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NK-Dependent Increases in CCL22 Secretion Selectively Recruits Regulatory T Cells to the Tumor Microenvironment

Adam W. Mailloux2* and M. Rita I. Young3†‡§

Tumor-induced immune suppression involves the accumulation of immune-suppressive infiltrates in the microenvironment. This study demonstrates increased numbers of CD4+CD25+FoxP3+ regulatory T cells (Tregs) in the lungs of C57BL/6 mice bearing a metastatic Lewis lung carcinoma (LLC) variant. These Tregs suppressed the proliferation of endogenous CD4+CD25− cells and expressed higher levels of the chemokine receptor CCR4 than other types of T cells. LLC-bearing lungs secreted elevated levels of the CCR4-associated chemokine CCL22 compared with normal lungs. However, CCL22 was not secreted by LLC or normal epithelial controls, suggesting that CCL22 is secreted by a nonepithelial component of the microenvironment. Migration assays revealed that medium conditioned by LLC-bearing lungs selectively recruited Tregs at higher frequencies than did medium conditioned by normal lungs. Neutralization of CCL22 significantly reduced this selective recruitment toward both conditioned media. A series of immunomagnetic isolations, FACS, and flow cytometric analyses were used to isolate different cellular fractions from both normal and LLC-bearing lungs. When isolated, only the NK-containing fractions secreted CCL22, and the same fraction isolated from LLC-bearing lungs secreted higher levels. Depletion of NK cells from both normal and LLC-bearing lung tissue significantly reduced CCL22 secretion, suggesting that a large portion of secreted CCL22 is NK cell dependent. Flow cytometric analysis of the lung NK compartments revealed no significant increase in NK cell numbers across LLC-bearing lung tissue as a whole compared with normal tissue. However, immunofluorescent staining revealed an increased frequency of NK cells at the tumor periphery that were closely associated with the elevated FoxP3+ infiltrate. The Journal of Immunology, 2009, 182: 2753–2765.

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4 Abbreviations used in this paper: Treg, regulatory T cell; LLC, Lewis lung carcinoma; MFI, mean fluorescence intensity; ND, not detected.

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The present study examines Treg levels and changes in chemokine secretion in a murine model of Lewis lung carcinoma (LLC). This aggressive squamous cell carcinoma readily establishes tumors in the lungs of injected mice and shares many pathological characteristics common to most squamous cell carcinomas. Although these studies reiterate the involvement of CCR4-associated chemokines in Treg recruitment, these studies also identify NK cells as a major source of CCL22 in the LLC microenvironment, providing a completely novel link between infiltrating NK cells and Treg recruitment.

**Materials and Methods**

**Cell culture**

A metastatic cell line of LLC (LN7) was used in all experiments. LLC and normal mouse lung epithelial cells (MLE-12) were kept in culture medium (RPMI 1640 medium (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.02 M HEPES buffer, and 5 × 10⁻⁵ M 2-ME). Cells were passed as needed just before confluence. MLE-12 cultures were detached before passage by brief trypsin treatment. LLC-LN7 cells are nonadherent in culture.

**Generation of LLC-bearing lung tissue**

Single-cell suspensions of 1.0 × 10⁵ cells/ml washed in HBSS (Invitrogen) were prepared from LLC cell cultures that were in log phase. Female C57BL/6 mice were injected i.v. in the tail vein with 0.2 ml of PBS or LLC cell suspension, resulting in the delivery of 2.0 C57BL/6 mice were injected i.v. in the tail vein with 0.2 ml of PBS or LLC cells. Cells were passed as needed just before confluence. MLE-12 cultures were detached before passage by brief trypsin treatment. LLC-LN7 cells are nonadherent in culture.

**Flow cytometry**

Nonselective staining was blocked with PBS and 1 μl per 10 cells of PBS (16–18 days later when signs of labored breathing first appeared in the LLC-injected animals. Lung tissue was harvested and placed in PBS before subsequent assays or was frozen in blocks in OCT (Sakura) compound using liquid N². The present study examines Treg levels and changes in chemokine secretion in a murine model of Lewis lung carcinoma (LLC). This aggressive squamous cell carcinoma readily establishes tumors in the lungs of injected mice and shares many pathological characteristics common to most squamous cell carcinomas. Although these studies reiterate the involvement of CCR4-associated chemokines in Treg recruitment, these studies also identify NK cells as a major source of CCL22 in the LLC microenvironment, providing a completely novel link between infiltrating NK cells and Treg recruitment.

**Immunomagnetic isolation**

M 2-ME). Cells were passed as needed just before confluence. MLE-12 cultures were detached before passage by brief trypsin treatment. LLC-LN7 cells are nonadherent in culture.

**Generation of conditioned medium**

Following harvest, entire sets of both normal and LLC-bearing lungs were used to measure their immune inhibitory activity toward CD4⁺CD25⁺ cells. The CD4⁺CD25⁺ cells were isolated from dissociated tissue and labeled with CFSE (Molecular Probes). Ninety-six-well plates were coated with 0.5 μl of anti-CD3 Ab in Ca²⁺/Mg²⁺-free PBS (Life Technologies) and then washed with PBS. Cells were plated at 2.0 × 10⁶ cells/well in culture medium with 10% FBS at CD4⁺CD25⁺ cell to CD4⁺CD25⁻ cell ratios of 1:1, 1:10, 1:100, and 1:1000. Control wells contained CD4⁺CD25⁻ cells without CD4⁺CD25⁺ cells. After 3 days, proliferation of the CD4⁺CD25⁺ cells was measured using flow cytometry and reported as a percentage of cells that have undergone at least one division as defined by CFSE fluorescent intensity. The immunofluorescent staining of frozen tissue sections

**Transwell migration assay**

Twelve-well Transwell plates with 6.5-μm insert filters (8-μm pore size; Costar) were used to measure migration. Seventy-five microliters of growth factor-reduced Matrigel (BD Biosciences) was coated on each insert and allowed to set for 2 h before being washed with serum-free culture medium. T cells isolated from normal splenocytes were reconstituted in culture medium with 1% FBS and seeded in the upper chamber (inserts) of the Transwell. Media placed in the bottom chambers contained 50% conditioned medium (serum free) and 50% culture medium with 2% FBS. In some cases, various amounts of neutralizing anti-CC122 (R&D Systems) were added to the conditioned medium before the assay. The T cells were left to migrate for 18–24 h at 37°C. Both the migratory and nonmigratory cell fractions were collected and analyzed using flow cytometry after immunofluorescent staining with CD4, CD8, CD25, and FoxP3.
FIGURE 1. Treg levels are increased in LLC-bearing lung tissue.
Immunofluorescent staining was performed on frozen sections from normal lung tissue (A) and LLC-bearing lung tissue (B) for nuclei (Hoechst, blue), CD4 (rhodamine, red) and FoxP3 (FITC, green). Tregs (white arrows) are considered CD4+ cells that also contain FoxP3 staining in the nucleus (merge). Tumor periphery is marked with a dashed white line. Insets are of a higher magnification of the indicated field. Immunofluorescent staining of CD4, CD25, and FoxP3 was also performed on normal and LLC-bearing lung tissue dissociates. Flow cytometric analysis shows an increase in total Treg numbers (C) and an increase in the Treg percentage of CD4 infiltrate (D) in LLC-bearing lung tissue. Tregs that were immunomagnetically isolated from both normal and LLC-bearing lung tissue displayed the ability to suppress the proliferation of endogenous CD4+CD25− cells isolated from the respective tissue types (E).

Generation of activated NK cells

NK cells were isolated from normal splenocytes via negative immunomagnetic separation (Miltenyi Biotec). The NK cells were then subjected to a range of IL-2 doses for 48 h in RPMI 1640 culture medium. IFN-γ ELISA was used to confirm NK cell activation.

Statistics

Data are reported using the mean as a measure of central tendency ± SEM. To compare one variable condition between groups, the two-tailed Student’s t test was used. Significance was reported in the 95% confidence interval.
IL-2. Although Treg levels were increased in LLC-bearing lungs, tissue samples. Proliferation was then stimulated using anti-CD3 and the CD8\(^+\) CFSE-labeled CD4\(^+\) and LLC-bearing lungs and coculturing in various ratios with normal lung tissue. However, in LLC-bearing lung tissue, an immunomagnetically isolating CD4\(^+\) expressed FoxP3, but are not immune suppressive (21). To address type, some studies have found rare populations of T cells that though Foxp3 expression is a good indicator of the Treg phenotype, some studies have found rare populations of T cells that expressed the proliferation of endogenous CD4\(^+\)CD25\(^-\) cells in a dose-dependent manner (Fig. 1E). This validated their identity as Tregs.

**Tregs express more CCR4 than other types of T cells**

Because previous studies had shown that chemokines that bind to CCR4 are able to chemotact Tregs, cells from dissociated normal and LLC-bearing lungs, along with normal splenocytes, were immunofluorescently stained for CD4, CD8, CD25, and CCR4 in an effort to profile T cell subpopulations in terms of their CCR4 expression. The mean fluorescent intensity (MFI) of CCR4 staining was significantly higher on Tregs compared with their CD8\(^+\) and CD4\(^+\)CD25\(^-\) counterparts in both normal and LLC-bearing lung tissue and in normal splenocytes (Fig. 2, A and B). Although this increased CCR4 expression on Tregs was not as pronounced in normal splenocytes as it was in normal and LLC-bearing lungs, splenic Treg CCR4 MFI was still between 7- and 8-fold higher than for CD8\(^+\) and CD4\(^+\)CD25\(^-\) cells. Regardless of CCR4 staining intensity, Tregs comprised the majority of CCR4\(^+\) T cells in both normal and LLC-bearing lungs and in normal splenocytes (Fig. 2C). Taken together, these data suggest that Tregs inherently express higher levels of CCR4 than do other T cells and that this CCR4 expression is unaffected by LLC tumor presence. However, the number of CCR4\(^+\) Tregs in LLC-bearing lungs (564 ± 146 Tregs/1.0 × 10\(^6\) lung cells) was greater than in normal lungs (109 ± 11 Tregs/1.0 × 10\(^6\) lung cells; Fig. 2D).

**LLC-bearing lung tissue secretes elevated levels of CCL22**

Having shown that Tregs express more CCR4 than other T cell populations, ELISA was used to measure the levels of the CCR4-associated chemokines CCL22 and CCL17 in medium conditioned by both LLC and normal mouse lung epithelial (MLE-12) cultures in addition to medium conditioned by intact normal and LLC-bearing lung tissue. No detectable levels of

![Figure 2](http://www.jimmunol.org/DownloadedFrom/May3,2017)
either CCL22 or CCL17 were secreted by MLE-12 or LLC cell cultures (Fig. 3). However, the conditioned medium taken off of normal lung tissue secreted both CCL17 (257 ± 17 pg/ml) and CCL22 (855 ± 29 pg/ml). LLC-bearing lung tissue secreted similar levels of CCL17 (292 ± 4 pg/ml) compared with normal tissue, but secreted significantly higher amounts of CCL22 (1313 ± 108 pg/ml; p = 0.016). Another chemokine, CXCL12, has been implemented in the chemoattraction of Tregs toward bone marrow (22). However, no CXCL12 was detected in any of the conditioned medium.

CCL22 secreted from LLC-bearing lung tissue selectively attracts more Tregs than normal lung tissue

After identifying CCL22 as a candidate factor for selective Treg recruitment, migration assays determined whether the elevated level of CCL22 secreted from LLC-bearing lungs selectively stimulates the migration of Tregs. Transwell migration assays were used with splenic T cells seeded in the upper chamber and medium conditioned by normal or LLC-bearing lungs placed in the lower. Following the overnight migration, flow cytometry was used to analyze the Treg content in the cell fractions that migrated into the lower chambers. The baseline Treg percentage of the normal CD4+ splenocytes used in the assay was 6.7 ± 0.2%. Therefore, migratory cell fractions whose Treg percentage of CD4+ cells was higher than this baseline would indicate selective Treg recruitment toward those conditioned media. The fraction of cells that migrated toward normal lung conditioned medium contained a 53.4 ± 8.1% increase in Treg content over the assay’s baseline (Fig. 4A). The fraction of cells that migrated toward LLC-bearing lung conditioned medium contained a significantly higher increase in Treg content over baseline at 92.1 ± 2.6% (p = 0.018). Addition of a neutralizing Ab against CCL22 into the conditioned medium reduced the selective Treg recruitment toward normal lung-conditioned medium to only 22.2 ± 6.4% (p = 0.019) and reduced the selective Treg recruitment toward LLC-bearing lung-conditioned medium to only 51.1 ± 8.2% (p = 0.018). The ratio of CD4+ cells to CD8+ cells remained unchanged in all fractions (data not shown). The nonmigratory cell fractions left in the top of the Transwell assay were also analyzed for Treg content. The change from baseline in Treg percentage of CD4+ cells in the nonmigratory fractions were all between −30 and −40%. In all cases, the

FIGURE 3. LLC-bearing lung tissue secretes higher levels of CCL22. Medium conditioned on normal mouse epithelial cell cultures (MLE-12), LLC cell cultures, normal lung tissue, or LLC-bearing lung tissue was tested for CCL17, CCL22, and CXCL12 using ELISA.

FIGURE 4. CCL22 mediates selective recruitment of Tregs toward conditioned medium. Whole T cells were isolated from normal splenocytes and used in Transwell migration assays to measure selective Treg recruitment toward normal (light bars) and LLC-bearing lung (dark bars)-conditioned medium with (striped bars) or without (solid bars) neutralizing Ab against CCL22. Following the assay, the Treg percentage of CD4+ cells was measured using flow cytometry in both the migratory fractions in the bottom chamber (A) and the nonmigratory fractions left in the top chambers (B). Inhibition of selective Treg recruitment using neutralizing Ab against CCL22 in a Transwell migration assay is dose dependent in both normal and LLC-bearing lung-conditioned medium and Ab specific (C).
Treg content in these fractions was less than the assay’s baseline level, suggesting that Tregs moved away from these fractions and toward the conditioned medium (Fig. 4B).

To ensure that the impact of CCL22 neutralization on selective Treg recruitment is anti-CCL22 dose dependent, increasing concentrations of neutralizing anti-CCL22 Ab and isotype control were added to both normal and LLC-bearing lung-conditioned medium and used in Transwell migration assays as described above (Fig. 4C). Increasing concentrations of anti-CCL22 reduced selective Treg recruitment toward both normal and LLC-bearing lung-conditioned medium. Increasing concentrations of isotype control had no effect.

CD11b⁺ cells isolated from LLC-bearing lung tissue secrete increased levels of CCL22. A. Following immunomagnetic isolation, CD11b⁺, CD11c⁺, CD31⁺, and CD3⁺ fractions from both normal and LLC-bearing tissue each contained a population enriched for each cell type as determined by immunofluorescent staining. B. These populations were then incubated overnight at 1.0 × 10⁶ cells/ml to create conditioned medium. ELISA was then used on the various conditioned media to measure the levels of secreted CCL22. C. Immunofluorescent staining and flow cytometric analysis was used to measure the endogenous levels of each cell type found in the lung homogenates before the isolations.

CD11b⁺ cells isolated from LLC-bearing lungs secrete more CCL22 than those isolated from normal lungs

CCL22 is secreted from normal and, to a greater extent, from LLC-bearing lung tissue. However, it is not secreted by normal epithelial cells or by the primary LLC cells in culture, suggesting that some other component of the microenvironment is responsible for the secretion seen in the solid lung tissues. In an effort to identify the CCL22-secreting components of the microenvironment, both normal and LLC-bearing lungs underwent immunomagnetic fractionation based on several broad-spectrum cellular markers: CD11b⁺, CD11c⁺, CD31⁺, and CD3⁺. The resulting cellular fractions were enriched for each cellular marker (Fig. 5A) and were plated overnight. ELISA was then used to measure CCL22 secretion into the medium. The CD31⁺ and CD3⁺ fractions isolated from both normal and LLC-bearing lungs secreted low levels of CCL22 (Fig. 5B). Interestingly, the CD11c⁺ cells isolated from normal lungs secreted moderate amounts of CCL22 (98.31 ± 14.75 pg/ml), but this secretion was significantly reduced when compared with CD11c⁺ cells isolated from LLC-bearing lungs (19.42 ± 11.15 pg/ml; p = 0.026). In contrast, the CD11b⁺ cells isolated from normal lungs secreted low levels of CCL22 (38.43 ± 28.74 pg/ml), while CD11b⁺ cells isolated from LLC-bearing lungs secreted elevated levels of CCL22 (271.94 ± 5.25 pg/ml; p = 0.003).

All of the fractionated cells were plated equally at 1.0 × 10⁶ cells/ml, allowing for the direct comparison of CCL22 secretion between the cell types in this assay. However, CCL22 secretion in vivo may also be impacted by the relative numbers of each cell type found in both normal and LLC-bearing lungs. To address this, flow cytometry was used to analyze the relative frequency of each cellular fraction in both normal and LLC-bearing lungs (Fig. 5C). Although moderate amounts of CD31⁺, CD4⁺, and CD8⁺ cells were present in both tissue types, the most abundant cell type was CD11b⁺ cells. This fraction comprised 10.4 ± 0.8% of cells from

FIGURE 5. CD11b⁺ cells isolated from LLC-bearing lung tissue secrete increased levels of CCL22. A, Following immunomagnetic isolation, CD11b⁺, CD11c⁺, CD31⁺, and CD3⁺ fractions from both normal and LLC-bearing tissue each contained a population enriched for each cell type as determined by immunofluorescent staining. B, These populations were then incubated overnight at 1.0 × 10⁶ cells/ml to create conditioned medium. ELISA was then used on the various conditioned media to measure the levels of secreted CCL22. C, Immunofluorescent staining and flow cytometric analysis was used to measure the endogenous levels of each cell type found in the lung homogenates before the isolations.
dissociated normal lungs and 27.9 ± 3.2% of cells from dissociated LLC-bearing lungs. Taken together, not only are CD11b⁺ cells the most abundant cellular fraction, but they display CCL22 secretion abilities consistent with the observed increases in total CCL22 levels seen in LLC-bearing lungs. Hence, any increase in CCL22 secretion seen from LLC-associated CD11b⁺ cells would be magnified by their increase in cell number inside LLC-bearing lung tissue.

**FIGURE 6.** Endogenous CD11b⁺ cells are comprised of multiple populations in normal and LLC-bearing tissue. A. Normal and LLC-bearing tissue homogenates were immunofluorescently stained with CD11b and analyzed with flow cytometry. CD11b⁺, CD11b⁺⁺, and CD11b⁺⁺⁺ populations were gated as depicted in B. C. Representative histograms of NK1.1, CD49a, Gr-1, and F4/80 expression across the gated CD11b populations.
CD11b+ cell subpopulations in normal and LLC-bearing lung tissue

CD11b is expressed on many different cell types. After identifying CD11b+ cells as the primary cellular components responsible for the observed increase in CCL22 secretion from LLC-bearing lungs, studies were conducted to characterize the CD11b+ populations found in cell dissociates from both normal and LLC-bearing lungs. Immunofluorescent staining of CD11b alone shows two distinct populations of CD11b+ cells in normal lung tissue (CD11bdim, and CD11bbright). The number of CD11bbright cells was increased in LLC-bearing tissue, along with the appearance of another CD11b+ population (CD11bvery bright, Fig. 6A). Notably, this CD11bvery bright population somewhat overlaps the CD11bbright population in terms of CD11b staining intensity. However, the CD11bbright and CD11bvery bright populations are distinctly different from each other in terms of side scatter (Fig. 6B).

All of the CD11bbright cells in normal and LLC-bearing lung tissue coexpress the myeloid/granulocyte marker Gr-1 (Fig. 6C). Although normal granulocytes may express both CD11b (23) and

**FIGURE 7.** Normal and LLC-bearing lung dissociates were immunofluorescently stained with CD11b, NK1.1, Gr-1, and F4/80 and the frequency of each population was determined using flow cytometry.

**FIGURE 8.** Immunofluorescently stained normal (A) and LLC-bearing (B) dissociates were first gated on CD11b+ cells, and FACS was performed to isolate the CD11bdimNK1.1+, CD11bbGr-1+, and CD11bvery brightF4/80 populations. The isolated fractions were then plated overnight at 5.0 x 10^5 cells/ml and ELISA was used to measure secreted CCL22 in the medium.

**FIGURE 9.** Analysis of the NK compartments in normal and LLC-bearing lung tissue. Dissociates from both normal and LLC-bearing lung tissue were immunofluorescently stained for NK1.1 along with CD49b (DX5), CD11b, CD122, CD19, CD27, and CD3 and analyzed using flow cytometry.
Gr-1 (24), these markers are also suggestive of myeloid-derived suppressor cells (25). Additionally, previous reports of increased myeloid-derived suppressor cells in the tumor microenvironment (26–31) are consistent with our observed increases in this population. The CD11b\textsuperscript{very bright} cells, found only in LLC-bearing lungs, coexpress the macrophage marker F4/80, suggesting that these cells are tumor-associated macrophages. Finally, the CD11b\textsuperscript{dim} cells express both CD49b and NK1.1, suggesting that they are NK cells. None of the CD11b\textsuperscript{+} cells stained positive for CD31\textsuperscript{+} (data not shown).

Finally, each CD11b subpopulation was quantified in both normal and LLC-bearing lung dissociates using flow cytometry (Fig. 7). There is a significant increase in the frequency of CD11b\textsuperscript{bright}Gr-1\textsuperscript{+} cells in LLC-bearing lung dissociates as compared with normal lung dissociates ($p = 0.0015$). A large influx of CD11b\textsuperscript{very bright}F4/80\textsuperscript{−} cells is seen in LLC-bearing lung dissociates. No detectable levels of CD11b\textsuperscript{very bright}F4/80\textsuperscript{+} cells were found in normal lung dissociates. Although the average frequency of CD11b\textsuperscript{dim}NK1.1\textsuperscript{+} cells was higher in LLC-bearing lung dissociates ($58,272$ cells/1.0 $\times 10^6$ lung cells) as compared with normal lung dissociates ($44,518$ cells/1.0 $\times 10^6$ lung cells), this difference was not statistically significant.

**Isolation of CD11b\textsuperscript{+} populations using FACS**

To identify which of the CD11b\textsuperscript{+} populations are able to secrete CCL22, FACS was used to isolate CD11b\textsuperscript{dim}NK1.1\textsuperscript{+}, CD11b\textsuperscript{bright}Gr-1\textsuperscript{+}, and CD11b\textsuperscript{very bright}F4/80\textsuperscript{−} populations from dissociated normal and LLC-bearing lungs. Following FACS isolation, each fraction was plated overnight and ELISA was used to measure secreted CCL22 levels in the medium. No CCL22 secretion was detected from the CD11b\textsuperscript{bright}Gr-1\textsuperscript{+} cells isolated from either normal or LLC-bearing lungs. Likewise, no CCL22 was secreted from the isolated CD11b\textsuperscript{very bright}F4/80\textsuperscript{−} cells that appear in the LLC-bearing lung tissue (Fig. 8). Of all the CD11b\textsuperscript{+} cells found in both normal and LLC-bearing lungs, the only detectable amounts of CCL22 secretion came from the CD11b\textsuperscript{dim}NK1.1\textsuperscript{+} populations. Furthermore, the CD11b\textsuperscript{dim}NK1.1\textsuperscript{+} cells isolated from dissociated LLC-bearing lungs secreted significantly higher levels of CCL22 than did the CD11b\textsuperscript{dim}NK1.1\textsuperscript{+} cells isolated from dissociated normal lungs. These data indicate that these CD11b\textsuperscript{dim}NK1.1\textsuperscript{+} populations are capable of CCL22 secretion and may significantly contribute to the increased CCL22 secretion seen in LLC-bearing lungs.

**Analysis of NK1.1\textsuperscript{+} cells in normal and LLC-bearing lung tissue**

Although NK1.1 is one of the most specific NK markers available for murine NK cells, it can also be expressed on NKT cells, which themselves have reported immune-suppressive capabilities. Therefore, to confirm that the NK1.1\textsuperscript{+} cells isolated in these studies are true NK cells, we conducted a thorough analysis of the NK compartment in the lungs of both normal and LLC-bearing mice (Fig. 9). In both normal and LLC-bearing lung dissociates, NK1.1\textsuperscript{+} cells were also CD11b\textsuperscript{dim}, CD49b\textsuperscript{−}, CD122\textsuperscript{−}, CD27\textsuperscript{−}, CD19\textsuperscript{−}, and CD3\textsuperscript{−}, indicating that they are true NK cells. Additionally, this analysis demonstrates that no detectable levels of NKT cells reside in either normal or LLC-bearing lung tissue.

**Depletion of NK1.1\textsuperscript{+} cells results in decreased CCL22 secretion from both normal and LLC-bearing lung dissociates**

The fact that the isolated NK1.1\textsuperscript{+} cells from both normal and LLC-bearing lung dissociates were the only CD11b\textsuperscript{+} subpopulations to secrete CCL22 suggests that NK cells may be the major CCL22-secreting components of both normal and LLC-bearing lung tissues. To test this hypothesis, we immunomagnetically depleted NK1.1\textsuperscript{+} cells from both normal and LLC-bearing lung dissociates and incubated the resulting fractions alongside un fractionated dissociates overnight before analyzing the resulting medium for CCL22 secretion using ELISA (Fig. 10). Immunomagnetic depletion of NK1.1\textsuperscript{+} cells resulted in reduced numbers of NK cells in both normal and LLC-bearing lung dissociates (Fig. 10A). Unfractionated LLC-bearing lung dissociates secreted more CCL22 than did normal lung dissociates ($902 \pm 34$ pg/ml vs $595 \pm 22$ pg/ml, respectively; Fig. 10B). Depletion of NK cells resulted in a significant reduction of CCL22 secretion for both normal and LLC-bearing lung dissociates (325 $\pm$ 14 pg/ml and 457 $\pm$ 12 pg/ml, respectively), indicating that a large portion of secreted CCL22 from both normal and LLC-bearing lungs is dependent on the presence of NK cells.

**FoxP3\textsuperscript{+} cells colocalize with large numbers of NK cells at the tumor periphery**

If NK cells play an active role in the selective recruitment of Tregs toward the tumor microenvironment, then it is reasonable to predict that NK cells and Tregs colocalize in the tumor microenvironment. Excised normal and LLC-bearing lungs were cryosectioned and stained with Hoechst dye (blue), anti-NK1.1 (PE, red), and anti-FoxP3 (FITC, green) (Fig. 11). Although both NK1.1\textsuperscript{+} cells coexpress the macrophage marker F4/80, suggesting that these cells are tumor-associated macrophages. Finally, the CD11b\textsuperscript{dim} cells express both CD49b and NK1.1, suggesting that they are NK cells. None of the CD11b\textsuperscript{+} cells stained positive for CD31\textsuperscript{+} (data not shown).
and FoxP3$^+$ cells can be found associated with each other in normal lung tissue (Fig. 11A), each cell type is distributed homogenously across the tissue section with modest frequency. In contrast, increased numbers of both FoxP3$^+$ cells and NK1.1$^+$ cells appear just inside the tumor periphery (white line, Fig. 11B). Furthermore, nearly all visible FoxP3$^+$ cells were located adjacent to NK cells (merge). Tumor periphery is marked with a dashed white line. Insets are of a higher magnification of the indicated field.

**FIGURE 11.** Increased numbers of FoxP3$^+$ cells associate with increased NK cell numbers at the tumor periphery. Immunofluorescent staining was performed on frozen sections from normal lung tissue (A) and LLC-bearing lung tissue (B) for nuclei (Hoechst Blue), NK1.1 (Texas Red, red), and FoxP3 (FITC, green). Most FoxP3$^+$ cells are located adjacent to NK cells (merge). Tumor periphery is marked with a dashed white line. Insets are of a higher magnification of the indicated field.

IL-2 induces CCL22 secretion from normal NK cells

Despite the apparent increase in NK cell numbers at the tumor periphery (Fig. 11B), large increases in NK cell numbers in LLC-bearing lung dissociates as a whole were not seen (Fig. 7). This fact suggests that an influx of NK cell numbers alone may not be responsible for increased NK-derived CCL22. Another explanation could be an increase in NK cell activation. As such, we treated normal splenic NK cells with increasing doses of IL-2 and measured CCL22 secretion responses to ascertain the effect of NK activation on CCL22 secretion. Without IL-2 treatment, NK cells did not secrete detectable levels of CCL22. Low-dose IL-2 induced appreciable amounts of CCL22 secretion, as did higher doses (Fig. 12). Interestingly, CCL22 secretion was the same in response to both low and high doses. This demonstrates that although CCL22 secretion is
CCL2 secretion is induced by IL-2. Splenic NK cells were treated with increasing doses of IL-2. After 48 h, CCL2 and IFN-γ levels were measured using ELISA.

**Discussion**

Tumor-induced immune suppression continues to impede the effectiveness of immune-based therapies against cancer. Solid tumors utilize numerous mechanisms designed to evade host antitumor immune responses. Many of these mechanisms involve the accumulation of immune-suppressive infiltrates. Elucidation of the mechanisms by which these immune-suppressive phenotypes accumulate in the tumor microenvironment could produce attractive therapeutic targets with which to combat tumor-induced immune suppression. One of the most potent and well-studied immune-suppressive cell types is the Treg. The studies presented here demonstrate that elevated secretion of the chemokine CCL2 can elicit the selective attraction of Tregs toward LLC-bearing lung tissue. However, these studies also indicate that CCL2 is not secreted by either the LLC cells themselves or normal mouse lung epithelial controls. Instead, these studies show that NK cells not only have the ability to secrete CCL2, but display secretion patterns consistent with the overall increases in CCL2 levels seen in tumor-bearing lungs. Together with the fact that a large portion of the total CCL2 secretion from both normal and tumor-bearing lungs is NK dependent, these studies collectively suggest that NK cells may represent a major contributing source toward basal CCL2 levels in normal lung tissue and toward the increased secretion levels of CCL2 seen in tumor-bearing lungs. This demonstration is a novel link between Treg recruitment and infiltrating NK cells.

The migration studies presented here demonstrate that selective Treg recruitment is higher toward medium conditioned by LLC-bearing lungs and that CCL22 contributes significantly to this recruitment. These studies were performed using unfractionated T cells isolated from normal splenocytes. It is possible that Tregs located in lung tissue may represent a slightly different Treg population, although both splenic and lung-associated Tregs have elevated levels of CCR4 expression. Although this elevated expression level is not as high as that of lung-associated Tregs, the fact that their CCR4 expression is significantly higher compared with other types of splenic T cells suggests that splenic Tregs are a conservative model for selective migration studies along the CCL22/CCR4 axis. It is noteworthy that even in excess concentrations of neutralizing anti-CCL22 Ab, selective Treg recruitment toward the conditioned medium was not entirely reduced to baseline levels. This suggests that other factors in the conditioned medium may also contribute to Treg recruitment. Recently, the chemokine MCP-1(CCL2) has been shown to have biologically significant signaling activity through CCR4 (19) and that this activity can attract Tregs in vitro (32). CCL2 has traditionally been associated with CCR2 and CCR5 due to its high affinity for these receptors. However, chemokines have long been described as "promiscuous" due to their tendency to have basal affinity with a wide range of receptors. Although these studies focused on the traditional CCR4 chemokines, it is possible that the selective recruitment of Tregs unaccounted by CCL22 is due to alternative CCR4 ligands such as CCL2.

CCL2 has previously been shown to induce the chemotaxis of Tregs in vitro (15), as well as the selective recruitment of Tregs toward ovarian carcinoma (17). However, unlike ovarian carcinoma, the data presented here show that neither LLC cells nor normal epithelial lung cells secrete CCL2. CCL2 secretion from a solid tumor in which the tumor cells do not secrete CCL2 has not been described, raising the possibility that this scenario may be somewhat unique to LLC. However, NK infiltration is known to be common in most solid cancers (for review, see Ref. 33), meaning that NK cells may contribute toward CCL2 secretion in many cancer types. Although these studies suggest that a significant amount of CCL2 secretion is NK dependent in LLC, it is not known whether NK cells represent a significant source of CCL2 in cancers that contain CCL2-producing tumor cells.

The initial observation that LLC-bearing lungs secreted elevated levels of CCL2 was made using ELISA analysis of conditioned medium. Therefore, the focus of these studies was on CCL2 secretion. An alternative approach to measure the contribution of NK cells toward CCL2 levels would be to immunofluorescently stain for intracellular CCL2. However, it is important to distinguish between CCL2 production and CCL2 secretion. It has been previously observed that cell lines that stain positively for intracellular CCL2 did not secrete CCL2 (19). Hence, although immunofluorescent detection of CCL2 would provide information about CCL2 production, it may be misleading in terms of CCL2 secretion.

The aim of the present studies was to introduce NK cells as a significant source of elevated CCL2 levels in the lungs of LLC-bearing mice and to demonstrate that increased CCL2 secretion leads to selective Treg recruitment. Future studies will focus on the direct impact that NK cells have on CCL2-mediated Treg recruitment in vivo through the use of depleting Abs against NK cells. Such studies will confirm the novel link between NK-secreted chemokines and Treg recruitment toward LLC introduced here. However, in vivo NK depletion has been shown to adversely affect other immune cell populations aside from NK cells, including T cells (34) and dendritic cells (35). This is likely due to the central role that NK cells have in establishing baseline levels and phenotypes of other immune cell populations (36). Similar disparities in infiltrate populations are seen in NK knockout mice (37–39). Therefore, extensive studies analyzing the impact of NK depletion on major groups of immune infiltrate in the lungs of both normal and LLC-bearing mice are also necessary to draw any conclusions gained from NK-depletion studies.

The idea that NK cells may play a role in the recruitment of Tregs to the tumor microenvironment is a novel yet troubling concept. NK cells are able to elicit rapid cytotoxic activity against certain tumors without prior exposure to any specific stimulus (40), and their activation is elicited by the lack of MHC class I on target cells (41). Along with the fact that the hallmark of many cancer types is the lack of MHC expression (42–48), NK cells have great potential for use in immune-based therapies against cancer and indeed have well-documented antitumor activity (49, 50). However, NK cells are vulnerable to tumor-induced immune suppression, particularly from Tregs (51–54). The relationship between NK cells and Tregs is complex, and further studies are needed to fully understand the role of NK cells in Treg recruitment.
NK cells and Tregs is further complicated by the recent observation that NK cells are able to kill Tregs (55). The ultimate outcome of NK cell and Treg interaction in the tumor microenvironment is assistance with cell sorting. We are also grateful to Jennifer Konopa-Muligan and Jarrett Walsh for support and assistance.

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References


