Video Article

Selective Harvesting of Marginating-pulmonary Leukocytes

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Abstract

Marginating-pulmonary (MP) leukocytes are leukocytes that adhere to the inner endothelium of the lung capillaries. MP-leukocytes were shown to exhibit unique composition and characteristics compared to leukocytes of other immune compartments. Evidence suggests higher cytotoxicity of natural killer cells, and a distinct pro- and anti-inflammatory profile of the MP-leukocyte population compared to circulating or splenic immunocytes. The method presented herein enables selective harvesting of MP-leukocytes by forced perfusion of the lungs in either mice or rats. In contrast to other methods used to extract lung-leukocytes, such as tissue grinding and biological degradation, this method exclusively yields leukocytes from the lung capillaries, uncontaminated with parenchymal, interstitial, and broncho-alveolar cells. In addition, the perfusion technique better preserves the integrity and the physiological milieu of MP-leukocytes, without inducing physiological responses due to tissue processing. This unique MP leukocyte population is strategically located to identify and react towards abnormal circulating cells, as all circulating malignant cells and infected cells are detained while passing through the lung capillaries, physically interacting with endothelial cells and resident leukocytes,. Thus, selective harvesting of MP-leukocytes and their study under various conditions may advance our understanding of their biological and clinical significance, specifically with respect to controlling circulating aberrant cells and lung-related diseases.

Video Link

The video component of this article can be found at https://www.jove.com/video/53849/

Introduction

Leukocytes adhering to the capillaries of the lungs (*i.e.*, marginating-pulmonary (MP) leukocytes)[1] were shown to exhibit distinct leukocyte composition and unique activity compared to leukocytes from other immune compartments (*e.g.*, circulation, spleen, bone-marrow)[2-4]. For example, MP-leukocytes exhibit higher natural killer (NK) cells cytotoxicity against various tumor cells, compared to circulating and splenic NK cells, as well as differentiated messenger RNA (mRNA) levels and increased secretion of pro- and anti-inflammatory cytokines. The composition of cells is also differentiated from circulating leukocytes as MP-leukocytes have a higher ratio of innate/adaptive immunity compared to circulating leukocytes (50% vs. 30%, respectively). The goal of the method presented herein is to enable selective harvesting of MP-leukocytes, in order to study this important and unique immune compartment (cell population), and to elucidate the impact of various manipulations (*e.g.*, immune activation) on these specific cells.

To understand the significance of this unique population, it is important to note that the immune system can control circulating tumor cells, micrometastases, and residual disease through *in vivo* functions of cell-mediated immunity (CMI). This ability is evident despite the precedent failure of the immune system to control the primary tumor, and supported by ample *in vivo* evidence in cancer patients and animal models[5]. Importantly, these findings are often inconsistent with *in vitro* and *ex vivo* studies, which report that most autologous tumor cells are resistant to cytotoxicity by circulating leukocytes in blood samples from humans and animals (measured by cytotoxicity assays) [6,7]. This discrepancy may be attributed to the *in vivo* existence of distinct leukocyte populations, such as the aforementioned MP leukocyte population, and specifically its subpopulation of activated NK cells[3]. Indeed, syngeneic tumor cells (MADB106), which were found to be resistant to circulating and splenic leukocytes, were shown to be lysed by MP-NK cells[3,8]. Thus, the allegedly 'NK-resistant' MADB106 cells that metastasize to lungs of fischer344 (F344) rats are controlled by MP-NK cells, but not by circulating or splenic NK cells, which are commonly studied given their ease of access.

Purified and active MP-leukocytes are inaccessible through the standard harvesting methods of leukocytes from the lungs, which are based on lung tissue grinding or biological degradation[9]. Our approach has two major advantages compared to tissue processing approaches. First, the perfusion approach selectively harvests MP-leukocytes, separating them from other cells that originate from the lung parenchymal, interstitial, and broncho-alveolar compartments. Second, the perfusion technique better preserves the integrity and the physiological milieu of MP-leukocytes, unlike the grinding and biological processing approaches that damage cells, alter their morphology, and induce the production and release of various factors that modulate immune activity and specifically suppress NK cytotoxicity[10].

The lungs are a major target organ for cancer metastasis and for various infectious diseases. All circulating malignant cells and infected cells pass through the lung capillaries, where they need to deform and interact with capillary endothelial cells and resident leukocytes. Under these

conditions, circulating cells can be easily targeted by resident MP-leukocytes. It thus seems biologically advantageous to have activated leukocytes in this immune compartment, and it is important to study this unique MP-population in different biological, experimental, and clinical settings. It is worthy to note that systemic immune activation by various biological-response-modifiers (*e.g.*, polyinosinic-polycytidylic acid (poly I:C) or type-C CpG oligodeoxynucleotides (CpG-C ODN)) have been shown to activate MP-leukocytes more than circulating leukocytes[3,8,11].

Protocol

Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at Tel-Aviv University.

1. Rat Protocol

1. Preparations
    1. Arrange 2 butterfly 21 G needles. Optionally, prepare 2 blunt-edged butterfly 21 G needles by filing the sharp edge of the needle.
    2. Sterilize surgical tools: 2 pairs of scissors, blunted-edged forceps, hemostat, tooth-tissue forceps sterilized by autoclave at 121 °C for at least 30 min on gravity (dry) setting.
    3. Prepare heparinized PBS (30 units/ml) by adding 30 units of heparin per ml of phosphate buffered saline (1x PBS) solution. Use at RT. 35 ml is the minimal volume needed per animal.
    4. Stream heparinized PBS into the peristaltic pump lines and the butterfly needles to avoid air bubbles.

2. Perfusion of the Lungs and Collection of MP-leukocytes
    1. Euthanize the animal by an overdose of 8% isoflurane. Confirm euthanasia by monitoring breathing cessation.
    2. Upon cessation of respiration, immediately open the peritoneal and chest cavities using the sterile scissors and tooth-tissue forceps. Perform a midline abdominal incision along the abdomen up to the xiphoid process.
    3. Lift the sternum using the forceps, cautiously cutting the rib cage on both sides, without puncturing any internal organ or large blood vessels. Clamp a hemostat on the sternum, and rostrally fold the rib cage to expose the cardiopulmonary complex.
    4. Insert a butterfly needle into the right ventricle of the heart, and within approximately a minute collect the maximal volume of blood into a syringe (~6 ml from a 250 g animal).
    5. Clamp the vena cava with a hemostat to avoid backward perfusion of the liver (see **Discussion**).
    6. Hold the butterfly needle within the right ventricle while replacing the syringe containing the blood with the outflow pipe of the peristaltic pump.
    7. Insert a second butterfly needle connected to a 5 ml harvesting syringe into the left ventricle, avoiding penetration of the interventricular septum.
    8. Turn on the peristaltic pump at a speed of approximately 5 ml/min and gently collect into the syringe the first milliliters of perfusate that are contaminated with blood. Continue until the perfusate color turns from dark to pale red. (Discard the blood-contaminated perfuste).
    9. Without cessation of the peristaltic pump, rapidly replace the 5 ml harvesting syringe with a 20 ml harvesting syringe and collect 20 ml of lung perfusate employing a higher speed of perfusion (up to 7 ml/min). Continuously monitor the perfusate flow into the collecting syringe, while avoiding vacuum formation.
    10. Cease the flow of the peristaltic pump.

3. Leukocytes Extraction
    1. Centrifuge the perfusate for 10 min at 400 × g.
    2. Aspirate the fluid.
    3. Add 10 ml PBS or medium, centrifuge at 400 x g for 10 min, and aspirate the fluid. Repeat this step twice. The use of PBS or medium depends on the consequent use of the sample.
    4. Add 20 ml PBS or medium, centrifuge at 400 x g for 10 min, and aspirate the fluid.
    5. Reconstitute the cells to the desired concentration, based on the sample usage (FACS, PCR, *etc.*).

2. Mouse Protocol

1. Preparations
    1. Arrange 2 butterfly 25 G needles. Optionally, prepare 2 blunt-edged butterfly 25 G needles by filing the sharp edge of the needle.
    2. Sterilize surgical tools: 2 pairs of scissors, blunted-edged forceps, hemostat, tooth-tissue forceps sterilized by autoclave at 121 °C for at least 30 min on gravity (dry) setting.
    3. Prepare heparinized PBS (30 units/ml) by adding 30 units of heparin per ml of 1x PBS solution. Use at RT. 25 ml is the minimal volume needed per animal.
    4. Stream heparinized PBS into the peristaltic pump lines and the butterfly needles to avoid air bubbles.

2. Perfusion of the Lungs and Collection of MP-leukocytes
    1. Euthanize the animal by an overdose of 8% isoflurane. Confirm euthanasia by monitoring breathing cessation.
    2. Upon cessation of respiration, immediately open the peritoneal and chest cavities using the sterile scissors and tooth-tissue forceps. Perform a midline abdominal incision along the abdomen up to the xiphoid process.
    3. Lift the sternum using the forceps, cautiously cutting the rib cage on both sides, without puncturing any internal organ or large blood vessels. Clamp a hemostat on the sternum, and rostrally fold the rib cage to expose the cardiopulmonary complex.
    4. Clamp the vena cava with a hemostat to avoid backward perfusion of the liver(see Discussion).
    5. Insert a butterfly needle connected to the outflow pipe of the peristaltic pump into the right ventricle of the heart, and a second butterfly needle connected to a 2 ml harvesting syringe into the left ventricle of the heart, avoidingpenetration of the interventricular septum.

6. Turn on the pump to a speed of approximately 2 ml/min and collect the first milliliters of blood-contaminated perfusate into the harvesting syringe until the perfusate turns from dark to pale red (Discard the blood-contaminated perfuste).
7. Without cessation of the peristaltic pump, rapidly replace the 2 ml harvesting syringe with a 10 ml harvesting syringe and collect 10 ml of lung perfusate employing a higher speed of perfusion (up to 4 ml/min). Continuously monitor the perfusate flow into the collecting syringe, while avoiding vacuum formation.
8. Cease the flow of the peristaltic pump.

3. Leukocytes Extraction
   1. Centrifuge the perfusate for 10 min at 400 × g.
   2. Aspirate the fluid.
   3. Add 10 ml PBS or medium, centrifuge at 400 x g for 10 min, and aspirate the fluid. Repeat this step three times. The use of PBS or medium depends on the consequent use of the sample.
   4. Add 10 ml PBS or medium, centrifuge at 400 x g for 10 min, and aspirate the fluid.
   5. Reconstitute the cells to the desired concentration, based on the sample usage (FACS, PCR, *etc.*).

Representative Results

The MP-compartment exhibit a different leukocyte subset composition compared to circulating leukocytes. Using flow cytometry analysis, leukocyte subpopulations were identified and quantified to characterize the composition of both circulating and MP leukocytes. Granulocytes and lymphocytes were identified based on forward and side scatters. Within the lymphocytes, NKRP-1^{bright} cells were identified as NK cells, CD3$^+$ as T cells, RM1$^+$ as monocytes, and CD4$^+$/CD8$^+$/CD25$^+$ were used for T cell subpopulations identification. Innate immunocytes, including granulocytes, monocytes, and NK cells, constituted 52% (± 1.52%) of the MP-leukocytes population, compared to 27.9% (± 0.66%) in the circulation ($p < 0.05$). Contrary, the T cell subpopulations, including CD4$^+$, CD4$^+$CD8$^+$ T cells, and regulatory (CD4$^+$ CD25$^+$) T cells, as well as dendritic cells, exhibited lower percentages in the MP compartment compared to the circulation ($p < 0.005$; $n = 8$). Cell yield in the MP compartment of this strain constitute of approximately 3 million leukocytes (**Figure 1**).

Within NK cells, two subpopulations are evident, based on cell size. Using a scatter plot of forward scatter by NKRP-1 labeling, NK cells were divided into small and large NK cells. The MP compartment exhibits a 3-fold higher percentage of large NK cells (~30%) compared to the circulation (~10%), while the total number of NK cells is similar (**Figure 2**). Moreover, using the standard 4 hr ^{51}Cr release assay, NK anti-tumor cytotoxicity against several target tumor cells was measured[8]**.** Shortly, this assay assesses the amount of ^{51}Cr released from tumor cells as a result of specific cytotoxicity by NK cells. The ability of NK cells from the MP compartment to lyse tumor cells is greater than circulating NK cells (**Figure 3**), as evident against both the MADB106 syngeneic cell line (**Figure 3A**), and the YAC-1 standard cell line (**Figure 3B**). In addition, immune stimulation through *in vivo* poly I:C administration produces a more profound impact on NK cytotoxicity of MP-NK cells than on circulating NK cells when MADB106 target cell line is used (**Figure 3A**).

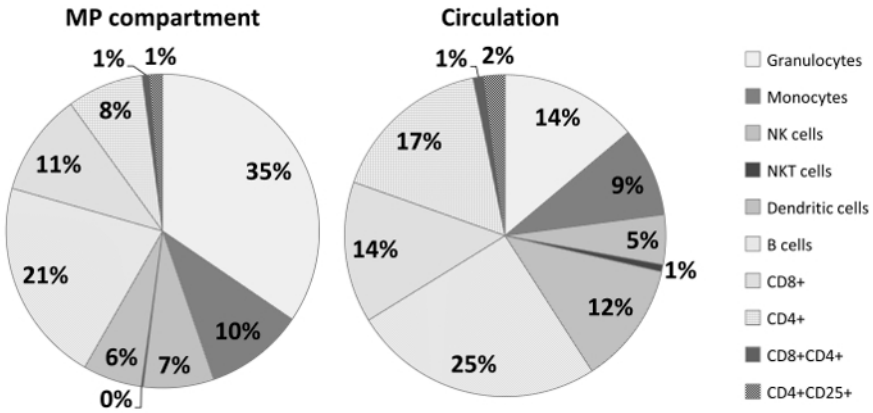

Figure 1. Different leukocyte subset composition characterizes the MP-compartment compared to the circulation in F344 rats. In the MP compartment, the fraction of innate immunity is larger compared to its equivalent in the circulation (52% vs. 28%), while the prevalence of T cells exhibit an opposite difference ($p < 0.005$; $n = 8$). Please click here to view a larger version of this figure.

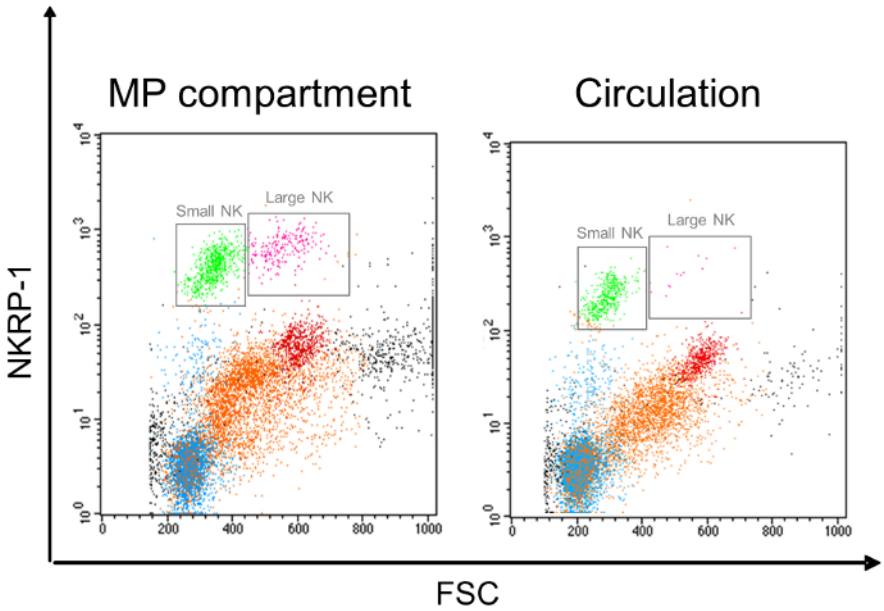

Figure 2. Increased percentage of large NK cells in the MP compartment compared to the circulation. MP-NK cells exhibited a three-fold higher percentage of large NK cells compared to circulating NK cells (30% vs. 10%, respectively; $p < 0.05$), while the total number of NK cells is similar. Please click here to view a larger version of this figure.

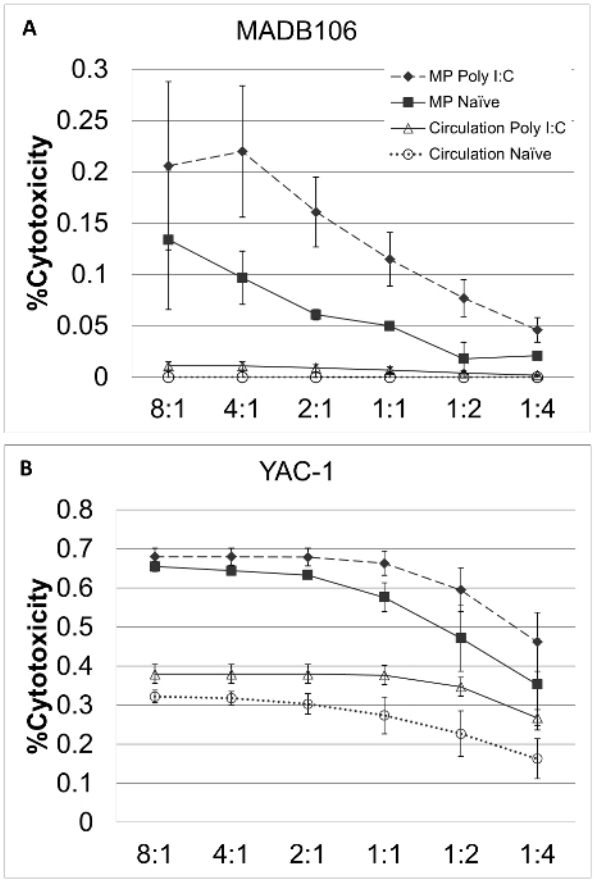

Figure 3. Higher NK cytotoxicity in the MP compartment compared to the circulation. (**A**) MADB106 target cells - NK cells harvested from the MP compartment exhibited a marked cytotoxicity against this cell line, while circulating NK cells show no cytotoxicity. *In vivo* immune stimulation by poly I:C increases NK cytotoxicity in the MP compartment, but not in the circulation. (**B**) YAC-1 target cells - NK cells harvested from the MP compartment exhibited a higher cytotoxicity compared to circulating NK cells against this standard cell line. Immune stimulation by poly I:C increases NK cytotoxicity in both the MP compartment and the circulation. NK cell numbers were controlled for cytotoxicity assessment. Data are expressed as mean ± SEM. Please click here to view a larger version of this figure.

Discussion

The method presented herein enables the selective harvesting and studying of the unique population of MP-leukocytes. Compared to circulating or splenic leukocytes, the MP population is characterized by a distinct composition of leukocyte subpopulations, higher activation levels, higher release of various cytokines, and higher mRNA levels of pro- and anti-inflammatory cytokines[2,3]. Specifically, we have shown that MP-NK cells are more cytotoxic than circulatory NK cells against various target cells[3,4], and express higher levels of interferon-γ. Overall, our results suggest that MP-NK leukocytes have a greater cytotoxicity than circulating NK cells, and thus may imply a greater biological significance in controlling blood-borne metastases and pulmonary metastases. Therefore, splenic or circulating leukocytes should not be considered as the gold standard or default population for reflecting immunological status, and human studies that commonly rely solely on circulating leukocytes should be interpreted cautiously. Moreover, as various diseases specifically inflict the lungs, immunity should be studied within this organ, and the MP compartment should be distinguished from the interstitial, parenchymal, and bronco-alveolar sub-compartments, as indeed is the case using our perfusion approach.

To assure the success of this procedure, it is crucial to (i) open the abdominal and chest cavities rapidly following complete cessation of respiration, to maintain a blood pressure that enables maximal cardiac blood collection, and (ii) to refrain from an undesirable additional perforation of the heart ventricle through the use of blunt-edged butterfly needles. Another key obstacle is the potential contamination of blood within the lung perfusate. As the first milliliters of lung perfusate are contaminated with cardiac blood, they should be discarded as demonstrated. To achieve standardization between animals, the criterion to start collecting the MP perfusate is based on the color transition from dark red to pale red. In addition, it is recommended to clamp the *vena cava* with a non-serrated hemostat, to avoid backward perfusion of the liver, especially if one wishes to also collect marginating hepatic cells through successive perfusion of the liver. While collecting the perfusate from the left ventricle, it is crucial not to penetrate the interventricular septum. Last, the heparin in the PBS used for the perfusion is crucial for disconnecting the MP-leukocytes population from the capillaries, and therefore must be used.

Several malfunctions may occur when performing this procedure, but most of them are correctable. If there is a leakage from the penetration site of the needle into the heart, it is suggested to seal the ruptured cardiac muscle using a blunt-nosed forceps with serrated tips surrounding the butterfly needle. If a needle slips out, stop the peristaltic pump, re-insert the needle into its original penetration site, and restart the pump. If collection flow ceases, halt the collecting vacuum pressure, relocate the needle within the ventricle, and restart the collection. If the left atrium

is inflated, and is not reduced by a continuous collection, lower the flow rate of the pump. In general, a high peristaltic flow rate may harm the capillary formation, and the lungs may become swollen.

The method described herein enables a selective harvesting of the MP leukocyte population in a relatively simple procedure. Given that this population exhibits unique characteristics in comparison to leukocytes from other immune compartments, and that this population may have biological and clinical significance to the organism, we propose the use of this method when feasible, especially when studying pulmonary-related processes and diseases. It is yet unknown whether MP cells, which reside in the lung capillaries upon cells harvesting, have matured in the lungs, or whether they would maintain their characteristics upon leaving the lungs. As leukocytes from other compartments may not reflect the profile of the MP population, and as it is not accessible in humans, we further suggest utilizing this method to elucidate specific circulating biomarkers that correlate with the unique MP characteristics.

Disclosures

The authors have nothing to disclose.

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References

1. Kuebler, W. M., & Goetz, A. E. The marginated pool. *Eur Surg Res.* **34**, 92-100 (2002).
2. Benish, M. *et al.* The marginating-pulmonary immune compartment in mice exhibits increased NK cytotoxicity and unique cellular characteristics. *Immunol Res.* **58**, 28-39 (2014).
3. Melamed, R. *et al.* The marginating-pulmonary immune compartment in rats: characteristics of continuous inflammation and activated NK cells. *J Immunother.* **33**, 16-29 (2010).
4. Shakhar, G. *et al.* Amelioration of operation-induced suppression of marginating pulmonary NK activity using poly IC: a potential approach to reduce postoperative metastasis. *Ann Surg Oncol.* **14**, 841-852 (2007).
5. Smyth, M. J., Godfrey, D. I., & Trapani, J. A. A fresh look at tumor immunosurveillance and immunotherapy. *Nat Immunol.* **2**, 293-299 (2001).
6. Talmadge, J. E., Meyers, K. M., Prieur, D. J., & Starkey, J. R. Role of NK cells in tumour growth and metastasis in beige mice. *Nature.* **284**, 622-624 (1980).
7. Grimm, E. A., Mazumder, A., Zhang, H. Z., & Rosenberg, S. A. Lymphokine-activated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. *J Exp Med.* **155**, 1823-1841 (1982).
8. Melamed, R. *et al.* Marginating pulmonary-NK activity and resistance to experimental tumor metastasis: suppression by surgery and the prophylactic use of a beta-adrenergic antagonist and a prostaglandin synthesis inhibitor. *Brain Behav Immun.* **19**, 114-126 (2005).
9. Dea, K. P. *et al.* Lung-marginated monocytes modulate pulmonary microvascular injury during early endotoxemia. *Am J Respir Crit Care Med.* **172**, 1119-1127 (2005).
10. Sorski, L., Melamed R., and Ben-Eliyahu. A unique role for marginating-pulmonary and marginating-hepatic NK cells in cancer anti-metastatic surveillance and in immunotherapy. *Annual Meeting of the Psychoneuroimmunology Research Society, Philadelphia, USA.*(2014).
11. Levi, B. *et al.* Continuous stress disrupts immunostimulatory effects of IL-12. *Brain Behav Immun.* **25**, 727-735 (2011).

