Splenectomy inhibits non-small cell lung cancer growth by modulating anti-tumor adaptive and innate immune response

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Keywords: lung cancer; splenectomy; tumor immunology; MDSC; metastases

It has been shown that inhibitors of the immune system reside in the spleen and inhibit the endogenous antitumor effects of the immune system. We hypothesized that splenectomy would inhibit the growth of relatively large non-small lung cancer (NSCLC) tumors by modulating the systemic inhibition of the immune system, and in particular Myeloid Derived Suppressor Cells (MDSC). The effect of splenectomy was evaluated in several murine lung cancer models. We found that splenectomy reduces tumor growth and the development of lung metastases, but only in advanced tumors. In immune-deficient NOD-SCID mice the effect of splenectomy on tumor growth and metastatic spread disappeared. Splenectomy significantly reduced the presence of MDSC, and especially monocytic-MDSC in the circulation and inside the tumor. Specific reduction of the CCR2+ subset of monocytic MDSC was demonstrated, and the importance of the CCL2-CCR2 axis was further shown by a marked reduction in CCL2 following splenectomy. These changes were followed by changes in the macrophages contents of the tumors to become more antitumorigenic, and by increased activation of CD8+ Cytotoxic T-cells (CTL). By MDSC depletion, and adoptive transfer of MDSCs, we demonstrated that the effect of splenectomy on tumor growth was substantially mediated by MDSC cells. We conclude that the spleen is an important contributor to tumor growth and metastases, and that splenectomy can blunt this effect by depletion of MDSC, changing the amount and characteristics of myeloid cells and enhancing activation of CTL.

Introduction

The field of cancer immunology is based on the understanding that, despite the presence of nonself-antigens, tumors evade immune surveillance by developing a complex immunosuppressive network that paralyzes the effector arm of the immune system and induces immune tolerance.¹ Furthermore, this tolerance is believed to be a major factor in the limited success of cancer immunotherapy in general, and specifically in thoracic malignancies.²,³ Various cell types have been described as contributing to this immune tolerance by direct immune suppression as well as production of angiogenic factors, matrix-degrading enzymes, and/or growth factors.⁴ Among these are MDSC,⁵ T regulatory cells (T-regs), tumor-associated macrophages (TAM), tumor-associated neutrophils (TAN), natural-killer T cells (NKT cells), and dendritic cell (DC) subtypes.⁶ Some of these cell subtypes (e.g. MDSC) inhibit the endogenous antitumor effects of the immune system ⁷ and reside in the spleen.

The spleen is the largest lymphoid organ in the body and is known to have a complex role in immune surveillance and immune defense against infections.⁹ The role of the spleen in tumororogenesis and tumor growth is less well understood, although it has been studied throughout the years. Effects of splenectomy on tumor growth and spread of metastases were studied using excision of the spleen (splenectomy) performed at different time points,⁸ and using different cancer models,⁹-¹² with conflicting results. While in some cases tumor growth was delayed following splenectomy,⁸,¹² in other cases there was no effect on tumor growth,⁹,¹³ or even enhanced tumor growth ⁸,¹⁴-¹⁶. This diversity in results was probably due to lack of uniformity in the timing of splenectomy, the cancer model evaluated, and other differences in methodologies. Interestingly, no previous work has clearly elucidated the mechanism by which splenectomy is capable of inhibiting tumor growth. Based on recent advances in understanding tumor immunology and the role of the spleen in tumor development, there is now growing interest in this field.¹⁷,¹⁸ We aimed to examine the effects of splenectomy on growth of non-small cell lung cancer (NSCLC) tumors in mice at different time points and different tumor sizes and elucidate the mechanisms by which the tumor induces systemic immune suppression through modulation of splenic activity. We compared the effects of splenectomy and sham surgery in immune-
competent and immune-deficient mice with implanted lung tumors, hypothesizing that splenectomy can affect the immune system in a way that inhibits the growth of large tumors and the spread of metastases. We then assessed immunologic changes induced in the tumor following splenectomy, and by flow analysis, depletion, and adoptive transfer studies investigated the mechanism by which splenectomy is capable of inhibiting tumor growth.

Results

Splenectomy inhibits tumor growth in murine lung cancer models

We evaluated the effect of splenectomy on tumor growth using three models of flank murine NSCLC. Splenectomy performed at advanced stages of tumor growth (mean tumor volume of 500 mm³, approximately 21 d after tumor injection) significantly inhibited tumor growth in mice injected with the Lewis lung carcinoma (LLC) and TC1 cell lines (Fig. 1A and B), and even caused temporary tumor regression in the LKR-M cell line (Fig. 1C). Representative tumors from mice undergoing splenectomy or sham surgery are shown in Fig. 1D.

In order to examine whether this inhibitory effect is dependent on tumor volume at time of splenectomy, we used the LKR-M cell line and performed splenectomy at different time points. Splenectomy was performed prior to tumor injection, at an early stage of tumor growth (mean tumor volume of 200 mm³, approximately 10 d after tumor injection), at an advanced stage of tumor growth (mean tumor volume of 500 mm³, approximately 20 d after tumor injection), and at very advanced tumor growth (mean tumor volume of 1000 mm³, approximately 30 d after tumor injection). As shown in Fig. 2, the inhibitory effect of splenectomy was noted in established tumors only. Splenectomy performed prior to tumor injection or at an early stage of tumor development had no effect on growth (Fig. 2A–B). Splenectomy at more advanced stages of tumor growth was clearly associated with regression in tumor growth (Fig. 2C–D).

Splenectomy reduces the number of lung metastases without affecting the mean size of metastases

We next investigated whether splenectomy would have similar inhibitory effect on the spread of metastases and their development. We used the LKR-M model, a variant of the LKR cell line, which we have previously modified to have reproducible spontaneous lung metastases. Splenectomy or sham surgery was performed with mean tumor volume of 500 mm³. At 10–14 d following splenectomy, a sufficient time interval for metastases to develop, we observed a significant reduction in the number of metastases in the lungs of mice that had splenectomy compared with sham-operated mice (Fig. 3A). There was no difference in the size of individual metastases between the two groups (Fig. 3B). The total area in a representative histologic section of lung metastases as a percentage of lung area in the section had a tendency to be reduced following splenectomy, but this trend did not reach significance (Fig. 3C). Taken together, the reduced number of metastases following splenectomy but the lack of
difference in their mean size may indicate that the main effect of splenectomy was reduction in the seeding of metastases but not in their growth. Photomicrographs and hematoxylin and eosine (H&E)-stained sections from representative lungs of splenectomized and sham operated immune competent mice are shown in Fig. 3D and E, respectively.

The effect of splenectomy on tumor growth is blunted in SCID mice and disappears in NOD-SCID mice.

In order to elucidate the immunologic mechanism affecting tumor growth following splenectomy, we performed splenectomy on SCID mice (defective adaptive immunity20) and on NOD-SCID mice (defective adaptive and innate immunity21) at advanced stages of tumor growth (i.e. tumor volume of approximately 500 mm³). As described above, splenectomy performed at this stage in immune-competent mice had an inhibitory effect on tumor growth. Splenectomy in SCID mice showed an inhibitory effect of tumor growth as well (Fig. 4A), but this effect existed for a shorter time period in comparison with immune-competent mice (Fig. 1A–C). This observation suggests that tumor inhibition following splenectomy is at least partially dependent on components of the adaptive immune system. Splenectomy in NOD-SCID mice had no inhibitory effect on tumor growth compared to sham surgery (Fig. 4B). This observation further supports the involvement of components of adaptive immunity in the process, but suggests that components of innate immunity are also involved in the mechanism of tumor inhibition following splenectomy.

In order to examine whether the anti-metastatic activity presented in Fig. 3 requires an intact adaptive immunity, we repeated the studies on the effect of splenectomy on lung metastases in immune-compromised NOD-SCID mice. In these mice, no difference was observed in the number of metastases (Fig. 4C), mean metastases size (Fig. 4D) or in the total area of metastases as a percentage of lung area following splenectomy (Fig. 4E) compared to sham operation, suggesting that the effect on metastatic spread is indeed immune-mediated.

Since tumor necrosis factor α (TNFα) is an important component in the activation of CD8⁺ cells and in the light of our observation shown below that TNFα mRNA levels increase following splenectomy, we examined whether the effect of splenectomy is blunted when using TNFα-knockout (TNF-KO) mice. We injected tumor cells from the LLC cell line to TNF-KO mice and compared their growth following splenectomy or sham operation. We found that the effect of splenectomy on tumor growth was similar in TNF-KO mice, as seen in wild-type mice (data not shown).

Splenectomy reduced circulating and infiltrating monocytic-MDSC, and increased intratumoral macrophages, and activated CD8⁺ T cells, involving the CCR2-CCL2 axis

To further characterize the involvement of innate and adaptive immunity, we analyzed the entire tumors using flow cytometry. We found that splenectomy significantly reduced circulating and tumor infiltrating monocytic (M)-MDSC (i.e., CD11b⁺/Ly6C⁺) (Fig. 5A, and representing flow traces in Fig. 5B). Following the recent manuscript by Ugel et al. suggesting that the main type of M-MDSC reduced is CCR2⁺18, we examined the
found a significantly higher percentage of M0 out of the total number of tumor cells in splenectomized mice (12.8% vs. 4.1%, \( p = 0.004 \)), comparable percentages of M1 (3.4% vs. 4.4%, \( p = 0.12 \), and a significantly lower percentage of M2 out of all tumor cells (1.7% vs. 2.7%, \( p = 0.03 \), Fig. 6A), suggesting that there are differences in the type of macrophages recruited to the tumor and/or in the tumor macrophage maturation process following splenectomy. Tumor macrophages tended to be more antitumor, with a decrease in the ratio of M2 to non-M2 macrophages from 0.3 to 0.1 (Fig. 6A). In a complete blood count and differential analysis, splenectomy had no effect on hemoglobin and platelets level, neither on the percentage of circulating neutrophils or macrophages.

The effect of splenectomy on CD8\(^+\) T cell activation was measured using expression of T-cell surface activation markers, CD25 or CD69.\(^{22,23}\) We found no difference in the percentage of lymphocytes in general, or CD8\(^+\) cells specifically in the blood following splenectomy (data not shown). However, the percentage of activated CD8\(^+\) T cells in the blood increased from 5.8% in the sham-operated mice to 17.9% in splenectomized mice. Furthermore, the level of activated CD8\(^+\) T-cells inside the tumor was \( \sim 2 \)-fold higher following splenectomy compared with sham surgery (14% vs. 6%, \( p = 0.04 \), Fig. 6B). A similar trend was obtained using the surface activation marker 4-1BB (CD137),\(^{24}\) but the difference did not reach statistical significance (data not shown). No change was observed in the number of T-helper cells (CD4\(^+\)CD25\(^-\)) or T-regulatory cells (CD4\(^+\)CD25\(^+\)) out of total tumor.

Splenectomy alters the tumor microenvironment to be more pro-inflammatory

We evaluated changes in the tumor microenvironment induced by splenectomy that could explain or reflect the increased numbers of neutrophils and M0 macrophages, and the activation of CD8\(^+\) T-cells. We used real-time RT-PCR of whole tumor explants to profile a set of relevant cytokines, chemokines, and cell adhesion molecules. Changes following splenectomy were unremarkable except for a 1.5, 2, and 3 fold increase in
TNFα, IFNγ, and MIG, respectively, all products of the activation of CD8 T-cells (data not shown).

The effect of splenectomy on tumor growth is mediated by MDSC

In order to evaluate the relationship between MDSC depletion and the changes induced following splenectomy, we used anti-GR1 monoclonal antibodies (mAb) to deplete MDSC in mice injected with the LKR cell line. Proper depletion was confirmed by measuring the amount of MDSC in the blood and tumor. The percentage of M-MDSC (CD11b+/Ly6C+) in the blood was reduced from 19.3% in the control group to 2.3% in the GR1-depleted mice, and the percentage of G-MDSC (CD11b+/Ly6G+) was reduced from 13.4% to 0. In the tumor M-MDSC went down from 25.7% of the leukocytes (CD45+) to 12.1%, and G-MDSC from 6.4% to 0.3%. Fig. 7A shows the effect of splenectomy vs. anti-GR1+ mAb or their combination on tumor growth. All three treatment arms significantly slowed tumor progression compared with sham in a similar manner (p < 0.05 vs. sham for each). There were no significant differences in tumor growth between the three arms of treatment, and the combination of splenectomy with anti-GR1+ mAb did not provide added value compared to either treatment alone. However, once anti-GR1+ mAb injections were halted, this group was the first to escape the effect of tumor inhibition.

We next examined whether adoptive transfer of MDSC can abrogate the effect of splenectomy on tumor growth. GR1+ cells, isolated from spleens of tumor-bearing mice, were injected into the tail vein of other tumor-bearing mice, in parallel to splenectomy or sham operation (Fig. 7B). Splenectomy had the regular effect, slowing the growth of flank tumors. However, two injections of MDSC cells, prevented this effect, comparing the growth of the tumors to sham-operated mice. Adoptive transfer of MDSC in sham-operated mice had no significant effect on tumor growth (not shown). These combination of depletion and adoptive transfer studies, suggest that the mechanism by which splenectomy can inhibit tumor growth is mediated at least partially by MDSC.

Discussion

The role of the immune system in tumor development has been the focus of a vast amount of research in the past decade. It is increasingly well recognized that tumors develop a complex immunosuppressive network that can paralyze the effector arm of the immune system, enabling their escape from immune surveillance despite the presence of nonself antigens. This complex immunosuppressive network is directed by inhibitory cytokines and a variety of different cell populations that include “redirected” myeloid cells functioning at the systemic level, e.g., MDSC, as well as inside the tumor, e.g., TAM or TAN. In healthy individuals, MDSC reside primarily in the bone marrow; however, in pathological conditions such as cancer or inflammation, their main reservoir is in the lymphatic organs, notably the spleen. We hypothesized that splenectomy could enhance the immune response by reducing the population of MDSC, which have an inhibitory effect on the immune system.

Splenectomy and the role of the spleen in immune regulation and oncogenesis has been the subject of research for several decades. Studies that examined the effect of splenectomy on tumor behavior used different hosts and different tumor models and showed conflicting results. A few studies actually found that splenectomy could enhance tumor growth and spread of metastases, some indicated no effect on tumor growth, and...
other studies showed inhibition of tumor growth and the spread of metastases following splenectomy.\textsuperscript{8,12} It should be emphasized that most studies assessed the effects of splenectomy performed prior to tumor inoculation or a short time after tumor injection.\textsuperscript{9,12-14,16,37-40} The effect of splenectomy on established tumors did not gain much attention.

Prehn et al.\textsuperscript{8} suggested that the ratio of spleen to tumor mass at time of splenectomy explains this controversy. According to this theory, small proportions of spleen cells can stimulate tumor growth, in which case splenectomy is inhibitory. Larger proportions of the same cells will usually inhibit tumor growth, in which case splenectomy results in tumor stimulation; thus, the effect of splenectomy on tumor growth and metastases spread is dependent upon tumor mass at the time of splenectomy.\textsuperscript{8,37,38} This theory did not gain much support and was not further investigated. Several other explanations were suggested in order to settle these apparent controversies. Ge et al.\textsuperscript{41} suggested that the involvement of different cell populations residing in the spleen at different stages of tumor development can be an explanation. A third explanation was that the response to splenectomy is dependent on tumor type.\textsuperscript{40} Interestingly, no previous work has clearly elucidated the exact mechanism by which splenectomy is capable of inhibiting tumor growth.

In our current work, we demonstrated in several models of NSCLC, that splenectomy performed at advanced stages of tumor growth has a significant inhibitory effect on tumor growth, and may even cause tumor regression (Fig. 1). This inhibitory effect is significant but temporary. If splenectomy is performed prior to tumor inoculation or at early stage of tumor growth, this inhibitory effect is absent (Fig. 2). We show therefore that the timing of splenectomy is a crucial component in determining the effect of splenectomy on tumor behavior, which may explain some of the contradictions in previous studies.\textsuperscript{8,37,38} Interestingly, we found that not only can splenectomy inhibit tumor growth at late stages, it can even reduce and delay the seeding of distant metastases, further demonstrating the clinical potential applicability of such an approach (Fig. 3).
Our mechanistic studies showed that splenectomy can inhibit tumor growth in SCID mice, but this effect is transient and the lack of adaptive immunity (mainly B and T lymphocytes), prevents it from continuing, suggesting a partial involvement of these cells in the process of growth inhibition. In NOD-SCID mice, no inhibition of tumor growth and no effect on metastases were observed, suggesting that the inhibitory effect of splenectomy in immune-competent mice has a combined mechanism of inhibition involving lymphocytes as well as cells of the myeloid lineage and the innate immune system (Fig. 4). As a support to these mechanistic findings, we found that the level of MDSC, and mainly M-NDSC, both in the circulation and in the tumors, is significantly reduced following splenectomy. Furthermore, as previously suggested by Ugel et al., we found an involvement in the process of the CCR2-CCL2 axis. Splenectomy reduced both the percentage of CCR2+ M-MDSC, and the level of CCL2. It is well established that the different subsets of MDSC have differential suppressive effect on T-lymphocytes, and it is generally accepted that M-MDSC are more suppressive than G-MDSC. This was further expanded by Ugel et al., showing the even higher suppressive effects of CCR2+ M-MDSC. These observations support the notion in our work, that the cells most affected from splenectomy were indeed the M-MDSC, and especially the CCR2+ M-MDSC, suggesting that these are the main mediators of the effect of splenectomy (Fig. 5).

When the tumor itself was evaluated for infiltration of other leukocytes (Fig. 6), we found that the total percentage of intratumoral neutrophils and macrophages increased following splenectomy. These macrophages tended to have stronger antitumor effects, as seen by the increase in the ratio of non-M2 to M2 macrophages. An increase in the activation of intratumoral CD8+ CTL was also noted, but no change was observed in either the absolute number of CD8+ CTL, or in the number of T helper (CD4+CD25+), T-reg (CD4+CD25−) or dendritic cells. These changes reflect the transition from a protumor to an antitumor microenvironment following splenectomy, which is also indicated by the significant upregulation of IFNγ, MIG, and TNFα. Interestingly, no change was observed in overall white blood count or differential count following splenectomy. This observation is supported by another study that investigated the influence of splenectomy on leukocyte counts and differential values in mice. This is somewhat surprising in light of the known leukocytosis observed in humans following splenectomy.

An important mechanistic finding in our study was that depleting MDSC, which are known to migrate from the bone marrow to the spleen during tumor progression, had the same effect on tumor progression. Adoptive transfer of MDSC abrogated the effect of splenectomy on tumor growth (Fig. 7). Taken together, we believe that our data support the hypothesis that splenectomy inhibits tumor growth in a murine model of NSCLC by removing a significant proportion of the MDSC population, hence reducing their overall capacity to suppress the immune response of CD8+ CTL, which in turn enables the latter to better inhibit tumor growth and seeding of metastases. This
observation is consistent with the recently published data by Ugel et al. suggesting that the spleen is fundamentally important for tumor-induced tolerance, and that splenectomy restores lymphocyte function and can induce tumor regression, especially when coupled with immunotherapy.45

Our finding that splenectomy inhibits tumor growth in a manner dependent both on the innate and the adaptive immune system is in line with the recent understanding that the generation of CTL is necessary, but not sufficient for an effective response to immunotherapy.25,46 The antitumor effectiveness of the immune system, either endogenous or induced, may be limited by systemic and local tumor-induced immunosuppression such as that imposed by MDSC. It is thus logical that a successful antitumor immune reaction requires a decrease in tumor-induced immune-suppression, an “inhibition of the inhibitors”.47 Splenectomy can potentially support this approach. Interestingly, we have recently shown a parallel outcome through manipulating TAM as a support to CTL-mediated vaccine immunotherapy.48

Recently, Cortez-Retamozo et al. published a comprehensive work on the origins of TAM and TAN.17 They demonstrated that a high number of TAM and TAN precursors physically relocate from the spleen to the tumor stroma, promoting tumor progression. Moreover, they show that splenectomy, either before or after tumor initiation, reduced the TAM and TAN responses and delayed tumor growth. Although shedding the light on a slightly different aspect of the subject, we believe their findings further support our evidence regarding the inhibitory effect of splenectomy on tumor growth, and further elucidate some of the mechanisms involved in this process. It also supports our observation that the inhibitory effect of splenectomy appears at advanced stage of tumor growth (after a minimum of 8 weeks in their model). Both our studies thus suggest that the spleen is an important source of cells inhibiting the adaptive immune system. It is
our study raises the question of whether further research is indicated to understand the potential role of splenectomy in therapy for advanced human NSCLC. Several retrospective population-based studies\(^{49-51}\) did not show a reduced tendency to develop new cancer cases in patients who underwent splenectomy due to traumatic injuries. Nevertheless, facing a disparity of evidence, we believe that splenectomy could be evaluated in the future in humans. Since splenectomy seems to be a somewhat too aggressive approach in advanced cancer, we conducted preliminary assessment of the possibility of spleen ablation in mice using ligation of the splenic artery. We found that this approach had similar effect of inhibiting tumor growth in advanced murine lung cancer models as splenectomy (data not shown). These results raise the question of feasibility of splenectomy in humans using less invasive techniques such as radiofrequency ablation or vessel ligation. Moreover, it is possible that splenectomy or partial splenectomy can be combined with other modalities of therapy that enhance the antitumor effects of the immune system, first and foremost immunotherapy, but also radiation and chemotherapeutic drugs (e.g., gemcitabine).\(^{52,53}\) This of course is not a simple task, taking under consideration the complexity of the patients and their advanced stage of disease. We hope that future studies could shed a light on this important issue of splenectomy in human cancer patients in general and specifically in advanced lung cancer.

### Materials and Methods

**Animals**

129SV, C57BL/6, and SCID (defective adaptive immunity\(^{20}\)) mice were purchased from Harlan Laboratories (Jerusalem, Israel). 129SV and C57BL/6 mice were crossed to create F1 hybrid B6/129 mice. NOD-SCID (defective adaptive and innate immunity \(^{21}\)) mice, and TNF\(\alpha\) knockout on a background of C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were housed under specific pathogen-free conditions at the Hebrew University School of Medicine Animal Resource Center. Research protocols were approved by the Animal Research Committee of the Hebrew University School of Medicine.

**Cell lines**

Murine NSCLC cell lines TC1 and LKR were kindly given to us by Dr. Steven Albelda from the University of Pennsylvania. TC1 cells were originally derived from the lung epithelium of a C57BL/6 mouse, immortalized with human papillomavirus (HPV) type 16 E6 and E7, and transformed with the c-Ha-ras oncogene, as previously described.\(^{54}\) LKR cells were derived from a lung adenocarcinoma that was derived from an activated Kras G12D mutant mouse and induced in an F1 hybrid of 129SvJ and C57BL/6 mice.\(^{55}\) Based on this line we have developed a tumor cell subline, LKR-M, which we have shown is capable of inducing lung metastases in more than 90% of animals.\(^{19}\) The LLC NSCLC cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). All cell lines were regularly tested and maintained negative for Mycoplasma spp.

**Animal model**

Mice were injected on their right flank with a \(2 \times 10^6\) TC1, LKR-M, or LLC tumor cells in the appropriate syngeneic host. Flank tumors were allowed to reach an average volume of 200/500/1000 mm\(^3\) (lengthxwidth\(^2\)x3.14/6). The mice then underwent splenectomy or sham operation. Experiments were performed with at least five mice per group and repeated at least two times. In the mechanistic experiments, blood was collected and tumors harvested 2–5 d following operations. In order to evaluate the effect of splenectomy on lung metastases, we used LKR-M cells in either syngeneic immune-competent mice or NOD-SCID immune-deficient mice. Operations were done at approximately 500 mm\(^3\), and metastases evaluated 10–14 d following surgery. In a subset of mice, splenic artery ligation was done instead of splenectomy.

**Splenectomy, splenic artery ligation, and sham surgeries**

Surgires were performed under general anesthesia induced with intraperitoneal injection of ketamine and xylazine using sterile techniques. Splenectomy was performed via a 2-cm left subcostal lateral laparotomy incision. The spleen was removed outside of the incision wound, the splenic vessels clamped, splenectomy performed, and the vessel stump was ligated with 6–0 thread (coated vicryl, Ethicon/Johnson and Johnson, Somerville, NJ, USA). Splenic artery ligation was performed via a 2-cm left subcostal lateral laparotomy incision. The spleen was removed outside of the incision wound, the splenic artery was clamped and ligated with 6–0 thread (coated vicryl, Ethicon), the spleen was returned, and the laparotomy was closed. Sham surgeries were performed via a 2-cm laparotomy. The spleen was carefully removed outside of the incision wound, remained extracorporeal for 5 min and returned, and the laparotomy was closed. At the end of all procedures Hartman solution and tramadol were injected subcutaneously to all mice.

**Metastases evaluation**

Ten to fourteen days following surgery, a sufficient time for metastases to develop and a time window at which a gap in tumor volume between groups still exists, mice were sacrificed and lungs were excised, separated into discrete lobes, and weighed. Sections were cut from each of the five lobes and were stained with H&E. In each lung, the total number of nodules was counted and total tumor area was measured and divided by total area of the lung section, using Image J software (National Institutes of Health, Bethesda, MD, USA). These evaluations were performed by a technician blinded to the group origin of the lung.

**Flow cytometry tumor analysis**

At indicated times, the mice were sacrificed. Flank tumors were harvested, minced, and digested with 1.5 mg/mL Dnase I

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\(^{50}\) Mycoplasma spp

\(^{51}\) Mycoplasma spp

\(^{52}\) Mycoplasma spp

\(^{53}\) Mycoplasma spp

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(Roche Applied Science, Penzburg, Germany) and 3 mg/mL collagenase type IV (Sigma Aldrich, St. Louis, MO, USA) at 37°C for 1 h. In addition, 1 mL of blood was pooled by cardiac puncture and transferred into heparinized tubes. Tumor and blood cells were suspended in flow cytometry buffer (PBS supplemented with 2% fetal calf serum (FCS) and 0.01% sodium azide). Cells were blocked with anti-mouse CD16/CD32 antibodies (BD Biosciences, San Jose, CA, USA) and stained with FITC-conjugated CD11b (CD11b), FITC-conjugated CD45, FITC-conjugated CD25, FITC-conjugated CD69, PE-conjugated Ly6G (1A8), PE-conjugated CD206, PE-conjugated CD11c, PE-conjugated 41BB (CD137), APC-conjugated CD4, APC-conjugated CD8, APC-conjugated F4/80, APC-conjugated MHCII, Pacific-blue conjugated CD80, or matched isotype controls (all from BioLegend, San Diego, CA), and APC-conjugated CCR2 (from R&D systems, Minneapolis, MN). For intracellular cytokine analysis, tumor cells were stimulated with 1 µg/mL LPS (Sigma Aldrich) for 3 h, treated with GolgiPlug TM (BD Biosciences) and stained with antibodies to CD11b and Ly6G. After fixation and permeabilization, cells were stained with allophycocyanin-conjugated anti-TNFα (all from BioLegend). Immunos- tained cells were analyzed with LSRII flow cytometry (BD Biosciences), and analysis was performed using FlowJo software (Tree Star, Ashland, OR, USA).

CCL2 ELISA

Blood was collected and tumors harvested 5 d following splenectomy. One × 10⁶ tumor cells were incubated in 24-well plate with 1 mL RPMI. After 24 h, the media was collected, and spun to remove cellular debris (5 min, 1500 rpm). The amount of CCL2 secreted by tumors and found in serum was quantified using an ELISA kit according to the instructions of the manufacturer (Murine JE/MCP-1 Standard ELISA Development Kit. Peprotech, Rocky Hill, NJ).

Blood count

At the indicated times, mice were sacrificed. Blood was pooled by cardiac puncture and transferred into heparinized tubes. Blood cell counts were determined using a hematology analyzer (Sysmex KX21, Sysmex Asia Pacific Pte Ltd, Tampines Grande, Singapore).

RNA isolation from different groups and real-time RT-PCR

Tumor RNA was isolated from whole tumor using the TRI Reagent® (Sigma Aldrich) according to manufacturer’s protocol. Neutrophils were isolated from whole tumors that were harvested and digested as described above. Ly6G⁺ cells were isolated using the EasySep PE Selection Kit (STEMCELL Technologies, Vancouver, BC, Canada) according to the to the manufacturer’s protocol. RNA was isolated from neutrophils using the PerfectPure TM RNA Cell and Tissue kit (5 PRIME, Hamburg, Germany). Macrophages were isolated from tumors that were harvested and digested as described above. CD11b⁺ cells were isolated using EasySep FITC kit (STEMCELL Technologies) according to the manufacturer’s protocol. RNA was isolated from macrophages using the PerfectPure TM RNA Cell and Tissue kit (5 PRIME). For real-time RT-PCR, absorbance at 260/280 nanometers for mRNA purity at a ratio above 1.9 was achieved for all samples used. cDNA was made using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Gene expression was assessed using relative quantification (ΔΔCt) RT-PCR and is shown as relative expression in whole tumor or neutrophils vs. control. RNA level was normalized to GAPDH levels. Each sample was run in triplicate and experiments were repeated three times. Primers used can be given upon request.

GR1 depletion

Systemic GR1 depletion was achieved by intraperitoneal injection of 300 µg of purified monoclonal anti-Gr1 antibody RB6-8C5 (BioXCell, West Lebanon, NH, USA) at days 1, 3, 5, and 7 of splenectomy, and tumor size was followed.

Adoptive transfer of MDSC

Gr-1⁺ cells were separated from spleens of tumor-bearing mice, using the EasySep PE Selection Kit (STEMCELL Technologies, Vancouver, BC, Canada) according to the manufacturer’s protocol. Three X 10⁶ Gr-1⁺ cells were injected IV at days 3 and 4 following splenectomy, and tumor size was followed.

Acknowledgments

We wish to thank Shifra Fraifeld, a research associate and medical writer in Pulmonary Medicine, for her editorial contribution to the preparation of this manuscript.

Funding

This work was supported in part by The Dr. Edward H. Kass fellowship from The American Physicians Fellowship for Medicine in Israel, a grant from The Joint Research Fund of the Hebrew University and Hadassah, and a grant from The G. Baum Foundation, Tel-Aviv Clinic for TB & Lung Disease.

Author Contributions

ZGF conceived the research concept, supervised the project and made critical revisions to the manuscript. LL conceived the research concept and strategies, designed and supervised the study, analyzed the data and wrote the manuscript. JM analyzed the data, performed and supervised all the experiment, and planned the revision. RB participated in the experiments described in Figs. 1–6. JM participated in the experiments described in Figs. 4–7. LZ performed all surgical procedures. All the authors discussed the results and implications, commented on the manuscript at all stages, and reviewed the final version prior to submission.
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