Synergistic Effects of IL-12 and IL-18 in Skewing Tumor-Reactive T-Cell Responses Towards a Type 1 Pattern

Qiao Li, Abbey L. Carr, Elizabeth J. Donald, Joseph J. Skitzki, Ryugi Okuyama, Lloyd M. Stoolman, and Alfred E. Chang

Abstract

We have previously described the antitumor reactivity of tumor-draining lymph node (TDLN) cells after secondary activation with antibodies. In this report, we examined the effects of interleukin (IL)-12 and IL-18 on modulating the immune function of antibody-activated murine TDLN cells. TDLN cells were activated with anti-CD3/anti-CD28 monoclonal antibody followed by stimulation with IL-12 and/or IL-18. IL-18 in combination with IL-12 showed a synergistic effect in augmenting IFNγ and granulocyte macrophage colony-stimulating factor secretion, whereas IL-18 alone had minimal effect. Concurrently, IL-18 prevented IL-12–stimulated TDLN cells from producing IL-10. The IL-12/IL-18–cultured TDLN cells therefore manifested cytokine responses skewed towards a Th1/Tc1 pattern. IL-12 and IL-18 stimulated CD4+ TDLN cells and enhanced IFNγ production by CD4+ cells to a greater extent than by CD8+ cells. Use of NFκB p50−/− TDLN cells suggested the involvement of NFκB in the IL-12/IL-18 polarization effect. Furthermore, a specific NFκB inhibitor significantly suppressed IL-12/IL-18–induced IFNγ secretion, thus confirming the requirement for NFκB activation in IL-12/IL-18 signaling. In adoptive immunotherapy, IL-12– and IL-18–cultured TDLN cells infiltrated pulmonary tumor nodules and eradicated established tumor metastases more efficiently than T cells generated with IL-12 or IL-18 alone. Antibody depletion revealed that both CD4+ and CD8+ cells were involved in the tumor rejection induced by IL-12/IL-18–cultured TDLN cells. These studies indicate that IL-12 and IL-18 can be used to generate potent CD4+ and CD8+ antitumor effector cells by synergistically polarizing antibody-activated TDLN cells towards a Th1 and Tc1 phenotype.

Introduction

Ex vivo generation of large numbers of tumor-reactive effector T cells remains a critical step for the successful clinical application of adoptive immunotherapy of cancer. Accumulating literature suggest that cytokine elaboration to specific tumor stimulation is associated with the ability of effector T cells to mediate in vivo tumoricidal effects (1–7). We reported that type 1 cytokine release (i.e., IFNγ) and granulocyte macrophage colony-stimulating factor (GM-CSF) correlates with in vivo tumor eradication whereas type 2 cytokine release [i.e., interleukin 10 (IL-10)] was found to suppress antitumor reactivity (3, 7). The inhibitory character of IL-10 on antitumor responses has been reported by multiple groups (8–11). Thus, methodologies that can up-regulate type 1, whereas down-regulating type 2 cytokine responses of effector T cells should increase their therapeutic potential.

The principle of T-cell activation using monoclonal antibody (mAb) ex vivo in the absence of antigen has been used to expand tumor-primed T cells contained within tumor-draining lymph nodes (TDLN) or vaccine-primed lymph nodes (VPLN). Our initial efforts involved the use of anti-CD3 mAb as a surrogate antigen to activate tumor-primed lymphoid cells, followed by expansion in IL-2 (12, 13). This approach resulted primarily in the generation of CD8+ effector cells that mediated tumor regression in vivo. Subsequent clinical studies using this method to activate VPLN cells showed that this cellular therapy can result in achieving durable tumor responses in subjects with advanced cancer (1, 14). We have extended our investigations in the animal models by examining other mAbs that deliver costimulatory signals in concert with anti-CD3 to activate tumor-primed lymphoid cells. These other antibodies have involved anti-CD28 and anti-CD137 (4-1BB; refs. 15, 16). The results of these investigations have indicated that costimulation can increase the activation of tumor-primed lymphoid cells and their ability to mediate tumor regression in vivo.

Although effector T cells can be generated through antibody activation to mediate tumor regression in animal models, clinical responses in adoptive immunotherapy have been confined to a minority of patients. One potential reason for these limited responses is that antibody activation procedures generally stimulate T cells broadly without discriminating between type 1 and type 2 cells, presumably due to the polyclonal expansion characteristics of antibodies directed to the TCR common chain (e.g., CD3ε of the TCR/CD3 complex or CD28). Thus, both type 1 cytokines (e.g., IL-2, IFNγ) and type 2 cytokines (e.g., IL-4, IL-5, and IL-10) are up-regulated (17–19). Alternative protocols need to be defined that will preferentially stimulate the type 1 cytokine profile to generate more potent tumor-reactive T cells for cancer immunotherapy. Towards this end, various ex vivo strategies have been investigated using additional signaling stimuli to promote Th1/Tc1 cell proliferation and antitumor reactivity (20–23).

The proinflammatory cytokine IL-18, either alone or in combination with IL-2 or IL-12, has shown immune modulatory effects in the induction of IFNγ production and the promotion of different effector cells such as CD8+ cells (24), natural killer (NK) cells (25, 26), and neutrophils (27). However, its effects on type 2 cytokine secretion, on CD4+ effector cells, as well as the involved signaling molecule(s) are either not characterized or minimally documented. In this study, we used IL-18 and IL-12 as a modulatory stimulus to antibody-activated TDLN cells and presented evidence that IL-18 in concert with IL-12 could significantly inhibit IL-10 production and generate both CD4+ and CD8+ effector cells. In addition, NFκB was identified as one

Requests for reprints: Qiao Li, Division of Surgical Oncology, Comprehensive Cancer Center, University of Michigan, Ann Arbor, MI 48109-0932. Phone: 734-936-4392; Fax: 734-647-9647; E-mail: qiaoli@umich.edu.

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of the molecular components involved in the IL-12/IL-18 signaling pathways in anti-CD3/anti-CD28 activated TDLN cells.

Materials and Methods

Mice. Female C57BL/6 (B6) mice and NF-κB p50−/− knockout mice on B6 background were purchased from the Jackson Laboratory, Bar Harbor, ME. They were used for experiments at age ≥8 weeks. The University of Michigan Laboratory of Animal Medicine approved all protocols.

Murine Tumor Cells. MCA 205 and MCA 207 murine tumors are 3-methylcholanthrene–induced fibrosarcomas that are syngeneic to B6 mice. These tumors have been previously characterized to be weakly immunogenic with distinct tumor-specific transplantation/rejection antigens (28). Tumors were maintained in vivo by serial s.c. transplantation in B6 mice and were used within the eighth transplantation generation. Tumor cell suspensions were prepared from solid tumors by enzymatic digestion as described previously (15, 16).

TDLN Preparation and T-Cell Activation and Expansion. To prepare tumor-draining lymph nodes (TDLN), wild-type B6 mice or NF-κB p50−/− mice were inoculated with 1 × 10⁶ tumor cells in 0.1 mL of PBS s.c. in the lower flank. Nine days later, inguinal TDLN were removed aseptically. Lymphoid cell suspensions were prepared as previously described (15, 16). TDLN cells (4 × 10⁶ cells per 2 mL per well) were activated with 1.0 μg/mL anti-CD3 mAb (BD PharMingen, San Diego, CA) plus 1.0 μg/mL anti-CD28 mAb (BD PharMingen) immobilized in 24-well plates (Costar, Cambridge, MA) for 2 days. After antibody activation, the cells were harvested and counted. The cells were then resuspended in complete media containing recombinant human IL-2 (Chiron Therapeutics, Emeryville, CA) starting at a concentration of 3 × 10⁶ cells per mL in 6-well culture plates (Costar) for 3 days. The concentration of IL-2 was 60 IU/mL. Recombinant mouse IL-12 (BD PharMingen) and rmIL-18 (MBL, Watertown, MA) were added at the beginning of the 3-day cell expansion period, either alone or jointly, at concentrations as indicated in the experiments. At the end of the cell expansion in IL-2 ± IL-12 and/or IL-18, cells were harvested, counted, and used for adoptive immunotherapy or cytokine secretion assessment.

Assessment of Cytokines (IFNγ, GM-CSF, and IL-10). Culture supernatants were collected at the end of cell expansion for cytokine quantification using ELISA kits (BD PharMingen). To measure cytokine release in response to tumor stimulation, 1 × 10⁶ activated and expanded TDLN cells were cocultured with 0.25 × 10⁶ irradiated MCA 205 in 2 mL volumes per well for 24 hours at 37°C. The culture supernatants were then collected and analyzed for cytokine production using ELISAs. The tumor stimulator cells were irradiated to 6,000 rGy by a 137Cs source. MCA 207 tumor cells were used for coculture as specificity controls.

CD4+ Cells and CD8+ Cell Fractionation and Fluorescence-Activated Cell Sorting Analysis. T cells were enriched by passing TDLN cell suspensions through nylon wool (Cellular Products, Inc., Buffalo, NY) column. CD4+ or CD8+ T cells were positively selected from T cell–enriched cell suspensions by treatment with super paramagnetic microbeads conjugated with anti-mouse CD4 or anti-mouse CD8 mAbs, followed by separation using the MACS Separator (Miltenyi Biotec, Inc., Sunnyvale, CA). The fractionated CD4+ or CD8+ cells were confirmed by fluorescence-activated cell sorting staining (>95% CD4+ or CD8+, respectively; data not shown). CD3, CD4, and CD8 molecules were assessed by direct fluorescence staining with anti-CD3, anti-CD4, and anti-CD8 mAbs or isotype-matching controls (all from BD PharMingen) and analyzed as previously described (15, 16).

Adoptive Immunotherapy. B6 mice were inoculated i.v. via tail vein with 2 × 10⁶ tumor cells to establish pulmonary metastases. Three days after tumor inoculation, mice were infused i.v. with cultured TDLN cells. Commencing on the day of cell transfer, IL-2 (30,000 IU) were given (i.p.) in 0.5 mL of PBS and continued twice daily for eight doses unless otherwise indicated. Five mice were used in each experimental group. On days 14 to 16, all mice were randomized and sacrificed for enumeration of pulmonary metastatic nodules. Metastatic foci too numerous to count were assigned an arbitrary value of <250. To deplete CD4+ and/or CD8+ T cells in vivo, mice were given 800 μg of anti-CD4 (GL1.4, rat IgG2a) and/or 400 μg of anti-CD8 (2.43, rat IgG2a; both from Ligocyte, Inc., Bozeman, MT) i.p. in 1 mL PBS immediately following cell transfer. Fluorescence-activated cell sorting analysis of splenocytes showed complete depletion of CD4+ cells or CD8+ cells for as long as 7 days. Therefore, in subsequent experiments the depletion procedure was repeated once on day 8 after cell transfer. Immunocytochemical evaluation of adoptively transferred TDLN cells accumulating in pulmonary tumor nodules was performed as previously described (29).

NF-κB Inhibition. We used an intracellularly targeted peptide inhibitor of nuclear translocation as an approach to NF-κB inhibition (30). The inhibitor, BMS-205820, was synthesized by Bachem Bioscience, Inc., King of Prussia, PA. BMS-205820 has the following peptide sequence PKKKRKVAAVALLPAVLLALLA PKKKRKV. Sequence in italic is the membrane-translocating hydrophilic sequence; sequence underlined is the nuclear localization sequence. The two control peptides we used were SV40 (GPGGPKKKRKV) and JBC (AAVALLPAVLLALLAPKRKKLMP). TDLN cells were activated with anti-CD3/anti-CD28 mAbs for 2 days, washed, and then incubated with BMS-205820 or control peptides in IL-2-containing medium for two hours before the addition of IL-12 and IL-18.

Statistical Analysis. The significance of differences in numbers of metastatic nodules between experimental groups was determined using the nonparametric Wilcoxon rank sum test. Two-sided P < 0.05 was considered statistically significant between two groups. Student's t test was used to analyze cell expansion and cytokine release data.

Results

Synergistic Effects of IL-12 and IL-18 in Skewing Antibody-Activated TDLN Cell Responses Toward a Type 1 Pattern. We examined the cytokine profile released by TDLN cells stimulated with IL-12 and/or IL-18 following antibody activation with anti-CD3 and anti-CD28. As shown in Fig. 1, IL-12 alone enhanced IFNγ, GM-CSF, and IL-10 secretion in a dose-dependent manner, whereas IL-18 alone had minimal effect on the levels of these cytokines. However, the use of IL-18 in combination with IL-12 showed a synergistic effect in augmenting IFNγ and GM-CSF secretion; whereas inhibiting IL-10 production induced by IL-12 alone. Specifically, when 10 ng/mL of IL-12 and 100 ng/mL of IL-18 were used simultaneously, both IFNγ and GM-CSF production was notably enhanced in a synergistic fashion. When IL-12 (10 ng/mL) and IL-18 (100 ng/mL) were used separately, the sum of secreted IFNγ was less than 10,000 pg/mL. However, when IL-12 (10 ng/mL) and IL-18 (100 ng/mL) were used jointly, nearly 30,000 pg/mL of IFNγ were produced, showing a synergy rather that an additive effect between IL-12 and IL-18. By contrast, the IL-10 secretion induced by IL-12 exposure at different concentrations was significantly down-regulated when IL-18 was added. In separate experiments, we used intermediate doses (25 and 50 ng/mL) of IL-18 between 10 and 100 ng/mL in combination with 10 ng/mL of IL-12. We found that IFNγ production by TDLN cells cultured in IL-12 (10 ng/mL) plus IL-18 at 10, 25, and 50 ng/mL were comparable. However, when IL-18 was used at 100 ng/mL, IFNγ production was found to be statistically higher than any other groups, demonstrating optimal synergistic effect with IL-12 (data not shown). Jointly, these experiments confirmed that 10 ng/mL of IL-12 plus 100 ng/mL of IL-18 were optimal within the concentration ranges we tested to selectively stimulate IFNγ and GM-CSF production by activated TDLN cells without up-regulating IL-10 secretion. Therefore, these concentrations of IL-12 and IL-18 were used to stimulate the antibody-activated TDLN cells for the rest of the experiments described in this study.

We subsequently measured the cytokines released by IL-12/IL-18 stimulated TDLN cells in response to tumor antigen. IL-12/IL-18 stimulated MCA 205 TDLN cells were cocultured with irradiated...
regardless of the culture conditions (data not shown). However, CD8+ cells being 15% to 20% and 30% to 35%, respectively (15, 16).

F-activated with anti-CD3 plus anti-CD28 for 2 days and then cultured in IL-2 secretions of anti-CD3/anti-CD28 activated TDLN cells. TDLN cells were

Figure 1. Synergistic polarization effects of IL-12 and IL-18 on cytokine secretion of anti-CD3/anti-CD28 activated TDLN cells. TDLN cells were activated with anti-CD3 plus anti-CD28 for 2 days and then cultured in IL-2 and/or IL-18 for 3 days. Culture supernatants were collected at the end of cell expansion and cytokines released into the culture media were assessed. The data are representative of three independently done experiments. a, P < 0.05 compared with no IL-12, no IL-18; b, P < 0.05 compared with IL-12 (1 ng/mL) alone or IL-18 (10 or 100 ng/mL) alone; c, P < 0.05 compared with IL-12 (10 ng/mL) alone or IL-18 (10 or 100 ng/mL) alone; d, P < 0.05 compared with IL-12 (1 ng/mL) alone; e, P < 0.05 compared with IL-12 (10 ng/mL) alone.

MCA 205 tumor cells. Culture supernatants after tumor cell stimulation were collected and analyzed for cytokines. As shown in Table 1, the combination of IL-12 and IL-18 resulted in an elevation of IFN-γ and GM-CSF secretion in response to MCA 205 tumor cells by an average of 28- and 13-fold respectively in seven independent experiments (P < 0.05 comparing fold change with IL-12/IL-18 versus no IL-12/IL-18), whereas IL-10 production minimally changed by only 1.3-fold (P = 0.30 comparing fold change with IL-12/IL-18 versus no IL-12/IL-18). We also used MCA 207 tumor cells as a specificity control. As shown in Fig. 2, the combined use of IL-12 and IL-18 in up-regulating IFN-γ secretion and concomitantly down-regulating IL-10 production was immunologically specific.

IL-12 and IL-18 Skewed T-Cell Response toward the Type 1 Pattern by Enhancing Both Th1 and Tc1 Responses. Typically, nonactivated TDLN cells contain ~50% CD3+ cells with CD4+ and CD8+ cells being 15% to 20% and 30% to 35%, respectively (15, 16). Phenotype examination revealed that at the end of IL-12 and/or IL-18 stimulation, >95% of the TDLN cells were CD3+ cells regardless of the culture conditions (data not shown). However, IL-12 preferentially stimulated the growth of CD4+ T cells either by itself or in combination with IL-18. The percentage of CD4+ T cells in the final CD3+ T-cell population increased significantly from ~15% in the absence of IL-12/IL-18 or when IL-18 was used alone, to ~30% with the use of IL-12 alone or in combination with IL-18. By contrast, the percentage of CD8+ T cells declined from nearly 80% to ~60% under these respective conditions (data not shown). Hence, the enhanced type 1 cytokine production induced by IL-12 and IL-18 seems to be associated with the preferential expansion of CD4+ cells within the whole CD3+ TDLN cell population. Using fractionated CD4+ or CD8+ TDLN cells for cytokine secretion analysis (Fig. 3), we found that IL-12 + IL-18 significantly enhanced IFN-γ production by CD4+ cells in a similar manner as un fractionated TDLN cells. In addition, we also found that IL-18 synergistically enhanced IFN-γ production by CD8+ cells, although IL-12/IL-18 enhanced CD4+ cells to a much higher extent than CD8+ cells when an equal number (1 × 10^6) of cells were examined (Fig. 3). Also, as seen with un fractionated TDLN cells, the IL-12--induced IL-10 production of both CD4+ and CD8+ cell subpopulations was significantly inhibited by IL-18. These experiments indicate that IL-12 and IL-18 synergistically skewed T-cell responses toward the type 1 pattern by enhancing both Th1 and Tc1 responses, and IL-18 was capable of inhibiting both Th2 and Tc2 cytokine production of IL-12--cultured TDLN cells.

In vivo Antitumor Activity of IL-12/IL-18--Stimulated TDLN Effector Cells. The in vivo antitumor reactivity of antibody-activated TDLN cells cultured in IL-12/IL-18 was examined in an adoptive immunotherapy model of mice bearing 3-day established MCA 205 pulmonary metastases. Varying numbers of activated TDLN cells were transferred along with the administration of IL-2. As illustrated in Fig. 4A, the administration of IL-2 alone had no therapeutic effect compared with control mice receiving no therapy. The mean number of metastases was significantly reduced after the transfer of TDLN cells cultured with IL-12 + IL-18 compared with an equivalent number of TDLN cells generated with IL-12 alone, IL-18 alone, or with neither (P < 0.05). We examined the overall proliferation of MCA 205 TDLN cells in IL-2 with the presence or absence of IL-12 and/or IL-18 following anti-CD3/anti-CD28 activation. In several independently done experiments, there were no significant changes in resultant total CD3+ T-cell numbers with the use of IL-12 or IL-18 alone, or both compared with that when IL-12 or IL-18 was not used (data not shown). These studies therefore suggested that the use of IL-12 and IL-18 did not change the potential for the whole TDLN populations to proliferate, but it may result in the generation of effector cells with increased therapeutic efficacy on a per cell basis. Additional adoptive transfer experiments were done to identify the effector cells responsible for tumor regression after exposure of antibody-activated TDLN cells to IL-12 and IL-18. Groups of mice bearing 3-day established MCA 205 pulmonary metastases received IL-12/IL-18--cultured TDLN cells followed by the i.p injection of RlgG, anti-CD4, anti-CD8, or anti-CD8+ plus anti-CD8 depleting mAbs. All mice received 3 × 10^6 cells unless otherwise indicated. As shown in Fig. 4B, administration of either anti-CD4 or anti-CD8 partially abrogated the antitumor activity mediated by the transferred cells. Anti-CD4 plus anti-CD8 completely abolished the tumor regression response. These experiments indicated that both CD4+ and CD8+ TDLN cells mediated antitumor reactivity in vivo and that they contributed additive effects.

The infiltration of lung metastases by adoptively transferred TDLN cells grown in IL-2 alone or in combination with IL-12 + IL-18 following antibody activation was investigated by
immunocytochemistry to localize labeled TDLN cells in tissue sections (29). Briefly, TDLN cells were labeled with CFSE immediately before infusion into animals bearing 10-day old MCA 205 metastases. Ten-day metastases were used since these are large enough to clearly visualize infiltration by the adoptively transferred cells. In contrast, preliminary studies of 3-day metastases (not shown) indicated that these lesions were too small for unequivocal microscopic identification. Twenty-four hours after infusion, lungs were harvested, sectioned and examined for TDLN cell infiltration as described (29). Representative sections from animals treated with TDLN cells cultured in IL-2 alone (Fig. 5A) or in IL-2 + IL-12 + IL-18 (Fig. 5B) are shown. In both cases, infused TDLN cells were observed to: (1) attach to venules, (2) mix with host leukocytes in perivascular collections, and (3) infiltrate tumor nodules. Quantitative comparison of TDLN cell trafficking into tumor nodules was beyond the scope of this study but is under active investigation.

The Synergistic Effect of IL-12 and IL-18 in Skewing T-Cell Responses toward the Type 1 Pattern Was NF-κB Dependent.

To evaluate potential signaling pathways involved in the IL-12/IL-18 skewing effect, we examined the role of the transcription factor NF-κB. We used NF-κB p50−/− knockout mice and compared the immune response of IL-12/IL-18–cultured NF-κB p50−/− TDLN cells with similarly treated wild-type TDLN cells. Using B6 NF-κB p50−/− knockout mice as TDLN donors, NF-κB p50−/− TDLN cells were induced. The wild-type TDLN or NF-κB p50−/− TDLN cells were then activated by anti-CD3/anti-CD28 followed by cell culture in IL-2 ± IL-12 and/or IL-18. Supernatants at the end of cell culture were collected to examine the IFNγ and GM-CSF production (Fig. 6, T cells alone). The IL-12 and IL-18 induced IFNγ and GM-CSF secretion seen with wild-type TDLN cells was nearly absent in similarly cultured NF-κB p50−/− TDLN cells. In addition, IL-12/IL-18–stimulated TDLN cells were cocultured with irradiated MCA 205 tumors to assess cytokine secretion in response to tumor antigen. In this setting, MCA 205–stimulated NF-κB p50−/− TDLN cells produced comparable levels of IFNγ (≈1,000 pg/mL) and GM-CSF (≈200 pg/mL) compared with wild-type TDLN cells when cultured in IL-2 alone after antibody activation (open bars). This indicated that the TDLN cells from NF-κB p50−/− knockout mice were generally responsive to the effects of anti-CD3/anti-CD28 and IL-2 in vitro. However, the enhanced IFNγ and GM-CSF production by IL-12/IL-18–stimulated wild-type TDLN cells in response to MCA 205 tumor antigen was minimal for similarly stimulated NF-κB p50−/− TDLN cells. In adoptive transfer experiments, IL-12 + IL-18–cultured NF-κB p50−/− TDLN cells mediated tumor regression, but the efficacy was significantly decreased when compared with IL-12 + IL-18–cultured wild-type TDLN cells (Fig. 7A). Collectively, these experiments indicated the requirement of NF-κB in IL-12/IL-18–induced immune modulation.

We proceeded to use an intracellularly targeted peptide inhibitor (BMS-205820) of nuclear translocation as an approach to NF-κB inhibition in order to confirm that the role of NF-κB was less global but more specific for IL-12/IL-18. The inhibitor was used after the completion of anti-CD3/anti-CD28 activation but right before the addition of IL-12/IL-18. As shown in Fig. 7B, BMS-205820, at 5 μmol/L, significantly inhibited IL-12/IL-18–induced IFNγ secretion to a level comparable to that achieved without the use of those cytokines. Control peptides SV40 or JBC had no effects.

### Discussion

**Ex vivo** antibody activation of T cells is a viable approach for the cellular treatment of cancer. However, the nondiscriminating character of antibodies in activating both type 1 and type 2 responses may result in the generation of nontherapeutic effector cells. We evaluated the immunomodulatory properties of IL-12 and IL-18 in shifting the differentiation of antibody-activated TDLN cells in vitro. We have found that IL-12 and IL-18 synergistically potentiated IFNγ and GM-CSF production of antibody-activated TDLN cells while minimally effecting IL-10 production. The IL-12/IL-18 skewing of effector T cells towards a type 1 response resulted in more potent antitumor reactivity in vivo. IL-12 and IL-18 stimulated CD4+ TDLN cells that could mediate tumor regression independent of CD8+ cells. The synergistic effect of IL-12 and IL-18 in skewing T-cell responses towards the type 1 phenotype was found to be NF-κB dependent. These results have potentially broad applications for the generation of effector T cells for clinical therapy.

The use of IL-12 and/or IL-18 as a therapeutic agent for cancer, autoimmune diseases, and infectious diseases has been explored by

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**Table 1.** Cytokine secretion (pg/mL) in response to tumor antigen by anti-CD3/anti-CD28 activated TDLN cells that were cultured in IL-12 (10 ng/mL) + IL-18 (100 ng/mL) compared with no IL-12/no IL-18

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*Fold change was calculated by dividing the concentration when both IL-12 and IL-18 were used by the concentration when neither was used.

†Fold change comparing “IL-12+IL-18” versus “No IL-12/No IL-18”: P = 0.048 for IFNγ, P = 0.002 for GM-CSF, P = 0.300 for IL-10.
other investigators (27, 31–34). A recent study showed that IL-12 and IL-18 are central mediators orchestrating Th1 and/or Th2 immune responses to respiratory syncytial viral infection (34). Osaki et al. reported that the combination of IL-12 and IL-18 administration resulted in a more potent antitumor response compared with each cytokine alone (33). However, they also found that administration of IL-12 and IL-18 was associated with lethal organ damage, attributed in part to extremely high levels of host-induced IFN-γ production. In a different approach, the IL-18 gene has been transferred into dendritic cells, either alone (35, 36) or simultaneously with the IL-12 gene (24), resulting in more effective antitumor responses associated with dendritic cell–based vaccines. As revealed by our results, the skewing of the immune response to a Th1/Tc1 reaction seems to be an important mechanism as to why the combination of IL-12 and IL-18 can enhance antitumor reactivity. To document the levels of IL-12 and IL-18 that normally be present, we collected culture supernatants at various time points (days 1, 2, and 4) during standard TDLN cell activation with anti-CD3/anti-CD28 and expansion in IL-2. IL-12 was detected at marginal levels (about 0.1 ng/mL, data not shown) compared with the amount (10 ng/mL) we used.

The mechanisms for the synergistic effects of IL-12 and IL-18 have not been clearly defined. In the present study, the polarization effect of IL-12 and IL-18 in skewing tumor-reactive TDLN cell responses toward the type 1 pattern was found to be NF-κB dependent. NF-κB is an inducible transcription factor affecting cells of the immune system (37–39). NF-κB is expressed as a cytosolic protein that translocates to the nucleus following cell activation, where it regulates the expression of a number of genes whose products are involved in inflammation and lymphocyte activation (30). In this study, the TDLN cells from NF-κB p50/κB/κB knockout mice were found

![Figure 2](#)

Figure 2. The synergistic effect of IL-18 in up-regulating IFN-γ secretion, whereas down-regulating IL-10 secretion of antibody-activated TDLN cells was tumor antigen specific. After antibody activation, MCA 205 TDLN cells were stimulated in IL-2 containing medium with either IL-12 (10 ng/mL) or IL-18 (100 ng/mL) alone or with both. The cells were then stimulated with irradiated MCA 205 tumor cells for 24 hours. Culture supernatants after tumor cell stimulation were collected and analyzed for IFN-γ and IL-10 secretion. MCA 207 tumor cells were used as a specificity control. The data are representative of four independent experiments. *, P < 0.05 compared with any other groups; **, P < 0.05 compared with IL-12 (10 ng/mL) alone.

![Figure 3](#)

Figure 3. Cytokine secretion of IL-12 and/or IL-18 cultured CD4+ or CD8+ MCA205 TDLN cells. CD4+ or CD8+ T cells were positively selected as described in the Methods section, and activated by anti-CD3/anti-CD28 mAbs followed by cell culture in IL-2 with or without the presence of IL-12 and/or IL-18 as indicated. Cytokine secretion of 1 × 10⁶ of each cell group in response to MCA205 tumor stimulation was analyzed. Representative of two independently done experiments. *, P < 0.05 compared with any other groups of CD4+ or CD8+ cells respectively; **, P < 0.05 compared with IL-12 (10 ng/mL) alone for CD4+ or CD8+ cells.
responsive to the effects of anti-CD3/anti-CD28 and IL-2 in vitro. However, these cells were not responsive to IL-12/IL-18 in inducing IFNγ secretion or in augmenting their therapeutic efficacy. The use of NF-κB–specific inhibitor BMS-205820 confirmed that the role of NF-κB was less global but more specific for IL-12/IL-18.

CD8+ T cell responses have been reported when IL-12 + IL-18 was used to induce antitumor responses (24). It is noteworthy that in this study we were able to generate CD4+ T-cell responses in addition to CD8+ cells with the combination of antibody activation followed by IL-12/IL-18 culture. We have previously shown that both CD4+ and CD8+ tumor-reactive cells can be generated from TDLN after anti-CD3/anti-CD28 activation (15, 19). The subsequent exposure of these activated cells to IL-12 + IL-18 further increased the proliferation of CD4+ cells. In addition, the skewing of cytokine response to tumor antigen was more pronounced in the CD4+ subpopulation than in CD8+ cells.

Previous studies by Plautz et al. showed that cultured TDLN cells must infiltrate pulmonary nodules to suppress tumor growth (40). Immunohistochemical localization of the TDLN cells in the current study showed active migration of infused cells into pulmonary tumor nodules whether the TDLN cells were cultured with or without IL-12 + IL-18. Because TDLN cells cultured with IL-12 + IL-18 increased tumor antigen–specific IFNγ and GM-CSF secretion in vitro, it is likely that these cytokines contributed to the enhanced therapeutic efficacy in vivo.

Son et al. has reported that cell culture in IL-2 and IL-18 resulted in enhanced NK activity (25). The activation method we used for TDLN cells involved expansion in IL-2. However, NK cells were found to be dispensable in the tumor rejection response mediated by the adoptively transferred TDLN cells cultured in IL-12/IL-18. NK cells comprised ~5% of the freshly harvested whole TDLN cell populations. After anti-CD3/anti-CD28 activation followed by IL-12/IL-18 polarization, the percentage of NK cells was <2% (data not shown). We depleted NK cells in vivo after the adoptive transfer of antibody-activated and IL-12/IL-18 cultured TDLN cells and found no significant decrease in the therapeutic efficacy (data not shown).

Figure 4. IL-12/IL-18 polarized effector cells mediated tumor regression in vivo. A, TDLN cells cultured with IL-12 + IL-18 showed more potent therapeutic efficacy. B6 mice with 3-day established MCA 205 pulmonary metastases were treated by adoptive transfer of MCA 205 TDLN cells activated with anti-CD3/anti-CD28 mAbs followed by cell culture in IL-2 ± IL-12 and/or IL-18. Different numbers of TDLN cells were transferred. IL-2 (30,000 IU) was given i.p. bid for eight doses after cell infusion. Data are representative of four independent experiments. *, P < 0.05 compared with any other group when an equal number of cells were transferred. B, IL-12/IL-18 cultured CD4+ and CD8+ TDLN cells independently mediated tumor regression. Immediately following cell transfer as in A, groups of mice receiving IL-12/IL-18 cultured TDLN cells were given anti-CD4 and/or anti-CD8 mAbs to deplete T-cell subpopulations in vivo. RígG was given to a separate group of animals as a control for the depleting antibodies. Data are representative of three independent experiments. *, P < 0.05 compared with any other groups except for RígG control; **, P < 0.05 compared with the no treatment groups.

Figure 5. IL-12 and IL-18-polarized TDLN cells accumulated in pulmonary MCA205 tumor nodules. TDLN cells were cultured either in IL-2 alone (A) or in IL-2+IL-12+IL-18 (B) following antibody activation. The lymphoblasts were then labeled with CFSE and infused (i.v.) into mice with 10-day pulmonary MCA 205 metastases. After 24 hours, the lungs were processed as described in Materials and Methods. Examples of infiltration TDLN cells (black arrows) and tumor nuclei (white arrows) are shown.
shown). On the other hand, depletion of both CD4+ and CD8+ cells following adoptive transfer completely abrogated the antitumor reactivity. Hence, it was unlikely that either host NK cells or transferred NK cells played a significant role in the regression of tumor in these experiments.

During the generation of antitumor effector T cells, it is crucial to identify in vitro correlates of T-cell function that are associated with in vivo therapeutic efficacy. We have determined the importance of IFNγ secretion in mediating the antitumor effects of the TDLN cells both in animal studies (3, 16) and in clinical trials (7). In an animal study, we examined the TCR Vβ repertoire usage of TDLN cells with respect to IFNγ release and therapeutic efficacy. Enrichment of Vβ subsets of TDLN cells by in vitro activation with anti-Vβ mAb revealed that Vβ8+ cells released high amounts of IFNγ in response to tumor and mediated potent tumor regression in vivo. Depletion of Vβ8+ subset from the whole TDLN pool confirmed that IFNγ production correlated with in vivo tumor eradication (3). In a separate study, we found that co-stimulation of TDLN cells through newly induced 4-1BB and CD3/CD28 signaling can significantly increase antitumor reactivity. The augmented antitumor effect through 4-1BB and CD28 costimulation was dependent on IFNγ production, because tumor regression was abrogated by IFNγ neutralization after adoptive transfer of 4-1BB/CD28-costimulated TDLN cells (16). We have also shown that IFNγ secretion by the activated vaccine-primed lymph node node cells correlates with tumor response in a phase II adoptive cellular trial

![Figure 6. IL-12/IL-18-induced skewing of antibody-activated TDLN cells towards the type 1 pattern was NF-κB dependent. TDLN induced from wild-type or NF-κB p50−/− KO B6 mice were designated as wt TDLN or NF-κB−/−TDLN respectively. The wild-type TDLN or NF-κB−/− TDLN cells were then activated with anti-CD3/anti-CD28 followed by culturing in IL-2 and IL-12 +IL-18. B6 mice with 3-day established MCA 205 pulmonary metastases were treated by adoptive transfer of 1 × 10^6 activated and cultured TDLN cells. IL-2 was given i.p. bid for eight doses after cell transfer. Data are representative of two independent experiments. *, P < 0.05 compared with "No IL-12/IL-18" or "NF-κB−/− TDLN IL-12 + IL-18" groups. **, P < 0.01 compared with "WT TDLN No IL-12/IL-18" or "NF-κB−/− TDLN IL-12 + IL-18" groups. B, NF-κB inhibitor BMS-205820 abrogated IL-12/IL-18-induced IFNγ production. Wild-type TDLN cells were activated with anti-CD3/anti-CD28 mAb for 2 days, washed, and then incubated with BMS-205820, SV40 and JBC for 2 hours in IL-2 containing medium before addition of IL-12 and IL-18 for cell culture. Culture supernatants were then collected for IFNγ production. Wild-type TDLN cells were activated with anti-CD3/anti-CD28 mAb for 2 days, washed, and then incubated with BMS-205820, SV40 and JBC for 2 hours in IL-2 containing medium before addition of IL-12 and IL-18 for cell culture. Culture supernatants were then collected for IFNγ assay. Similar results were obtained in three independent experiments. *, P < 0.05 compared with all other groups except for the group without the use of IL-12/IL-18.

![Figure 7. NF-κB dependent IL-12 and IL-18 interactions in TDLN cells. A, generation of potent therapeutic effector cells with IL-12 + IL-18 required NF-κB. WT TDLN and NF-κB p50−/− TDLN cells were activated with anti-CD3/anti-CD28 followed by culturing in IL-2 and IL-12 +IL-18. B6 mice with 3-day established MCA 205 pulmonary metastases were treated by adoptive transfer of 1 × 10^6 activated and cultured TDLN cells. IL-2 was given i.p. bid for eight doses after cell transfer. Data are representative of two independent experiments. *, P < 0.05 compared with "No IL-12/IL-18" or "NF-κB−/− TDLN IL-12 + IL-18" groups. **, P < 0.01 compared with "WT TDLN No IL-12/IL-18" or "NF-κB−/− TDLN IL-12 + IL-18" groups. B, NF-κB inhibitor BMS-205820 abrogated IL-12/IL-18-induced IFNγ production. Wild-type TDLN cells were activated with anti-CD3/anti-CD28 mAb for 2 days, washed, and then incubated with BMS-205820, SV40 and JBC for 2 hours in IL-2 containing medium before addition of IL-12 and IL-18 for cell culture. Culture supernatants were then collected for IFNγ assay. Similar results were obtained in three independent experiments. *, P < 0.05 compared with all other groups except for the group without the use of IL-12/IL-18.

in patients with advanced renal cell cancers. We found that the IFNγ: IL-10 ratio of cytokine released by effector T cells in response to tumor antigen was associated with clinical outcomes. Specifically, activated T cells that have an increased IFNγ/IL-10 ratio correlated with tumor response (7). Therefore, the use of polarizing reagents to modulate T-cell function towards the type 1 response and/or down-regulate type 2 responses may offer a rational strategy in generating effector cells for cellular therapy.

The findings in this study indicate that proinflammatory cytokines IL-12 and IL-18 promote the differentiation of Th1 and Tc1 effector cells. Moreover, the observations that IL-18 significantly inhibited both Th2 and Tc2 cytokine production of IL-12–cultured TDLN cells represent novel findings. The mechanisms involved in this phenomenon remain to be defined.
Acknowledgments


Grant support: NIH grants CA82529 and CA69102, the Gillson Longenbaugh Foundation, and the Hashman Family.

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We thank Kerry Odneal for her excellent assistance in the preparation of this article.

References


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Qiao Li, Abbey L. Carr, Elizabeth J. Donald, et al.


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