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IL-15 is a cytokine that can affect many immune cells, including NK cells and CD8 T cells. In several tumor models, IL-15 delays primary tumor formation and can prevent or reduce metastasis. In this study, we have employed a model of breast cancer metastasis to examine the mechanism by which IL-15 affects metastasis. When breast tumor cells were injected i.v. into IL-15<sup>−/−</sup>, C57BL/6, IL-15 transgenic (TG) and IL-15/IL-15R<sub>e</sub>-treated C57BL/6 mice, there were high levels of metastasis in IL-15<sup>−/−</sup> mice and virtually no metastasis in IL-15 TG or IL-15–treated mice. In fact, IL-15<sup>−/−</sup> mice were 10 times more susceptible to metastasis, whereas IL-15 TG mice were at least 10 times more resistant to metastasis when compared with control C57BL/6 mice. Depletion of NK cells from IL-15 TG mice revealed that these cells were important for protection from metastasis. When NK cells were depleted from control C57BL/6 mice, these mice did not form as many metastatic foci as IL-15 TG mice, suggesting that other cell types may be contributing to metastasis in the absence of IL-15. We then examined the role of CD4 T cells and macrophages. In IL-15<sup>−/−</sup> mice, in vivo depletion of CD4 T cells decreased metastasis. The lack of IL-15 in IL-15<sup>−/−</sup> mice, and possibly the Th2-polarized CD4 T cells, was found to promote the formation of M2 macrophages that are thought to contribute to metastasis formation. This study reveals that whereas IL-15 effects on NK cells are important, it also has effects on other immune cells that contribute to metastasis. The Journal of Immunology, 2014, 193: 6184–6191.

Interleukin-15 is a cytokine that has effects on cells of the innate and adaptive immune system. It is most known for its effects in promoting the survival, differentiation, and activation of NK cells, as well as promoting memory CD8 T cell responses (1–3). Because both NK cells and CD8 T cells are important in tumor defense and correlate with survival in various cancers, there has been extensive interest in IL-15 as an immunotherapy (4–6). The effects of IL-15 have been assessed in many tumor models, and it has been found to be effective against primary tumors as well as metastasis (7–9). In many cases, the mechanism of protection from metastasis was not evaluated. When investigated in models of metastasis, NK cells were most frequently the cell type identified that was important for protection (7, 9).

In recent years, it has become apparent that macrophages are very important cell types involved in the promotion of metastasis. Classically activated macrophages, also known as M1, are able to promote tumor cell death due to their production of NO, proinflammatory cytokines, efficient Ag presentation, and phagocytic abilities (10, 11). In contrast, the alternatively activated group of macrophages (M2), of which tumor-associated macrophages (TAMs) are a member, has more of a role in tumor promotion (10, 12). It functions to promote angiogenesis, tumor cell invasion, and intravasation, and it plays a role at the metastatic site (11). Macrophages are very plastic and are able to travel between M1 and M2 states depending on the microenvironment in which they are found (13). M1 macrophages are created in environments with stimuli such as IFN-γ and LPS (14). M2 is a group of macrophages that can develop under the influence of IL-4/IL-13, immune complexes, IL-10, TGF-β, or glucocorticoids (14). Although the role of macrophages at the primary tumor is well established, a lot less attention has been paid to the role of macrophages at the metastatic site. Kaplan et al. (15) found that after primary tumors have been established, but before metastasis has occurred, there are premetastatic sites in which there are clusters of vascular endothelial growth factor receptor<sup>+</sup> hematopoietic bone marrow progenitors. CD11b<sup>+</sup> myeloid cells are also attracted to these premetastatic niches, and these cells are important for establishing metastasis (16). Recently, to investigate the role of macrophages at the metastatic site, several groups have used the injection of tumor cells i.v. to mimic tumor metastasis. In models such as this, macrophages are required for efficient tumor cell seeding as well as growth at the metastatic site (17, 18).

Macrophage polarization is dependent upon the signals it receives in its microenvironment. It is, of course, no wonder that CD4 T cells play a role in macrophage polarization. Th1 CD4 T cells produce IFN-γ, which promotes M1 macrophage polarization, whereas Th2 or regulatory T cells produce IL-10, IL-4, or IL-13, which promotes M2 macrophage polarization (19). In fact, in mouse models that are Th2 biased, knockout of CD4 T cells promotes the formation of macrophages with a more M1 phenotype (20, 21). Macrophages also influence the polarization of...
T cells themselves, with M2 macrophages promoting the formation of T regulatory cells (22). Recently, it was discovered that, in a spontaneous model of breast tumor formation, CD4 T cells expressing IL-4 were able to promote invasion and metastasis due to their effects on promoting M2 phenotype in TAMs (20). In this case, loss of CD4 T cells actually decreased the amount of metastasis observed (20). Other studies have also indicated that CD4 T cells may play a role in promoting metastasis, although via different mechanisms (23, 24).

The effect of IL-15 on macrophages and CD4 T cells is an area that has not been studied thoroughly. It is known that IL-15 is produced from human monocytes upon LPS activation (25). Several in vitro studies on various sources of macrophages (mouse peritoneal macrophages, RAW cells, human monocytes) indicate that IL-15 is able to stimulate proinflammatory cytokine release (TNF-α, IL-1, IL-6, IL-8, MCP-1) as well as NO production (26, 27). Others have also determined that IL-15 treatment promotes the formation of macrophages with higher costimulatory molecules (CD80/86) as well as increased MHCIi (28). The reported effects of IL-15 on CD4 T cells are rather contradictory. Early reports indicated IL-15 could prime CD4 T cells for Th1, although later it was found that it could induce Th1 or Th2 depending on other cytokines present (IL-12/IL-4, respectively) (29, 30). In addition, some report that it can prime naive T cells, whereas others report effects only on memory CD4 T cells (29–31). Some find that IL-15 can promote the formation of T regulatory cells, but that it also increases the proliferation and cytokine secretion of IFN-γ-producing CD4 and CD8 T cells so that the T regulatory cells were unable to inhibit the T cells (32, 33). In contrast, it was recently found that, in mice that lack the receptor for IL-15 signaling (IL-15Rα−/−), CD4 T cells that were primed in this environment produced high levels of IL-10 (34).

In view of the recent literature, we wanted to determine the effect of IL-15 on metastasis and the mechanism by which it promotes this effect. This will include whether IL-15 or the lack of IL-15, directly or indirectly, affects the polarization of macrophages and CD4 T cells and whether this would affect metastasis. We have employed a model of metastasis in which we inject breast tumor cells i.v. in mice that lack IL-15 (IL-15−/−) and mice that over-express IL-15 (IL-15 transgenic [TG]). IL-15−/− mice lack NK cells, whereas IL-15 TG mice have increased NK cells (2× in C57BL/6; data not shown) (35, 36). This model of metastasis is frequently used to examine the later stages of metastasis when tumor cells have already entered the bloodstream. In this model, we will examine the roles played by IL-15, NK cells, CD4 T cells, and macrophages in lung metastasis.

Materials and Methods

Animal models

IL-15−/− and IL-15 TG mice were bred and maintained in the McMaster Central Animal Facility in clean rooms with a 12-h day/night schedule and standard temperature controls. Control C57BL/6 mice were ordered from Charles River. Procedures performed in this study were approved by the McMaster Animal Research Ethics Board and comply with the guidelines set out by the Canadian Council on Animal Care. C57BL/6 (Charles River), IL-15−/− mice (Taconic, Germantown, NY), and IL-15 TG mice (provided by M. Caligiuri, Ohio State University, School of Medicine, Columbus, OH) on a C57BL/6 background were used.

Metastasis model

A cell line was established from a mouse mammary tumor virus/polypoma middle T (MT) C57BL/6 spontaneous breast tumor (Mayo Clinic, Scottsdale, AZ) grown in RPMI 1640 (10% FBS, 1% penicillin/streptomycin, 1% HEPES, and 1% l-glutamine). After brief trypsinization, cells were washed in PBS and resuspended as 0.5×10⁶–1×10⁸ per 400 μl sterile PBS. Cells with <5% cell death were injected via the tail vein of IL-15−/−, C57BL/6, or IL-15 TG mice. Mice were monitored for endpoint, and 13 d postinjection, mice were euthanized and lungs were harvested. Lungs were fixed in 2% paraformaldehyde for 48 h. Sections were then stained with H&E for analysis of lung tumor metastasis. The level of metastasis was assessed while blinded in ImageJ. In brief, in an entire lobe of lung, per each experimental animal, a threshold algorithm was applied to assess tumor area (manually checked for accuracy). Total lung area (with lumen areas subtracted) was also assessed, and percentage of tumor area was calculated by the following formula:

\[ \text{Percent tumor area} = \frac{\text{tumor area}}{\text{total lung area}} \times 100 \]

Melanoma cells (B16F10) were also used and were prepared in the same manner as MT cells. A total of 1×10⁶ cells was injected in the tail vein of IL-15−/− and C57BL/6 control mice. Lungs were harvested 14 d postinjection and fixed in paraformaldehyde, and the number of visible nodules was assessed with a stereoscope.

IL-15/IL-15Ra treatment

A dose of IL-15 (500 μg; PeproTech) and IL-15Ra (1 mg; PeproTech) in 200 μl PBS per mouse was incubated for 30 min at 37°C prior to injection i.p. into mice. C57BL/6 mice were treated with this dose of IL-15/IL-15Ra 4 and 3 d prior to tumor cell injection, on the day of tumor cell injection and every 3–4 d till the end of the experiment. This dosing schedule had been previously tested in our laboratory (data not shown). A total of 1×10⁶ MT cells was injected i.v. Mice were monitored for endpoint, and 13 d postinjection, mice were euthanized and lungs were harvested. Lungs were fixed in 2% paraformaldehyde for 48 h. Sections were then stained with H&E for analysis of lung tumor metastasis.

NO assay

Under anesthetic, the peritoneal cavity of the mice was washed with RPMI 1640 (10% FBS, 1% penicillin/streptomycin, 1% HEPES, and 1% l-glutamine). Resultant cells were plated for 2–4 h, and only adherent cells were collected to be used in subsequent assays. A total of 1×10⁶ or 2×10⁶ peritoneal macrophages was plated in 96-well plates with RPMI 1640 (10% FBS, 1% penicillin/streptomycin, 1% HEPES, and 1% l-glutamine) or media supplemented with LPS (100 ng/ml; Sigma-Aldrich). Assay was performed in triplicate. At 24 and 48 h, media was collected and combined with Greiss reagent (0.04 g/ml; Sigma-Aldrich). Absorbance was measured at 450 nm and compared with a standard curve of sodium nitrate.

FIGURE 1. IL-15−/− mice have increased lung metastasis. IL-15−/−, C57BL/6, and IL-15 TG mice were injected with 5×10⁵ MT cells i.v. After 13 d, lungs were harvested, fixed, sectioned, and stained for H&E (original magnification ×1.6) (A). Percentage of lung area occupied by metastasis is reported in (B) (n = 5/group). Representative of three experiments. ***p < 0.001.
Ab depletions

Mice were given two injections, 1 day apart of either anti-CD4 (GK1.5; American Type Culture Collection) (100 µg) or anti-NK1.1 (PK136) (200 µg) Ab i.p. On day 4 after the first injection, MT cells were injected in the tail vein. CD4 depletion was carried out once per week, and NK1.1 depletion was every 3–4 d to maintain the depletion for the course of the experiment. Efficiency of depletion was checked via flow cytometry. Spleens from each group were isolated, and stained for H&E. Each picture is representative of a different mouse in that group (numbered 1–5).

Nonspecific CD4 stimulation

Single-cell suspensions were created from spleens of IL-15−/−, C57BL/6, and IL-15 TG mice. CD4 T cells were isolated using CD4 T cell enrichment kit (Stem Cell) and plated at 5 × 10^5 in triplicate in 96-well plates that had been coated in purified CD3 (1 µg/ml)/CD28 (5 µg/ml) (eBioscience) Ab overnight at 4°C (washed in 2× PBS before plating). After 48 h, the supernatant was collected and analyzed for cytokines. Control wells were included. Purity of CD4 T cells after enrichment was >85% via flow cytometry.

ELISA

IL-10 (DY417) and IL-13 (DY413) ELISAs were performed using Quantikine Murine Duoset Kits from R&D Systems (Minneapolis, MN). ELISAs were performed as per manufacturer’s instruction.

Isolation of cells and RNA from lungs

IL-15−/− mice or C57BL/6 mice were injected with 5 × 10^5 MT tumor cells i.v. Two days postinjection, lungs were perfused and harvested, and one lobe was used for RNA isolation and the other for cell isolation. For cell isolation, lungs were minced and digested in 150 U/ml collagenase I (Life Technologies) in HBSS for 1 h at 37°C with shaking. Cells were then filtered and stained for CD16/32 (eBioscience) (1 in 100, 15 min, 4°C), followed by staining for CD45, F480, CD11b, CD11c, GR1 or CD45, CD4, CD3 (BD Biosciences or eBioscience). CD45 R− leukocytes were gated on first to begin analysis. To extract RNA, TRIzol (Invitrogen) was used initially (as per manufacturer’s instruction), followed by Qiagen RNeasy Mini Kit and on-column DNase treatment (RNase-Free DNase Set 79254; Qiagen). Subsequently, 1 µg RNA per sample was used to create cDNA following the procedure outlined in RT2 First Strand Kit 3304011 (Qiagen). The cDNA was then used in a RT2 Profiler PCR Array for Mouse Cytokines and Chemokines, as per manufacturers’ instruction (RT2 SYBR Green ROX PCR Mastermix; SABiosciences/Qiagen). This 96-well plate includes housekeeping genes, genomic DNA controls, reverse-transcription controls, and positive controls. The plate was run on the Applied Biosystems 7900 RT machine. Results were analyzed using the SABiosciences analysis tool, and all samples used passed the quality controls inherent in these assays. Results are reported as fold up- or downregulation, with two samples per group included in the analysis (accession GSE61858; link to data file: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE61858).

Statistical analysis

GraphPad Prisms was used for statistics. T tests or one-way ANOVAs were performed, depending on the number of groups to be compared. For ANOVAs, Bonferonni’s posttest was employed to compare groups.

Results

IL-15−/− mice have increased lung metastasis, whereas IL-15 TG mice and IL-15–treated C57BL/6 mice have decreased lung metastasis in comparison with control

IL-15 can impact, both directly and indirectly, many cell types that are thought to be important in the establishment of metastasis. To determine whether the absence or overexpression of IL-15 is able to affect the formation of metastasis, we used a mouse model mimicking breast tumor metastasis. IL-15−/−, C57BL/6, and IL-15 TG mice were injected i.v. with 5 × 10^5 MT cells (cell line established from a mouse mammary tumor virus-polyoma MT mouse breast tumor). The IL-15−/− mice had lungs with extensive metastasis (Fig. 1). At the same time point, there was greatly reduced metastatic tumor burden in control mice, and metastasis was virtually
absent in IL-15 TG mice (Fig. 1). The difference between IL-15−/− and C57BL/6 or IL-15 TG mice was statistically significant. To explore the susceptibility of IL-15−/− mice, we reduced the amount of tumor cells injected down to $1 \times 10^4$ (Fig. 2A). Tumors began to form in IL-15−/− mice at as low a dose as $5 \times 10^3$, whereas they did not form in C57BL/6 mice until a dose of $5 \times 10^4$. To delineate the differences between control and IL-15 TG mice, we increased the amount of tumor cells injected $(1 \times 10^5)$ (Fig. 2B). In this scenario, metastasis in the control mice was much higher and was still absent in IL-15 TG mice (Fig. 2B). Lastly, to confirm the high degree of susceptibility to MT metastasis observed in the IL-15−/− mice in an alternate tumor model, we injected $1 \times 10^5$ B16F10 melanoma cells i.v. Once again, a significantly higher degree of lung tumor formation was observed in the IL-15−/− mice in comparison with control C57BL/6 mice.

We also examined what would occur in our model with short-term treatment of control C57BL/6 mice with exogenous IL-15/IL-15Rα prior to and during the metastasis experiment. The combination of IL-15 with IL-15Rα was used for treatment, as it has been demonstrated to have higher activity, increased stabilization, and longer $t_{1/2}$ than IL-15 alone (37, 38). We found that, at a dose of $1 \times 10^6$ MT cells i.v., there was extensive lung metastasis in all C57BL/6 mice, but virtually no metastasis in IL-15/IL-15Rα–treated mice or IL-15 TG mice (Fig. 3). Thus, short-term administration of IL-15 was able to protect from metastasis formation.

Resistance to metastasis in IL-15 TG mice is NK cell dependent

IL-15 has very important effects on cells of both the innate and the adaptive immune system. It is known that IL-15−/− mice lack NK cells, whereas IL-15 TG mice have increased NK cells and CD8 T cells (35, 36, 39). NK cells and CD8 T cells function in tumor immunosurveillance and are able to kill tumor cells (39). To determine whether NK cells or CD8 T cells are important to the resistance observed in IL-15 TG mice, we depleted IL-15 TG mice with anti-NK1.1 or anti-CD8α Abs prior to and throughout the experiment (Fig. 4). Depletions with these Abs were effective (data not shown). A total of $5 \times 10^5$ MT tumor cells was injected i.v. The depletion of NK1.1+ cells increased metastasis to levels equal to those found in IL-15 TG mice

**FIGURE 4.** Depletion of NK cells in IL-15 TG mice promotes metastasis to a similar level as in IL-15−/− mice. (A and B) IL-15 TG mice were depleted of NK cells or CD8 T cells prior to injection with $5 \times 10^5$ MT cells. Depletion of CD8 T cells had no effect on metastasis, whereas depletion of NK cells promoted tumor formation (representative pictures shown, $n = 5$ depleted group, $n = 4$ IL-15−/−, $n = 3$ IL-15 TG). H&E, original magnification $\times 1.6$. (B) Percentage of lung area occupied by metastasis is reported. *p < 0.05.

**FIGURE 5.** NK cells decrease metastasis in C57BL/6 mice and contribute to type 1 cytokines in the lung environment, but do not recapitulate the IL-15−/− phenotype. (A) C57BL/6 and IL-15−/− mice were depleted of NK cells (anti-NK1.1 Ab) or treated with PBS prior to injection with $5 \times 10^5$ MT cells i.v. Depletion was continued throughout the experiment. After 13 d, lungs were harvested and fixed for H&E sectioning ($n = 5$ group). Original magnification $\times 1.6$. (B) Percentage of lung area occupied by metastasis is reported. (C) Lungs were isolated from IL-15−/− or C57BL/6 mice 2 d post tumor cell injection ($5 \times 10^5$). RNA was extracted, cDNA was synthesized, and a PCR array was performed for cytokines and chemokines ($n = 2$ group). Fold downregulation in IL-15−/− versus C57BL/6 is reported.
similar to that seen in IL-15−/− mice, whereas depletion of CD8 T cells had no statistically significant effect on metastasis (Fig. 4). Therefore, NK cells are key mediators of the protection seen in IL-15 TG mice.

**NK depletion in control mice increases metastasis, but not to the same level observed in IL-15−/− mice**

To determine whether the lack of NK cells in IL-15−/− mice was the factor that promoted increased metastasis in these mice, control mice were depleted of NK cells before being injected with 5 × 10⁵ MT cells. Depletions were maintained for the duration of the experiment. We found that, although depletion of NK cells in control mice promoted metastasis, the trend in two separate experiments was that there was still less metastasis than was observed in IL-15−/− mice (Fig. 5A, 5B). Thus, it seemed likely that there were other factors involved that promoted metastasis in IL-15−/− mice. We also assessed the differences in cytokine expression in the lung environment of IL-15−/− lung versus control C57BL/6 lung 2 d posttumor cell injection (accession GSE61858; link to data file: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE61858). The main difference between these lungs involved a decrease in CCL5 (RANTES; 4548), Fas ligand, IFN-γ, and XCL1 (lymphotactin) in IL-15−/− mice (Fig. 5C). CCL5, IFN-γ, and XCL1 have all been identified as being expressed when NK cells are activated in response to infections such as Listeria or murine CMV (40, 41).

**CD4 T cells in IL-15−/− mice promote metastasis**

Recently, several reports have indicated that CD4 T cells play a role in promoting metastasis (20, 23, 24). IL-15 is known to affect CD4 T cell phenotype, but conclusions regarding this have been mixed. To determine whether CD4 T cells impacted metastasis in vivo, IL-15−/− mice were depleted of CD4 T cells before being injected i.v. with 5 × 10⁵ MT tumor cells (Fig. 6). Depletion of CD4 T cells from IL-15−/− mice decreased tumor formation greatly in comparison with IL-15−/− mice (Fig. 6A, 6B). Therefore, CD4 T cells in IL-15−/− mice promoted tumor formation. It was also found that CD4 T cells were a higher proportion of CD45+ leukocytes in the lung of IL-15−/− mice 2 d posttumor cell injection in comparison with control lungs (Fig. 6C). Thus, they were a highly prevalent immune cell present in the IL-15−/− mouse lung microenvironment.

**IL-15−/− mice have M2-polarized macrophages, whereas IL-15 TG mice have M1-polarized macrophages**

TAMs or M2 macrophages are an important cell type for promotion of metastasis (17, 42). Many studies have shown that, at the primary site, TAMs promote both invasion and metastasis and are correlated with poor prognosis (11). It has been previously found in the same model of metastasis used in this work that genetic or chemical ablation of macrophages decreases both metastatic seeding and growth (17). To determine whether IL-15 affects the polarization of macrophages, we analyzed the ability of peritoneal macrophages from IL-15−/−, control, and IL-15 TG mice to produce NO in response to LPS (Fig. 7A). IL-15 TG macrophages produced the highest amounts of NO, which indicates a M1 phenotype, whereas IL-15−/− mice produced the lowest amount of NO. We also examined the myeloid population present in the lungs of IL-15−/− and C57BL/6 mice at 2 d posttumor cell injection (Fig. 7B). Cells in the lung were stained for F4/80 to
Peritoneal macrophages from IL-15 were contributing to the M2 macrophage phenotype in these mice, representative of four experiments. (A) Peritoneal macrophages were isolated from C57BL/6, IL-15−/−, and IL-15 TG mice. Cells were assessed via a NO assay for their ability to respond to LPS with the production of NO at 24 and 48 h. IL-15 TG mice consistently had higher levels of NO production in response to LPS. Lungs were isolated from IL-15−/− or C57BL/6 mice 2 d posttumor cell injection (5 × 105). Lungs were digested, and the resultant cells were analyzed via flow cytometry for CD45, F4/80, CD11b, CD11c, and Gr1. Results are reported as percentage of F4/80+ cells. *p < 0.05. (B) Lungs were isolated from IL-15−/− or C57BL/6 mice 2 d posttumor cell injection (5 × 105). Lungs were digested, and the resultant cells were analyzed via flow cytometry for CD45, F4/80, CD11b, CD11c, and Gr1. Results are reported as percentage of F4/80+ cells. *p < 0.05.

**FIGURE 7.** IL-15 affects macrophage polarization and recruitment. (A) Peritoneal macrophages were isolated from C57BL/6, IL-15−/−, and IL-15 TG mice. Cells were assessed via a NO assay for their ability to respond to LPS with the production of NO at 24 and 48 h. IL-15 TG mice consistently had higher levels of NO production in response to LPS. Representative of four experiments. (B) Lungs were isolated from IL-15−/− or C57BL/6 mice 2 d posttumor cell injection (5 × 105). Lungs were digested, and the resultant cells were analyzed via flow cytometry for CD45, F4/80, CD11b, CD11c, and Gr1. Results are reported as percentage of F4/80+ cells. *p < 0.05.

**FIGURE 8.** In the absence of IL-15, CD4 T cells are of a more Th2 phenotype and promote M2 macrophage polarization. (A and B) CD4 T cells were isolated from the spleen of IL-15−/−, C57BL/6, and IL-15 TG mice and stimulated nonspecifically (CD3/CD28) for 48 h. Supernatants were collected and assessed for IL-10 and IL-4 production via ELISA. (C) Peritoneal macrophages from C57BL/6, IL-15−/−, and IL-15−/− CD4-depleted mice were harvested, plated, and tested for production of NO. ***p < 0.001.

**Discussion**

The ability of IL-15 to affect tumor formation and its effects on both NK cells and CD8 T cells have been well studied in a variety of tumor models. Unfortunately, very few studies have examined extensively the role of IL-15 in metastasis and its effects on immune cells other than NK cells or CD8 T cells. In this study, we have shown in a breast cancer model of metastasis that IL-15 overexpression or IL-15/IL-15R treatment greatly decreases the formation of metastatic sites in the lung, whereas lack of IL-15 highly promotes the formation of metastasis. The susceptibility of IL-15−/− mice to metastasis was observed in both the breast cancer MT model and a melanoma B16F10 model, thus indicating that the effect is not specific to one particular cell line or tumor type. In IL-15 TG mice, this protection was found to be dependent on NK cells. This agrees with earlier studies in which the effects of IL-15 in metastatic models of colon carcinoma or melanoma are NK cell dependent, not CD8 T cell dependent (7, 9). We observed that the loss of IL-15, and hence NK cells, from IL-15−/− lungs led to a decrease in type 1 cytokines such as IFN-γ, CCL5, and XCL1. These cytokines have been described as the Th1-promoting cytokines expressed by activated NK cells in certain infections (40, 41). It is also possible that these molecules can be expressed by Th1 CD4 or CD8 T cells, as well as macrophages (40, 41, 44). These molecules can affect many cell types to prevent the formation of lung metastasis. For example, in a model of melanoma metastasis,
production of IFN-γ by lung NK cells was critical for resistance to metastasis (45). In addition, IFN-γ is known to promote the formation of M1 macrophages and Th1 T cells (46). Chemokines such as CCL5 and XCL1 have been found to play a role in antitumor defense by recruiting immune cells such as NK cells, Th1, CD4 T cells, and CD8 CTL into the site of expression, in this case the lung (47–49). Due to the significant susceptibility of IL-15 knockout mice to metastasis, it is likely that the lack of NK cells in these mice could be a significant contributor to susceptibility. Interestingly, when we depleted NK cells from control mice, it did not fully recapitulate the degree of metastasis seen in IL-15−/− mice. This indicates that the absence of IL-15 has more of an effect on metastasis than the loss of NK cells alone. When IL-15 is absent, there must be an effect on other factors that are important in promoting metastasis. Indeed, it has been suggested previously that IL-15 has antitumor activities that are not dependent upon NK cells or CD8 T cells (50).

It has recently come to light that macrophages not only have a protumoral role at the primary tumor, they are also very important for the establishment of metastasis (17, 18). They contribute both to the extravasation of the tumor cells and growth at the metastatic site (17, 18). Therefore, we were interested in determining whether overexpression or lack of IL-15 has an impact on macrophage phenotype. The macrophages that aid in tumor formation and metastasis have multiple subtypes, but most of the functions they exhibit are more of the M2 phenotype (11, 12, 18). Previously, there have been reports that IL-15 affects macrophages by promoting the secretion of proinflammatory cytokines and increasing MHCII (26, 28). In this study, we found that peritoneal macrophages from IL-15 TG mice have much higher levels of NO production in comparison with wild type, whereas IL-15−/− macrophages produced little to no NO. NO production is typical of M1 macrophages and is a mechanism by which macrophages can cause tumor cell death (51). Therefore, IL-15 overexpression promotes the formation of M1 macrophages, whereas lack of IL-15 changes macrophages into more of an M2 phenotype. It is unknown whether this is a direct effect of IL-15, or an effect due to how IL-15 affects other cell types. We also examined the myeloid cell environment in IL-15−/− and control mice 24 h post-tumor cell injections. We found that IL-15−/− lungs had an increased proportion of recruited macrophages from the circulation. An increase in this population, not lung-resident macrophages, has been shown to aid in the extravasation and establishment of lung metastasis (17).

It has been suggested that CD4 T cells play a role in polarizing macrophages and promoting metastasis (20, 52). Denardo et al. (20) found that, in a spontaneous mouse breast tumor model, CD4 T cells secrete Th2 cytokines that promote the formation of M2 macrophages in the primary tumor. Removal of these CD4 T cells promoted TAMs with a M1 phenotype, and, although this did not affect primary tumor growth, it did decrease the amount of metastasis (20). In our case, we are modeling metastasis by injecting MT tumor cells i.v., so we will only observe the effects of macrophages/CD4 T cells at the metastatic site. The first step was to determine whether IL-15 was able to affect the Th1/Th2 balance of CD4 T cells. It has been previously shown that IL-15 can cause proliferation of CD4 T cells (naive and/or memory), and there are contrasting results as to whether it promotes T regulatory cells or highly active Th1 CD4 T cells (29, 30, 32). This lack of consensus between studies is most likely due to the different in vitro and in vivo conditions under which they investigated the effect. We have shown that CD4 T cells isolated from IL-15−/− mice are capable of producing high levels of the Th2 cytokines IL-10 and IL-4. This indicates a more Th2 CD4 T cell in mice that lack IL-15. We have not established whether this change in CD4 T cell phenotype is directly due to loss of IL-15 or indirect due to its effects on other cells such as NK cells. As mentioned, the macrophages in IL-15−/− mice produce very little NO, but when we depleted CD4 T cells they produced wild-type levels of NO. Thus, the lack of IL-15 in IL-15−/− mice promotes CD4 T cells that are more Th2 biased and promotes the formation of M2 macrophages. It was confirmed that in vivo, CD4 depletion in IL-15−/− mice reduced the formation of metastasis. Therefore, CD4 T cells play an important role in promoting metastasis, and this most likely involves their impact on macrophage polarization.

Further examination into whether the effects of IL-15 loss/gain on CD4 T cell and macrophage phenotype are direct or indirect would be of interest.

In this model of metastasis, IL-15 acts to decrease metastasis, whereas the absence of IL-15 promotes metastasis. These effects are due to NK cells, but also to the effects of IL-15 on other immune cells such as macrophages and CD4 T cells. Because metastasis is a significant contributor to mortality in breast cancer, this study indicates that IL-15 may be an ideal immunotherapy, and perhaps its antitumor effects on immune cells are even more widespread than previously appreciated.

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Disclosures

The authors have no financial interests of interest.

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