Published in final edited form as:

Eur J Immunol. 2009 August ; 39(8): 2126–2135. doi:10.1002/eji.200839010.

Rapid release of cytoplasmic IL-15 from tumor associated macrophages is an initial and critical event in IL-12 initiated tumor regression

Stephanie K. Watkins^{1,*}, Bing Li¹, Katharine S. Richardson¹, Kimberly Head¹, Nejat K. Egilmez², Qun Zeng¹, Jill Suttles¹, and Robert D. Stout¹

¹ Department of Microbiology and Immunology and James Graham Brown Cancer Center, University of Louisville Health Sciences Center, Louisville, KY 40292

² Department of Microbiology and Immunology, State University of New York at Buffalo, Buffalo, NY

Summary

This study reveals that the IL-15 rapidly released into serum upon IL-12 injection into tumor-bearing mice is critical for the subsequent leukocytic infiltration of the tumor and tumor-bearing tissue. The increase in serum IL-15 occurs within 2 h after IL-12 injection concomitantly with a decrease in cytoplasmic IL-15 in tumor associated macrophages (TAMs). Injection of anti-IL-15 1h prior to IL-12 abrogates subsequent leukocytic infiltration into the tumor and prevents the IL-12 induced reduction of primary tumor mass and the clearance of metastases. Administration of anti-IL-15 18h after IL-12 did not have a detectable impact on IL-12 induced leukocytic infiltration of the tumor. Deletion of NK cells had no impact on the IL-12 induced change in the functional phenotype of TAMs or on the subsequent initiation of leukocytic infiltration of the tumor. In concert with our previous studies demonstrating that IL-12 reduces tumor supportive activities of TAMs, the current study supports the hypothesis that functional re-programming of TAMs not only undermines macrophage support for tumor growth but also contributes to a critical step in the initiation of anti-tumor immune responses. In this context, the functional plasticity and pro-immunogenic potential of TAMs may constitute a significant and unappreciated target in existing cytokine therapies.

Keywords

macrophages; cancer; IL-12; IL-15; inflammation

Introduction

Although tumor vaccines can induce protective immunity in naive mice, their efficacy in mice with established tumors has been disappointing [1]. The difficulty in inducing anti-tumor responses in tumor-bearing animals appears to be due to the strong immunosuppressive environment of the host, enforced predominantly by regulatory T cells

Address correspondence to: Robert D. Stout, Department of Microbiology and Immunology, School of Medicine, University of Louisville Health Sciences Center, 319 Abraham Flexner Way, Louisville, KY 40292, Phone: 502 852 5351, FAX: 502 852 7531, bobstout@louisville.edu.

*Current address: Cancer and Inflammation Program, Tumor Immunity and Tolerance Section, Laboratory of Immunoregulation, NCI-Frederick, Frederick, MD 21702

Disclosures

The authors have no conflicting financial interests.

and immunosuppressive Mφs and myeloid cells [2–5]. Mφs play very diverse roles in carcinogenesis and metastasis [6–8]. Tumor-modulated Mφs within primary tumor tissue and metastatic nodes promote angiogenesis, matrix re-modeling, and metastasis [6–8]. Tumor-modulated Mφs in distal tissues contribute to formation of pre-metastatic niches [9,10]. Tumor-modulated Mφs in lymphoid tissues or Mφs distal to the metastatic node but within the tissue contribute to suppression of immune responses [2–5,11–13]. Several approaches have been used to destroy or alter these suppressor cells with limited success [2,4]. Interestingly, the administration of microsphere encapsulated IL-12 has been reported to initiate a tumor destructive immune response in several murine tumor models even in the absence of overt vaccination [14–18]. IL-12 is known to promote NK and T cell activation in tumor-bearing animals [19] and, indeed, the cytotoxic anti-tumor effector response elicited by IL-12 treatment is dominated by NK cells and cytotoxic T cells 5–10 days post-treatment [16–18]. However, the mechanism by which IL-12 elicits the mobilization of an immune response to the tumor in the presence of the immunosuppressive environment created by the tumor remains unclear. Mφs have been reported to be responsive to IL-12 [11,20]. Although tumor-infiltrating Mφs (TIMs) and distal tumor-associated Mφs (TAMs) actively express tumor-promoting and immunosuppressive functions, respectively, [2,3,8,11,21] it has been established that they retain the functional plasticity characteristic of Mφs [22–24] insofar as they can be rapidly converted from predominantly tumor-supportive/immunosuppressive cells to predominantly inflammatory/pro-immunogenic cells either *in vitro* or *in vivo* [11,23]. Treatment of tumor-bearing mice with IL-12 results in a rapid down-regulation of tumor-supportive activities in TAMs, including CCL2, Migration Inhibitory Factor (MIF), and TGFβ expression, and an equally rapid up-regulation of IL-15 and IL-18 gene expression. Treatment of *ex vivo* purified TAMs with IL-12 *in vitro* results in the same functional conversion that is observed upon *in vivo* treatment of tumor-bearing mice, thus establishing that TAMs are capable of responding directly to IL-12 without mediation by other cell types [11].

The effect of IL-12 on expression and release of IL-15 by TAMs [11] is particularly interesting. IL-15 is an intriguing cytokine in that it plays multiple roles in the development and activation of the immune system. IL-15 is produced by Mφs, myeloid dendritic cells and some stromal cells [25]. Two isoforms of IL-15 exist, distinguished by either a complete (secreted isoform) or a truncated signaling sequence [26]. Like Mφ migration inhibitory factor (MIF) [27], the isoform of IL-15 with a truncated signaling sequence does not enter the endoplasmic reticulum but instead accumulates in the cytoplasm [26]. It has not been established whether this isoform of IL-15 can be exported by a non-conventional mechanism [28], as has been demonstrated for MIF [27,29]. IL-15 is known to play a role in chronic inflammation and innate immune responses [25,30,31] and is required for the development and survival of NK and NK T cells as well as for establishment and maintenance of long-term memory in the CD8+ T cell compartment [25,30,32,33]. In the context of cancer, soluble recombinant IL-15 has been demonstrated to induce immunogenic maturation of dendritic cells from monocytes [30,34], to activate normal resting NK cells [30] and to rescue tumor-specific anergic CD8+ T cells *in vitro* and to enhance their survival upon adoptive transfer [35,36]. Therefore, we investigated whether the early release of IL-15 played an early intermediary role in IL-12 initiated leukocytic infiltration of the tumor.

Results

IL-12 re-programs macrophage function in tumor-bearing mice

Our previous studies demonstrated that IL-12 treatment either *in vivo* or *in vitro* could change the functional phenotype of Mφs in tumor bearing mice [11]. The shift in functional activities induced by IL-12 was observed in Mφs within the primary tumor, in Mφs within tissue bearing metastases (lung), in tissue without detectable metastases (peritoneal lavage),

and in lymphoid tissue (spleen) (summarized in Fig. 1). The change in functional phenotype included a reduction in tumor supportive and immunosuppressive activities (MIF, TGF β , CCL2, and IL-10 expression) and an increase in inflammatory, pro-immunogenic activities (TNF α , IL-6, IL-15, and IL-18 expression). IL-12 has also been reported to inhibit angiogenic activity within the tumor, in part by reducing the activity of angiogenic factors such as VEGF [37–39]. To determine if treatment with IL-12 microspheres reduced VEGF secretion by M ϕ s present in the primary tumor mass, M ϕ s were purified from the primary tumor mass of mice treated with placebo microspheres or IL-12 microspheres 24 h previously and cultured overnight without stimulus. The culture supernatants of M ϕ s from IL-12 treated mice contained significantly less VEGF and CCL2 and significantly more TNF α than M ϕ s from mice treated with placebo microspheres (Table 1).

Tumor associated macrophages rapidly release IL-15 in response to IL-12 treatment in vivo

In our studies on the impact of IL-12 on tumor bearing mice, we observed an increase in serum IL-15 3 h after injection of IL-12 into tumor bearing mice [11]. To determine the duration of this effect, serum samples were collected at 2 and 24 hs after IL-12 injection into tumor-bearing mice. Serum IL-15 was significantly elevated at 2 hs post-IL-12 injection and had diminished at least 3-fold by 24 h (Fig. 2A). Analysis of the cytoplasmic store of IL-15 in the M ϕ s of these mice revealed a significant decrease in IL-15 in peritoneal and splenic M ϕ s 2 h after IL-12 treatment (Fig. 2B, C). M ϕ s within the primary tumor mass (TIMs) contained a lower cytoplasmic content of IL-15 and a less discernable decrease in cytoplasmic IL-15 upon IL-12 treatment (Fig. 2D). Curiously, although treatment of M ϕ s with IL-12 in vivo or in vitro induced a rapid change in functional phenotype in M ϕ s from both normal and tumor-bearing mice [11], little IL-15 release was observed upon *in vivo* IL-12 treatment (Fig. 2A) and no IL-15 release was observed upon *in vitro* IL-12 treatment (Fig. 3). To determine if tumor-derived factors contributed to IL-12 induction of IL-15 release, M ϕ s were cultured for 6 h with IL-12 in the presence or absence of tumor cell conditioned medium (CM). IL-15 was detected in the supernatant fluid from cultures containing M ϕ s plus both IL-12 and tumor cell-CM but not from cultures containing M ϕ s plus IL-12 or M ϕ s plus tumor cell-CM (Fig. 3).

IL-15 release is required for IL-12 induced leukocytic infiltration of the primary tumor and lungs and facilitates clearance of lung metastases

IL-12 therapy has been reported to induce activation and mobilization of an anti-tumor immune response dominated by NK cells and CD8 $^{+}$ cytotoxic T cells in a variety of syngeneic tumor models [16,17]. Several researchers have demonstrated the ability of IL-15 to rescue and activate anergic NK and CD8 $^{+}$ T cells from tumor-bearing animals [35,40]. We therefore sought to determine if the bolus of IL-15 released upon IL-12 treatment played a critical role in IL-12 initiation of a tumor destructive immune response. To measure the effects of IL-15 during IL-12 treatment, neutralizing anti-IL-15 antibody was injected i.p. into tumor bearing mice 1 h prior to intra-tumor IL-12 injection. A single dose of 5 μ g anti-IL-15 reduced the IL-15 detectable in serum 2 hs after IL-12 treatment of tumor bearing mice by 80–90% (data not shown). Tumors were then harvested 5 or 10 days after treatment to measure lymphocyte infiltration and tumor mass. Five days after IL-12 injection there was a 10 fold increase ($p=0.0046$) in infiltrating CD4 $^{+}$ and CD8 $^{+}$ T cells within the lung tissue and a 6–8 fold increase ($p=0.013$) in infiltrating CD4 $^{+}$ and CD8 $^{+}$ T cells within the primary tumor (Figs. 4AB,5AB). This increase was not observed in mice treated with anti-IL-15 1h before IL-12. To determine if antibody-mediated blockade of surface IL-15 on the membranes of stromal M ϕ s, which contributes significantly to CD8 $^{+}$ T and NK cell homeostasis [33], contributed to the anti-IL-15 mediated reduction in IL-12 induced leukocytic infiltration, anti-IL-15 was injected 18h after treatment with IL-12. There was no

significant difference in the degree of lymphocytic infiltration into the lungs and primary tumor mass in mice treated with IL-12 alone compared to mice treated with anti-IL-15 18h after IL-12 (Fig. 5AB). Tumor mass was significantly reduced ($p=0.027$) in the IL-12 treated group in comparison to the placebo control (Fig. 5C). Similar to the infiltration data, there was no significant difference in tumor mass between groups treated 10 days earlier with IL-12 alone or with IL-12 followed 18h later with anti-IL-15 (Fig. 5C) whereas the tumor mass of the group treated with anti-IL-15 1h prior to IL-12 was significantly larger ($p=0.015$) than the group treated with IL-12 alone and not significantly different from the placebo control.

It has been reported that IL-12 treatment not only reduces tumor volume, but also reduces the formation of metastatic nodes and/or induces destruction of existing metastatic nodules [16,37]. To determine whether IL-15 release was involved in the IL-12 initiation of metastatic destruction, lungs were harvested 10 days after IL-12 or anti-IL-15 + IL-12 treatment to assess the degree of metastases. Lungs from the IL-12 treated mice displayed a reduced frequency and size of metastatic lesions compared to the placebo-treated control mice (Fig. 6A). In contrast, tumor-bearing mice treated with anti-IL-15 1h before injection of IL-12 displayed increased number and size of metastatic lesions compared to the group treated with IL-12 alone (Fig 6A). However, the lungs of anti-IL-15 + IL-12 treated mice did not display the extensive metastatic tumor growth displayed by control (placebo) mice (Fig. 6). Cross section of the lungs revealed heavy metastatic areas and hemorrhage in the control untreated lungs (Fig. 6B, C). Mice treated with IL-12 alone or with isotype control + IL-12 contained few metastatic nodes compared to the group treated with anti-IL-15 (at -1h) + IL-12, which contained several areas of discrete tumor growth (Fig. 6B, C).

NK-depletion does not impact the in vivo response of TAMs to IL-12

Previous studies suggested that the efficacy of treatment with IL-12 was dependent on IFN γ produced by IL-12 activated NK cells and/or tumor-specific CD8 $^{+}$ T cells. Rescued @ by IL-12, events which occurred concomitantly with activation of M ϕ cytotoxic activities in tumor-bearing mice treated with IL-12 [41,42]. However, we have demonstrated that treatment of purified ex vivo TAMs in vitro with IL-12 rapidly altered their functional phenotype [11], demonstrating that IL-12 could act directly on TAMs in the absence of other cell types. To determine whether the IL-12 mediated conversion of TAMs to inflammatory phenotype and the subsequent leukocytic infiltration of the tumors were dependent on upon NK activation, mice were depleted of NK cells by 2 sequential daily injections of anti-NK 1.1 prior to IL-12 treatment. This regimen sustained NK-deficiency for at least 4 days (Fig. 7A). The impact of NK cell depletion on IL-12 induced leukocytic infiltration of the lungs of tumor-bearing mice was assessed. Five days after IL-12 treatment, the lungs of both NK-sufficient and NK-depleted mice displayed similar levels of infiltration by CD4 $^{+}$ and CD8 $^{+}$ T cells (Fig. 7B). To determine if NK cells contributed to the IL-12 induced change in functional phenotype of TAMs, TAMs purified from the peritoneal lavage of tumor-bearing mice 4 h after IL-12 treatment were assayed for cytokine gene expression. IL-12 treatment reduced or abrogated mRNA expression of tumor promoting TGF β and MIF genes, and elevated expression of IL-15 mRNA in NK-depleted tumor bearing mice (Fig. 8). No significant difference was observed in this early shift in gene expression between TAMs from NK-intact and NK-depleted tumor bearing mice.

Discussion

Research over the past decade has revealed the remarkably diverse roles M ϕ s play in the growth and metastasis of tumors [2–8,12]. One of these roles is to sustain a strong immunosuppressive environment, an activity that has contributed to the diminished efficacy of multiple attempts to establish anti-tumor responses [1–5,13]. Research on M ϕ biology

over the same time period has revealed that Mφs display a remarkable degree of functional plasticity in that they can alter the functional activities they express in response to changes in environmental signals [11,12,22,23,43–45]. In support of the functional plasticity of Mφs, we demonstrated that the functional profile of Mφs in primary tumors and from distal sites (lung, spleen, peritoneal cavity) could be altered *in situ* by treatment of the mice with IL-12 [11]. We therefore suggested that the efficacy of IL-12 in eliciting a cytotoxic anti-tumor response upon administration to tumor bearing mice was due, at least in part, to its ability to decrease the tumor-supportive and immunosuppressive activities of TIMs and TAMs and increase their inflammatory and pro-immunogenic activities, thus facilitating the induction of an immune response. The ability of IL-12 to alter the functional profile of TAMs when added to purified TAMs *in vitro* indicated that TAMs could respond directly to IL-12 without intervention of another cell type [11].

The rapid release of IL-15 from TAMs, and possibly other stromal cells, in response to IL-12 treatment appears to contribute significantly to the efficacy of IL-12 treatment, as evidenced by the reduced leukocyte infiltration as well as the reduced tumor destruction and metastatic clearance when the early bolus of IL-15 is neutralized. Although IL-12 up-regulates IL-15 gene expression in both normal and tumor-bearing mice, it induces the rapid release of stored IL-15 only in tumor bearing mice [11]. The reason for this remains to be resolved and is currently under investigation. We have not been able to detect significant differences in IL-12 receptor expression between normal peritoneal Mφs and peritoneal TAMs. One distinction between the Mφs in normal and tumor bearing mice is that TAMs are in an activated state [2,3,8,11,21]. We have observed that IL-15 is released from Mφs upon treatment with IL-12 in the presence of tumor-CM (Fig. 3). This suggests that the tumor environment has an as yet unresolved activating/priming effect on TAMs that sensitizes them for release of cytoplasmic IL-15 upon receipt of a pro-inflammatory signal such as IL-12. The ability of IL-12 to modulate Mφ function without inducing the release of IL-15 clearly indicates that, although IL-12 initiation of leukocytic infiltration of the tumor is dependent on IL-15, IL-12 modulation of tumor-supportive and immunosuppressive Mφ activities is independent of IL-15.

IL-15 has been reported to contribute significantly to inflammatory processes, both by supporting the survival and activation of NK cells as well as by stimulating IFN γ production and chemokine production by cells of both the innate and adaptive immune systems [25,30–34,46,47]. Treatment with IL-12 induces increases in serum IFN γ and increases in IFN γ -positive NK cells by 24 h with a peak activity at 24–72 h [16,42,48]. In the current study, a single 5 μ g dose of anti-IL-15, sufficient to significantly neutralize the early spike in serum IL-15 during the first 6 hours after IL-12 treatment, abrogated inflammatory infiltration of the tumors. Neither injection of the neutralizing anti-IL-15 after the bolus dissipated nor deletion of NK cells prior to IL-12 treatment impaired the change in TAM functional phenotype or the early leukocytic infiltration. This establishes TAMs as one of the initial targets of IL-12, reconciles the remarkable efficacy of IL-12 therapy in mobilizing CD8 $^{+}$ T and NK responses with the known synergy of IL-15 with IL-12/IL-18 induction of IFN γ production by CD8 $^{+}$ T and NK cells [47,49,50], and underscores the early role of IL-15 in mobilizing the subsequent NK and T cell responses which are critical for the successful cytotoxic assault against the tumor [16–18,41].

IL-15 is currently being examined as a potential candidate for cancer therapies [35,36]. Thus far, IL-15 appears to be most effective when administered in combined cytokine therapies and has only a modest effect when administered alone [47,49]. It is therefore likely that the IL-15 released from TAMs upon IL-12 treatment acts synergistically with the IL-12, and likely with other inflammatory cytokines such as IL-18, to promote the innate and adaptive anti-tumor responses [49,50]. In this context, it is interesting that, although neutralization of

IL-15 abrogated destruction of the primary tumor and clearance of metastases, the metastatic involvement of the lungs from IL-12 plus anti-IL-15 treated mice was substantially and consistently less than that of lungs from placebo-treated mice. Flow cytometric analysis and histological examination did not reveal evidence of destruction of the tumor in the lungs of mice treated with anti-IL-15. However, the lungs of these animals appeared to have fewer metastases. Given the role of functionally polarized Mφs in tumor metastasis [6–10], it is possible that modulation of Mφ function by IL-12 reduced the rate of tumor metastasis, thus reducing seeding of the lungs from animals treated with IL-12 plus anti-IL-15 antibody but not reducing the growth of, nor destroying, metastatic lesions existing at the time of treatment with IL-12 plus anti-IL-15. Indeed, IL-12 has been reported to interfere with tumor angiogenesis [37–39,51], which is known to be highly dependent on tumor-associated Mφ function [8]. We observed that IL-12 reduced the expression of VEGF, MIF, and TGFβ, all of which contribute to angiogenesis and/or metastasis (Fig. 1, Table 1). We are currently investigating whether cytokines in the cascade initiated by IL-12 selectively impact, independently or in concert, tumor viability, proliferation, and/or metastasis.

In conclusion, the initial event upon IL-12 treatment of tumor-bearing mice appears to be the conversion of TAMs to a potent inflammatory phenotype characterized by the up-regulation of IL-15 and IL-18 [11]. Within the first 2 hs of IL-12 treatment, the TAMs release IL-15, which is required for subsequent leukocytic infiltration of the primary and secondary tumors. This conversion of TAMs from tumor-supportive (VEGF, MIF, CCL2, TGFβ) and immunosuppressive (IL-10, TGFβ) activities to pro-inflammatory (IL-15, IL-18) activities appears to establish a pro-immunogenic window @, the duration of which may depend on several factors, including tumor burden and the immunogenicity of the tumor. As predicted by the hypothesis that Mφs are functionally plastic [22,23], immunosuppressive TAMs can be converted to pro-immunogenic allies in anti-tumor therapies. This not only has been demonstrated with IL-12 treatment [11,46] but also with IFNγ and retinoic acid treatment [2] and by neutralization of tumor-derived cytokines [24]. These observations underscore the potential benefit of developing co-therapeutic regimens which focus on altering and controlling the local and systemic functional activities of TAMs.

Materials and Methods

Animals

C57Bl/6J female mice were obtained from Jackson Laboratories. All animal protocols received prior approval of the Institutional Animal Care and Use Committee and all experiments were performed in accordance with relevant guidelines and regulations.

Tumor models

The Lewis Lung carcinoma (3LLC) line was obtained from the James Graham Brown Cancer Center, Louisville, KY. The tumor cells were passaged in vivo prior to use for the establishment of tumors in mice for experimentation. Tumors were established by subcutaneous injection of 10^5 cells. IL-12 therapy was initiated when a primary tumor volume of 0.4–0.6 cm³ was attained (15–18 days). Lung metastases were apparent at this time point upon gross examination of perfused lung tissue. Tumor-CM was prepared by seeding tumor cells at 4×10^5 cells per ml of RPMI 1640 supplemented with 5% fetal bovine serum and gentamicin. The culture supernatant was collected 72 hours later and centrifuged at 300xg to eliminate intact cells.

Macrophage isolation and purification

Mice were perfused with cold PBS/citrate prior to removal of primary tumor and lungs. Single cell suspensions spleen, lungs and tumor were prepared by gently pressing diced

tissue between the frosted ends of glass slides and passing the suspension through nylon screen. Mφs from lung and tumor tissue were enriched by centrifugation through a 30%/40%/70% Percoll gradient and collecting the cells at the 40%/70% interface. Mφs were purified from the single cell suspensions of peritoneal lavage, spleen, lung, and tumor by magnetic bead separation using a negative selection with anti-CD19, anti-CD5, and anti-CD49b followed by a positive selection with anti-CD11b (Mac-1) (Miltenyi Biotech, Auburn CA). Purity of >95% CD11b⁺/F480⁺ cells was confirmed by flow cytometry.

Real-time RT-PCR

For assay of gene expression, cDNA was produced from mRNA from 10⁶ cells from independent cell culture experiments by using a ΦMACS One-Step cDNA kit (Miltenyi Biotec). Samples were analyzed by real-time RT-PCR amplification with SYBR Green using gene-specific real-time primers for MIF (Superarray), IL-15 and TGFβ (Maxim Biotech). Beta-actin mRNA was analyzed using primers purchased from BD Clontech. Relative expression of mRNA transcripts was quantified using the relative expression software tool [52].

Cytokine ELISAs

IL-15 was analyzed by ELISA, using rat anti-mouse IgG2a IL-15 Ab at 4mg/ml as the capture Ab in combination with the biotinylated goat IgG anti-mouse IL-15 antibody at 400 ng/ml as the detection Ab (R&D Systems ELISA kit) in combination with color development reagents from the eBioscience IL-15 ELISA kit. CCL2 (MCP-1), TNFα, and VEGF were assayed using ELISA kits obtained from R&D systems.

IL-12 treatment

The preparation of IL-12 encapsulated PIN polylactic acid microspheres was described in detail previously [16,53]. Microspheres were re-suspended at 1mg/100μl in PBS (250 ng IL-12) for injection into the center of the tumor mass. For in vitro studies on IL-12, purified Mφs were plated at 5 × 10⁵ cells/ml RPMI 1640 supplemented with 5% fetal bovine serum and gentamicin and treated with 5 ng/ml soluble recombinant IL-12 (R&D Systems).

Infiltration Studies

Mice bearing subcutaneous 3LLC tumors were injected with microspheres (placebo or containing IL-12). Five to ten days later the mice were euthanized and perfused with 10ml cold PBS/citrate prior to removal of the primary tumor, spleen, lungs, and peritoneal lavage. Wet weights of the primary tumor masses were recorded and one lobe of the lungs from each animal was fixed in 10% buffered formalin for 16 hours, washed in 70% EtOH and subsequently sectioned and H&E stained by Pathology Research Services at the University of Louisville Health Sciences Center. The primary tumor mass and unfixed lung tissue were diced into small fragments and gently pressed between the frosted ends of glass slides to extrude the cells. The monodispersed cell suspension was washed with DPBS + 2% FBS and analyzed for infiltrating lymphocytes by flow cytometry.

Flow Cytometry

Cells were treated at 1μg/10⁶ cells with Fc block (BD-Pharmingen) for 15 minutes prior to, and during, incubation with fluorochrome conjugated anti-CD4, anti-CD8, and anti-CD45 or with anti-NK 1.1 and anti-pan-NK (CD49b), washed and analyzed on a BD FACS Calibur. Intracellular IL-15 was assayed using the BD Pharmingen cytofix/cytoperm kit per manufacturer's instruction along with biotinylated goat IgG anti-mouse IL-15 antibody (500ng/10⁶ cells, R&D Systems) and PE-conjugated streptavidin (BD Pharmingen). Prior to fixation and permeabilization, surface CD11b was labeled with APC-anti-CD11b (BD-

Pharmingen) and surface IL-15 was blocked using unlabeled goat IgG anti-IL-15 (500ng/10⁶ cells). Un-labeled goat IgG anti-IL-15 was used at a 10–20 fold excess as a cold blocking reagent to control for intracellular staining specificity. Biotinylated goat IgG anti-human thymopoietin was used as an isotype control. The cytometer was gated on viable CD11b+ cells and 10⁴ cells were analyzed for cytoplasmic IL-15 content.

In vivo neutralization and NK deletion

Neutralizing anti-IL-15 and IgG isotype control was obtained from eBioscience. Anti-IL-15 (5 µg), or isotype control, was injected i.p. 1 h prior to, or 18 h after, intra-tumor IL-12 microsphere treatment. For NK cell depletion, 25 µg anti-NK1.1 (BD Pharmingen) was injected i.p. on days -2 and -1 prior to IL-12 microsphere treatment. Efficacy of deletion was confirmed by dual staining of spleen cells with anti-NK 1.1 and anti-pan NK.

Statistical analysis

The mean and standard deviation of the frequency of infiltrating CD4+/CD45+ and CD8+/CD45+ cells were recorded from 3–5 mice and cytokine gene expression assayed per individual mouse by real-time RT-PCR. The significance between groups was determined by the two sample t test.

Acknowledgments

This research was supported by National Institutes of Health Grant CA100656 (N.K.E.), the Susan G. Komen Race for the Cure (R.D.S.), the Commonwealth of Kentucky Lung Cancer Research Program (R.D.S., J.S.), the American Lung Association of Kentucky (S.K.W.), and the Commonwealth of Kentucky Research Challenge Trust Fund (J.S., and R.D.S.)

Abbreviations

TAMs	tumor-associated macrophages
TIMs	tumor-infiltrating macrophages

References

1. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. *Nat Med.* 2004; 10:909–915. [PubMed: 15340416]
2. Serafini P, Borrello I, Bronte V. Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. *Semin Cancer Biol.* 2006; 16:53–65. [PubMed: 16168663]
3. Kusmartsev S, Gabrilovich DI. Effect of tumor-derived cytokines and growth factors on differentiation and immune suppressive features of myeloid cells in cancer. *Cancer Metastasis Rev.* 2006; 25:323–331. [PubMed: 16983515]
4. Nagaraj S, Gabrilovich DI. Myeloid-derived suppressor cells. *Adv Exp Med Biol.* 2007; 601:213–223. [PubMed: 17713008]
5. Huang B, Pan PY, Li Q, Sato AI, Levy DE, Bromberg J, Divino CM, Chen SH. Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. *Cancer Res.* 2006; 66:1123–1131. [PubMed: 16424049]
6. Lewis CE, Pollard JW. Distinct role of macrophages in different tumor microenvironments. *Cancer Res.* 2006; 66:605–612. [PubMed: 16423985]
7. van der Bij GJ, Oosterling SJ, Meijer S, Beelen RH, van EM. The role of macrophages in tumor development. *Cell Oncol.* 2005; 27:203–213. [PubMed: 16308469]
8. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell.* 2006; 124:263–266. [PubMed: 16439202]

9. Peinado H, Rafii S, Lyden D. Inflammation joins the “niche”. *Cancer Cell*. 2008; 14:347–349. [PubMed: 18977322]
10. Cheng P, Corzo CA, Luetsteke N, Yu B, Nagaraj S, Bui MM, Ortiz M, Nacken W, Sorg C, Vogl T, Roth J, Gabrilovich DI. Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein. *J Exp Med*. 2008; 205:2235–2249. [PubMed: 18809714]
11. Watkins SK, Egilmez NK, Suttles J, Stout RD. IL-12 rapidly alters the functional profile of tumor-associated and tumor-infiltrating macrophages in vitro and in vivo. *J Immunol*. 2007; 178:1357–1362. [PubMed: 17237382]
12. Stout RD, Watkins SK, Suttles J. Functional plasticity of macrophages: in situ re-programming of tumor-associated macrophages. *Journal of Leukocyte Biology*. 2009; 85 in press.
13. Valenti R, Huber V, Filipazzi P, Pilla L, Sovenia G, Villa A, Corbelli A, Fais S, Parmiani G, Rivoltini L. Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-beta-mediated suppressive activity on T lymphocytes. *Cancer Res*. 2006; 66:9290–9298. [PubMed: 16982774]
14. Brunda MJ, Luistro L, Warrier RR, Wright RB, Hubbard BR, Murphy M, Wolf SF, Gately MK. Antitumor and antimetastatic activity of interleukin 12 against murine tumors. *J Exp Med*. 1993; 178:1223–1230. [PubMed: 8104230]
15. Nastala CL, Edington HD, McKinney TG, Tahara H, Nalesnik MA, Brunda MJ, Gately MK, et al. Recombinant IL-12 administration induces tumor regression in association with IFN-gamma production. *J Immunol*. 1994; 153:1697–1706. [PubMed: 7913943]
16. Hill HC, Conway TF Jr, Sabel MS, Jong YS, Mathiowitz E, Bankert RB, Egilmez NK. Cancer immunotherapy with interleukin 12 and granulocyte-macrophage colony-stimulating factor-encapsulated microspheres: coinduction of innate and adaptive antitumor immunity and cure of disseminated disease. *Cancer Res*. 2002; 62:7254–7263. [PubMed: 12499267]
17. Nair RE, Jong YS, Jones SA, Sharma A, Mathiowitz E, Egilmez NK. IL-12 + GM-CSF Microsphere Therapy Induces Eradication of Advanced Spontaneous Tumors in her-2/neu Transgenic Mice But Fails to Achieve Long-Term Cure Due to the Inability to Maintain Effector T-Cell Activity. *J Immunother*. 2006; 29:10–20. [PubMed: 16365596]
18. Colombo MP, Trinchieri G. Interleukin-12 in anti-tumor immunity and immunotherapy. *Cytokine Growth Factor Rev*. 2002; 13:155–168. [PubMed: 11900991]
19. Del VM, Bajetta E, Canova S, Lotze MT, Wesa A, Parmiani G, Anichini A. Interleukin-12: biological properties and clinical application. *Clin Cancer Res*. 2007; 13:4677–4685. [PubMed: 17699845]
20. Fantuzzi L, Puddu P, Varano B, Del CM, Belardelli F, Gessani S. IFN-alpha and IL-18 exert opposite regulatory effects on the IL-12 receptor expression and IL-12-induced IFN-gamma production in mouse macrophages: novel pathways in the regulation of the inflammatory response of macrophages. *J Leukoc Biol*. 2000; 68:707–714. [PubMed: 11073111]
21. Lamagna C, urrand-Lions M, Imhof BA. Dual role of macrophages in tumor growth and angiogenesis. *J Leukoc Biol*. 2006; 80:705–713. [PubMed: 16864600]
22. Stout RD, Suttles J. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *J Leukoc Biol*. 2004; 76:509–513. [PubMed: 15218057]
23. Stout RD, Jiang C, Matta B, Tietzel I, Watkins SK, Suttles J. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J Immunol*. 2005; 175:342–349. [PubMed: 15972667]
24. Guiducci C, Vicari AP, Sangaletti S, Trinchieri G, Colombo MP. Redirecting in vivo elicited tumor infiltrating macrophages and dendritic cells towards tumor rejection. *Cancer Res*. 2005; 65:3437–3446. [PubMed: 15833879]
25. Fehniger TA, Caligiuri MA. Interleukin 15: biology and relevance to human disease. *Blood*. 2001; 97:14–32. [PubMed: 11133738]
26. Tagaya Y, Kurys G, Thies TA, Losi JM, Azimi N, Hanover JA, Bamford RN, Waldmann TA. Generation of secretable and nonsecretable interleukin 15 isoforms through alternate usage of signal peptides. *Proc Natl Acad Sci USA*. 1997; 94:14444–14449. [PubMed: 9405632]

27. Donn RP, Ray DW. Macrophage migration inhibitory factor: molecular, cellular and genetic aspects of a key neuroendocrine molecule. *J Endocrinol.* 2004; 182:1–9. [PubMed: 15225126]
28. Nickel W, Rabouille C. Mechanisms of regulated unconventional protein secretion. *Nat Rev Mol Cell Biol.* 2009; 10:148–155. [PubMed: 19122676]
29. Flieger O, Engling A, Bucala R, Lue H, Nickel W, Bernhagen J. Regulated secretion of macrophage migration inhibitory factor is mediated by a non-classical pathway involving an ABC transporter. *FEBS Lett.* 2003; 551:78–86. [PubMed: 12965208]
30. Ohteki T. Critical role for IL-15 in innate immunity. *Curr Mol Med.* 2002; 2:371–380. [PubMed: 12108948]
31. Ohteki T, Tada H, Ishida K, Sato T, Maki C, Yamada T, Hamuro J, Koyasu S. Essential roles of DC-derived IL-15 as a mediator of inflammatory responses in vivo. *J Exp Med.* 2006; 203:2329–2338. [PubMed: 16966429]
32. Sato N, Patel HJ, Waldmann TA, Tagaya Y. The IL-15/IL-15R α on cell surfaces enables sustained IL-15 activity and contributes to the long survival of CD8 memory T cells. *Proc Natl Acad Sci USA.* 2007; 104:588–593. [PubMed: 17202253]
33. Burkett PR, Koka R, Chien M, Chai S, Boone DL, Ma A. Coordinate expression and trans presentation of interleukin (IL)-15R α and IL-15 supports natural killer cell and memory CD8+ T cell homeostasis. *J Exp Med.* 2004; 200:825–834. [PubMed: 15452177]
34. Saikh KU, Khan AS, Kissner T, Ulrich RG. IL-15-induced conversion of monocytes to mature dendritic cells. *Clin Exp Immunol.* 2001; 126:447–455. [PubMed: 11737061]
35. Diab A, Cohen AD, Alpdogan O, Perales MA. IL-15: targeting CD8+ T cells for immunotherapy. *Cytotherapy.* 2005; 7:23–35. [PubMed: 16040381]
36. Klebanoff CA, Finkelstein SE, Surman DR, Lichtman MK, Gattinoni L, Theoret MR, Grewal N, Spiess PJ, Antony PA, Palmer DC, Tagaya Y, Rosenberg SA, Waldmann TA, Restifo NP. IL-15 enhances the in vivo antitumor activity of tumor-reactive CD8+ T cells. *Proc Natl Acad Sci USA.* 2004; 101:1969–1974. [PubMed: 14762166]
37. Coughlin CM, Salhany KE, Wysocka M, Aruga E, Kurzawa H, Chang AE, Hunter CA, Fox JC, Trinchieri G, Lee WM. Interleukin-12 and interleukin-18 synergistically induce murine tumor regression which involves inhibition of angiogenesis. *J Clin Invest.* 1998; 101:1441–1452. [PubMed: 9502787]
38. Albin A, Fassina G, Nicolo M, Dell'Eva R, Vene R, Cammarota R, Barberis M, Noonan DM. Inhibition of a vascular ocular tumor growth by IL-12 gene transfer. *Clin Exp Metastasis.* 2007; 24:485–493. [PubMed: 17653825]
39. Imagawa Y, Satake K, Kato Y, Tahara H, Tsukuda M. Antitumor and antiangiogenic effects of interleukin 12 gene therapy in murine head and neck carcinoma model. *Auris Nasus Larynx.* 2004; 31:239–245. [PubMed: 15364358]
40. Fehniger TA, Cooper MA, Caligiuri MA. Interleukin-2 and interleukin-15: immunotherapy for cancer. *Cytokine Growth Factor Rev.* 2002; 13:169–183. [PubMed: 11900992]
41. Tsung K, Dolan JP, Tsung YL, Norton JA. Macrophages as effector cells in interleukin 12-induced T cell-dependent tumor rejection. *Cancer Res.* 2002; 62:5069–5075. [PubMed: 12208763]
42. Hess SD, Egilmez NK, Bailey N, Anderson TM, Mathiowitz E, Bernstein SH, Bankert RB. Human CD4+ T cells present within the microenvironment of human lung tumors are mobilized by the local and sustained release of IL-12 to kill tumors in situ by indirect effects of IFN- γ . *J Immunol.* 2003; 170:400–412. [PubMed: 12496425]
43. Mylonas KJ, Nair MG, Prieto-Lafuente L, Paape D, Allen JE. Alternatively activated macrophages elicited by helminth infection can be reprogrammed to enable microbial killing. *J Immunol.* 2009; 182:3084–3094. [PubMed: 19234205]
44. Hume DA, Wells CA, Ravasi T. Transcriptional regulatory networks in macrophages. *Novartis Found Symp.* 2007; 281:2–18. [PubMed: 17534062]
45. Raz E. Organ-specific regulation of innate immunity. *Nat Immunol.* 2007; 8:3–4. [PubMed: 17179960]
46. Di Carlo E, Comes A, Basso S, De AA, Meazza R, Musiani P, Moelling K, Albin A, Ferrini S. The combined action of IL-15 and IL-12 gene transfer can induce tumor cell rejection without T and NK cell involvement. *J Immunol.* 2000; 165:3111–3118. [PubMed: 10975824]

47. Comes A, Di Carlo E, Musiani P, Rosso O, Meazza R, Chiodoni C, Colombo MP, Ferrini S. IFN-gamma-independent synergistic effects of IL-12 and IL-15 induce anti-tumor immune responses in syngeneic mice. *Eur J Immunol.* 2002; 32:1914–1923. [PubMed: 12115611]
48. Gu T, Kilinc MO, Egilmez NK. Transient activation of tumor-associated T-effector/memory cells promotes tumor eradication via NK-cell recruitment: minimal role for long-term T-cell immunity in cure of metastatic disease. *Cancer Immunol Immunother.* 2008; 57:997–1005. [PubMed: 18049819]
49. Smeltz RB. Profound enhancement of the IL-12/IL-18 pathway of IFN-gamma secretion in human CD8+ memory T cell subsets via IL-15. *J Immunol.* 2007; 178:4786–4792. [PubMed: 17404259]
50. French AR, Holroyd EB, Yang L, Kim S, Yokoyama WM. IL-18 acts synergistically with IL-15 in stimulating natural killer cell proliferation. *Cytokine.* 2006; 35:229–234. [PubMed: 17052916]
51. Wigginton JM, Gruys E, Geiselhart L, Subleski J, Komschlies KL, Park JW, Wiltout TA, et al. IFN-gamma and Fas/FasL are required for the antitumor and antiangiogenic effects of IL-12/pulse IL-2 therapy. *J Clin Invest.* 2001; 108:51–62. [PubMed: 11435457]
52. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* 2002; 30:e36. [PubMed: 11972351]
53. Egilmez NK, Jong YS, Mathiowitz E, Bankert RB. Tumor vaccination with cytokine-encapsulated microspheres. *Methods Mol Med.* 2003; 75:687–696. [PubMed: 12407772]

Figure 1. IL-12 re-programs TIMs and TAMs *in vivo*.

Mφs isolated from the indicated tissues 24h after injection of placebo or IL-12 microspheres were assayed for cytokine mRNA expression by Q-rt-PCR (solid black bars) immediately upon isolation or assayed for cytokine protein secretion by ELISA after overnight stimulation with LPS (light gray bars). n.t. = not tested. Fold increase/decrease induced by IL-12 is derived from Watkins et al. [11].

Figure 2. IL-12 induces release of cytoplasmic IL-15 from TAMs

Blood and/or peritoneal lavage were obtained from normal and tumor-bearing mice at 2 or 24 h after injection of placebo or IL-12 microspheres. **(A)** Serum was analyzed for IL-15. Data is presented as arithmetic mean $\pm$ s.d. Asterisk indicates $p < 0.01$ in comparison to placebo-treated tumor-bearing mice. **(B, C, D)** Mφs purified from peritoneal lavage, spleen, or primary tumor were analyzed for cytoplasmic IL-15. Histograms of 10^4 CD11b $^{+}$ cells are displayed. Data are representative of 3 independent trials.

Figure 3. Tumor influences IL-12 induced IL-15 release from Mφs in vitro
Purified peritoneal Mφs were cultured for 6 hours with IL-12, tumor-CM, neither or both. The culture fluid was collected and assayed by ELISA for IL-15. Data is presented as arithmetic mean $\pm$ s.d. Results are representative of 4 independent trials.

Figure 4. Neutralization of released IL-15 reduces IL-12 induced leukocytic infiltration

Tumor bearing mice received either empty microspheres (placebo) or microspheres containing IL-12 one h after, or 18 h before, injection of either neutralizing IgG anti-IL-15 or IgG isotype control. Five days later the lungs (**A**) or primary tumor mass (**B**) were harvested and analyzed for infiltration of CD45⁺ CD4⁺ and CD45⁺ CD8⁺ leukocytes. Histograms representative of 4 trials are displayed and statistical analysis is presented in Fig. 5.

Figure 5. IL-15 dependent leukocytic infiltration into tumors is associated with decreases in tumor mass

Data from 3 mice per group in the trial described in Fig. 4 are presented as arithmetic mean $\pm$ s.d. for (A) infiltration of lung (asterisk indicates $p \leq 0.0046$ for both CD4+ and CD8+ T cell infiltration compared to placebo control) and for (B) infiltration of primary tumor (asterisk indicates $p \leq 0.013$ for both CD4+ and CD8+ T cell infiltration compared to placebo control). (C) Primary tumor was weighed after perfusion and the total mass is presented as arithmetic mean $\pm$ s.d. of 3 mice (asterisk indicates $p \leq 0.027$ compared to placebo control). Data is representative of 4 trials.

Figure 6. Neutralization of IL-15 reduces the efficacy of IL-12 induced clearance of lung metastases

Mice were injected with placebo (PBS) microspheres, or with IL-12 microspheres 1 h after either anti-IL-15 or IgG isotype control. Ten days after IL-12 treatment, the lungs were removed (5 per group) and photographed (**A**). H&E sections of the lungs were prepared and representative sections are displayed at 2× (**B**) or 20× (**C**) magnification. Results are representative of 8 independent trials subjected to gross analysis (**A**) and 2 independent trials subjected to histological analysis (**B, C**).

A. Day 4 NK Depletion

B. Lung Infiltration

Figure 7. IL-12 induced leukocytic infiltration is not influenced by depletion of NK cells
(A) The regimen of anti-NK 1.1 used sustained depletion of NK cells for at least 4 days. **(B)** Lungs were removed from intact or NK-depleted mice 5 days after IL-12 injection and analyzed for infiltration of CD45+ CD4+ and CD45+ CD8+ leukocytes. Representative histograms are displayed. In 3 independent trials, no significant difference in leukocyte infiltration was observed between NK-sufficient and NK-depleted mice.

Figure 8.

Four hours after treatment with placebo or IL-12 microspheres, peritoneal Mφs were purified from NK-intact or NK-depleted mice and assayed for expression of cytokine mRNA by real-time RT-PCR. Arithmetic mean $\pm$ s.d. of triplicate samples are presented. The data is representative of 3 independent trials. Paired t test analysis of placebo microspheres to IL-12 microspheres indicated a high level of significance in all groups (ranging from $p < 0.0001$ to $p = 0.0046$). Differences between arithmetic means of intact controls versus NK-depleted mice were not significant (ranging from $p = 0.2220$ to $p = 0.4256$).

Table 1

Impact of IL-12 on cytokine release by intra-tumor macrophages

<u>Cytokine</u>	<u>Tumor-bearing mice treated with:</u>		
	<u>PBS</u>	<u>IL-12</u>	<u>P value</u>
MCP-1	4.6 ng/ml $\pm$.91	1.9 ng/ml $\pm$.52	0.0111
VEGF	388 pg/ml $\pm$ 61	101 pg/ml $\pm$ 38	0.0023
TNF α	308 pg/ml $\pm$ 46	471 pg/ml $\pm$ 81	0.0387

M ϕ s were purified from the primary tumor of mice 24 h after injection of empty (PBS) or IL-12 containing microspheres. M ϕ s isolated from 3 individual mice were cultured overnight without stimulus after which the culture fluid was assayed for the indicated cytokine. Results are representative of 2 independent trials.