFN-γ. Moreover, these results suggest that adoptive transfer of these potentially multifunctional Tc2 effector cell populations not only participate directly in primary antitumor responses but may also contribute to the induction and generation of secondary tumor Ag-specific and nonspecific immune cell responses in recipients with established malignancy. With the current capacity to isolate tumor Ag-specific T cell populations from cancer patients (63), ex vivo generation, propagation, and reinfusion of Tc2-like effector cell populations may offer a new strategy for successful tumor immunotherapy and vaccine development for cancer patients with select primary and metastatic disease.

References

