**Review**

# Isolation of Lymphocytes and Their Innate Immune Characterizations from Liver, Intestine, Lung and Uterus

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**In steady-state conditions, the number and distribution of lymphocyte populations are under homeostatic control. New lymphocytes are continuously produced in primary and secondary lymphoid organs and then achieve immune-competence within different tissues, and they must challenge with resident cells for survival. The first step in the study of tissue lymphoid cells is their isolation in intact and viable form appropriate for establishment of *in vitro* culture systems. For reasons of simplicity, cell purity, cell yields and various purposes, lymphocytes obtained from different tissues in different labs were subjected to diverse protocols. To fully elucidate the nature of the local immune system as well as to adequately study the innate role of lymphocytes in liver, intestine, lung and uterus, we briefly reviewed the characterization of resident lymphocytes, and additional information on those cells from non-lymphoid tissues by using the recommended operation procedure was also presented. *Cellular & Molecular Immunology*. 2005;2(4):271-280.**

## Introduction

Circulating lymphoid cells, because of their ready availability and relative ease of preparation, have been used almost exclusively in studies of the cellular immune system. However, to fully elucidate the nature of the local immune system as well as to adequately study its role in states such as cancer or inflammatory disease, the first step in the study of tissue lymphoid cells is their isolation in intact and viable form appropriate for establishment of *in vitro* culture systems. Therefore, a major objective of the present study is the development of a satisfactory isolation procedure. Monocyte isolation by adherence, although simple (1), has several disadvantages such as high lymphocyte contamination and low flexibility (2). Alternative methods are immune-selection, centrifugal elutriation and density gradients. Immune selection is too expensive for daily routine and for large volumes of blood. Several kinds of density gradients requiring specialized procedure, are available, both continuous and discontinuous. Although optimization of a cell isolation procedure for a particular cell type is dependent upon the adequate recovery of cells having various required characteristics, some guidelines can be established.

For many years after their discovery, and despite their relevance in defense mechanisms, major questions concerning the mode of action and exact nature of T, B, natural killer (NK) cells remained, and different results were obtained in different research systems. In recent years, unique leukocyte populations including dendritic cells (DC) (3), regulatory T (Treg) cells (4), etc. were emphasized on how to control the primary immune response in particular tissues or specific status. Also functional detecting and clinical application of low proportion of Treg cells (5) and DC (6) raised the questions of subpopulation of lymphocytes isolation. Meanwhile, many questions concerned about commonly accepted lymphocyte isolation approaches regarding to various tissues -- how to collect abundant these cells as their "natural" status. There will be tissue-biased expression of the different ligands and receptors depend on the ways of their enrichment. Fortunately, general protocols for isolating lymphocytes in different tissues were established in the efforts of worldwide labs in recent years. This paper describes the isolation and characterization of larger numbers of lymphocytes from the liver, intestine, lung and uterus of humans or rodents.
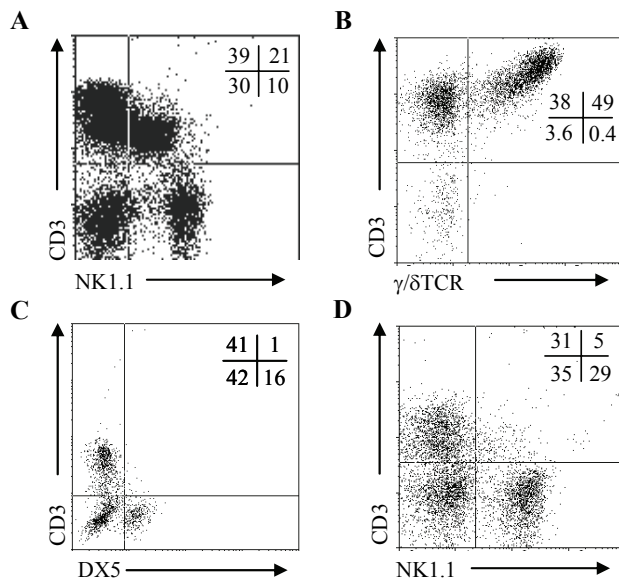
## Isolation of hepatic lymphocytes

*Components of hepatic lymphocytes*

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**Figure 1. Representative FACS assay of tissue associated lymphocytes.** Lymphocytes isolated from liver (A), intestine (B), lung (C) and uterus (D) (gd 8) of normal C57BL/6J mice then stained with two-color immunofluorescence.

Liver is one of the biggest digestive organs. On account of special location and function, the liver is continuously exposed to a large load of the intestinal antigens that include pathogens, toxins, tumor cells and harmless dietary antigens (7), making the liver not only to bear "internal depurating blood" function, but also to perform the function of removing the foreign matter of intestinal source. These characteristics provide with the possibility that liver is endowed with a unique fast immune response mechanism in response to the liver-specific potential dangers. Several lymphocyte subpopulations reside in the normal adult human liver (Figure 1A). In 1980s, a distinct kind of liver cells with NK -like large granular lymphocyte (LGL) was found and named as Pit cells (8). Later, these cells were shown to have natural cytotoxicity. In recent decade, isolated lymphocytes including NK cells from human or murine liver were universally used to explore the immune mechanisms in the defense of pathogens such as hepatitis virus (9, 10) and in the pathogenesis of liver diseases, especially in the autoimmune hepatitis (11) and liver transplantation (12). Liver lymphocytes mainly include a large number of T cells, B cells, NK cells and natural killer T (NKT) cells, which are distinct from the peripheral blood lymphocytes (PBL) (Table 1) (13). In these cell populations, NK cells are about 10-15% of total hepatic lymphocytes.

*General isolation procedure*
Up to now, mechanical dissection and enzymatic digestion are two main techniques for the isolation of hepatic lymphocytes. Isolation of hepatic lymphocytes with the mechanical dissection was carried out as follows:

Step 1, under deep methoxyfluran anesthesia, a needle was carefully inserted into the portal vein. The liver was perfused with 20 ml PBS (pH7.0), and then removed to a petri dish;

Step 2, the liver was thoroughly dissected and gently passed through a 200-gauge stainless steel mesh and then suspended in RPMI 1640 medium containing 100 ml/L fetal calf serum (FCS);

Step 3, the above cell suspension was centrifuged at 1,500 rpm. The pellet was resuspended in 40% Percoll solution containing 100 U/ml heparin, and then loaded on the layer of 70% Percoll solution followed by centrifugation at 2,000 rpm for 20 min at room temperature;

Step 4, the cells were aspirated from the Percoll interface and harvested by centrifugation and washed twice with Hanks' Balanced Salt Solution (HBSS) containing 50 ml/L FCS.

The procedures for the enzymatic digestions were as follows (14): under deep anesthesia, a needle was inserted into the portal vein. The inferior caval vein was cut to enable blood out-flow. The liver was perfused with 20 ml liver perfusion medium, followed by an injection of 5 ml liver digestion medium mainly containing 0.05% collagenase IV and 0.001% DNase I, then the liver was removed and gently pressed through a mesh. The liver cell suspension was collected, and parenchymal cells were separated from mononuclear cells (MNCs) by centrifugation at 500 g for 5 min. MNC populations were purified by centrifugation through a Percoll gradient as mentioned before. Cell pellet was collected, washed in PBS, and resuspended in 40% Percoll in complete RPMI 1640 medium. The cell suspension was gently overlaid onto 70% Percoll and centrifuged for 20 min at 750 g. MNCs were collected from the interface layer, washed twice in PBS, and resuspended in proper medium for further process.

*Comparison of mechanical and enzymatic digestion*
Liver mechanical dissection method was started in early 1980s. Reportedly, the viability of lymphocytes with this method is poor, and this method also leads to low yield. The latter method was through incubation with digestive enzymes, 0.05% collagenase IV and 0.001% DNase I (14) and was considered to have a relative low contamination of PBL. Because the manipulation of the method was difficult to handle for tenderfoots, the prevalent use was limited. Some investigators also discovered that two digestive enzymes used could influence and decrease the percentage of surface markers of human hepatic lymphocytes such as CD56 molecule (15). However, the effects of two digestive enzymes on the surface markers of murine hepatic lymphocytes especially NK1.1$^+$ cells, remain obscure. Yet there was not any report exclusively focused on the difference between above two methods. The enzymatic digestion was considered to be a satisfied method in the preparation of human organ-specific lymphocytes (16) used to isolate hepatocytes (17). However, results from different studies appeared to be inconsistent. For example, Trobonjaca et al. reported that NKT cells accounted for 35.9% of the

**Table 1.** Quantitative characterization of tissue associated lymphocytes

| Tissue | Source | Total lymphocytes ($\times 10^6$/ml) | Ratio of yield (%) | Time considerations (hour) | Subpopulation (%) | | |
|--------|--------|--------------------------------------|--------------------|----------------------------|-------------------|---|---|
| | | | | | CD3$^+$ | NK1.1$^+$ | CD3$^+$NK1.1$^+$ |
| PBMC | human | 1-2 | 80-95 | 1 | ~70 | 5-10* | 1-6* |
| Spleen | B6 mice | 5-10 | 85-95 | 1 | ~30 | 8-10 | ~2.5 |
| Liver | B6 mice | 3-5 | 70-80 | 3 | ~60 | 25-40 | 10-20 |
| Intestine | B6 mice | 2-10 | 60-75 | 5 | ~90 | 1-10 | 1-10 |
| Lung | B6 mice | 1-2 | 60-75 | 4 | ~40 | 10-20 | <1 |
| Decidua | B6 mice | 1-3 | 60-75 | 4 | ~35 | 10-50 | 2-5 |

# Recoveries might vary with age, gender, strain and stress, etc. * Refer to CD56$^+$ and CD3$^+$CD56$^+$, respectively.

total hepatic lymphocytes in normal mice (14) while Osman et al. reported that NKT cell accounted for only 16% (18). This difference may contribute to the different isolation methods applied. The roles of collagenase on surface makers of peripheral blood lymphocytes were investigated in 1996. It was found collagenase could disrupt the surface markers, such as CD3, CD4, CD8, αβ and γδ TCR by about 20%-40% (19). It was also reported that isolation of intestinal lymphocytes (details below) using collagenase released cytotoxic factors, which were found to suppress NK cell activity of isolated cells (20). We found that there was no difference in cell yields and viability between the mechanical dissection method and the enzymatic digestion method. We did not observe any decrease in the surface molecules of hepatic lymphocytes in all cases except NK1.1 and DX5, suggesting that enzymatic digestion using collagenase IV and DNase was unsuitable to isolation of hepatic lymphocytes for phenotypic analysis of NK1.1$^+$ cells mainly referring to NK cells and NKT cells (9).

## Isolation of intestinal intraepithelial lymphocytes

*Components of intestinal intraepithelial lymphocytes*
Intestinal intraepithelial lymphocytes (IELs) are mostly T cells (Table 1), which are continuously associated with gut epithelium. They are the first line of defense against invading pathogens that have entered the body *via* the local epithelial surface. The intestinal epithelium forms the largest surface area of the body, and as a consequence, the IELs that reside there represent one of the largest T-cell populations of the organism (21). It has been estimated that the IEL of the mouse small intestine alone amount to almost 50% of the total T cell number in all lymphoid organs (22), although the number that can be purified is somewhat less. IELs are likely to be exposed to a diverse range of luminal antigens. In the sterile core of the body, a non-self-based adaptive immune response can effectively protect against invasive pathogens, but in the intestine, an equivalent immune response against non-self food peptides or commensals would be wasteful, possibly leading to destructive inflammatory immune disorders. This unique immune challenge imposed by the intestinal microenvironment probably has contributed

significantly to the multifaceted nature of IEL populations.

Although all IELs exhibit several distinguishing characteristics, they were classified into just two cell types: "a" and "b". Type a includes the αβTCR$^+$ IELs that primarily recognize antigens presented by conventional MHC class I and II and are primed within the systemic circulation. Type b includes αβTCR$^+$CD8αα$^+$ IELs and γδTCR$^+$ IELs that respond to antigens not restricted by conventional MHC. Although TCRαβ$^+$CD8αα$^+$ and γδTCR$^+$ cells are clearly different from one to another, type b cells share many "unconventional" features that distinguish them from type a cells (23, 24). IELs exhibit various cytotoxic activities including alloreactive and virus-specific CTL activity (25), NK activity and spontaneous cytotoxicity (26), activities consistent with an immune surveillance or first line of defence role. IELs also appear activated *in vivo* and secrete a variety of cytokines (27). Subsets of IELs have been shown to provide B cell help, to play a role in the maintenance of oral tolerance and to regulate epithelial cell function (28).

*Systemic isolation method*
Unlike studies of lymphocytes from the spleens or lymph nodes, the study of IELs is complicated by the difficulties of obtaining pure preparations of cells and using isolation procedures by which the functional capabilities of the cells are not changed. As a result, different protocols for purifying IEL cause varying artifacts, resulting in wide discrepancies in reports of the phenotypes and functions of IELs. The most widely used approach to isolate IELs in humans and other species, involves several steps:

Step 1, individualization of epithelial content. The epithelial integrity is disrupted and IEL and intestinal epithelial cell (IEC) are liberated into the medium without altering the basement membrane. The original protocols developed for IEL isolation require dissection to remove Peyer's patches, and mincing of the tissue physically to disrupt the epithelium (29-32). This method is limited by variable contamination of the IEL preparation with lamina propria lymphocytes. These protocols for IEL isolation have required enzymatic digestion, chelation with agents like ethylene diamine-tetraacetic (EDTA), panning, or magnetic bead separation. None of the modifications have proven

entirely satisfactory. An alternative method for IEL isolation then is developed. Intestine is everted, ligated, distended and then incubated with dithioerythritol (DTE) and subjected to repeated rigorous vortexing (33, 34). Contamination of this IEL preparation with lymphocytes from lamina propria and Peyer's patches is minimal. The low cell yield, however, makes it uncertain that phenotypic and functional characteristics of IELs prepared in this way are representative of the entire, much larger, IEL compartment. To address these issues, a new technique for the rapid isolation of large numbers of highly purified IELs has been developed (35). The technique is based in part on the susceptibility of intestinal epithelial cells to hypoxic conditions that leave the basement membrane relatively undisturbed. The method is rapid and requires neither enzymatic digestion, nor surgical removal of Peyer's patches, nor vigorous mechanical manipulation of the intestine. The yield of rat IELs using this method is 5- to 10-fold greater than that reported by other methods. Morphological and phenotypic analyses demonstrated that the purified cell population is comprised of IELs and is not contaminated with lamina propria or Peyer's patch lymphocytes.

Step 2, density gradient was applied to enrich IELs. In the original protocols developed for IEL enrich, one-step Ficoll density gradients is commonly used. It is effective in separating lymphocytes from more dense cells but not from less dense cells, such as epithelial cells. So, the use of a separating medium such as Percoll which is adjusted to various densities by dilution is recommended (36). Usually, a first centrifugation in 30% Percoll is performed and then a discontinuous gradient is applied. The usual layers for the second Percoll are 20%-44%-67% for human cells and 30%-40/45%-75% for mouse or rat cells. This procedure implies a low cell recovery but purity is usually good (90% IELs) though lower for human samples (60%-70%).

Step 3, purification by immunomagnetic methods or fluorescence activated cell sorting. This final step involves staining IELs if their positive selection is preferred (with an antibody such as anti-CD45) or IEC if their depletion is favored (with anti-epithelial markers). In the case of magnetic methods, which offer better viability, anti-Ig-coated paramagnetic beads are generally used to label the cells to be retained in the magnet. Depletion of IEC provides with untouched IELs, and markers such as cytokeratin or BerEP47 have been used for the purpose (10).

*Characterization of intestinal intraepithelial lymphocytes*
IELs are a highly unusual population of T cells with many features distinct from cells of the lamina propria and systemic lymphoid tissues. The majority of IELs in the human small bowel are CD8$^+$, with fewer CD4$^+$ cells (37, 38). There are no B cells or macrophages in mucosal epithelium but it has been reported that dendritic cells are present in the rat gut epithelium (39). The majority of IEL in man use the $\alpha\beta$TCR (40). Only about 10% of IEL in healthy individuals use the $\gamma\delta$TCR (41). However in rodents and mice $\gamma\delta$ IELs are much more common (Figure 1B) (42, 43).

## Isolation of lung lymphocytes

*Components of lung lymphocytes*
Lymphocytes in lung are mostly composed of T cells and B cells, which are the major populations responding to the invading pathogens and the acute allergic diseases in the lung. Nearly all T cells bear the $\alpha\beta$TCR, most of which are stained with either CD4$^+$ or CD8$^+$, although small numbers of double-negative T cells are present；while all B cells bear surface IgM, and no IgA$^+$ cells are found, and approximately more than 80% of the plasma cells produce IgA (44). NK cell is the third large population as a protagonist of innate immunity here (Table 1). NK cells, NK1.1 T cells (NKT cells), and $\gamma\delta$ T cells have been the first line of defense to invasive pathogens (Figure 1C) (45-47). Histological examination of lung tissue has just shown that lung lymphocytes are primarily localized in the lymphoid tissue of the bronchial mucosa, at sites exposed to inhaled bacteria, and where phagocytic cells are poorly represented under normal conditions (48, 49). Because of this anatomic localization, with some other cells such as macrophages, eosinophil granulocytes, dendritic cells, etc, lung plays a very important role in the defense of the airborne pathogens and antigens. In addition, the innate immune lymphocytes give tentative roles in determining the nature of the acquired immune response (45, 50, 51). This possibility has been discussed particularly with regard to host response to infection airway responsiveness (AR), which is determined by complex mechanisms reflecting lung responses to airborne stimuli.

*Common isolation protocol*
Given the importance of lymphocytes in lung functions, it is necessary to isolate enriched populations of lymphocytes with sufficient viability and purity for functional studies. There are common used methods of pulmonary lymphocyte separation such as separation by density gradient, cell electrophoresis, cell adherence, affinity chromatography, isolation of cell populations by antisera and complement, by cell sorting, by monolayer and lectins. All these methods for purifying immune cells are well established and have been widely used to help characterize the function of these cells, because the complicated branching airways of the lung, there are quite a few distinct ways to isolation the subpopulations of lung lymphocytes.

To isolate intraparenchymal lung lymphoid cells, mice were sacrificed by cervical disolation. After the chest of the mouse was opened, the lung vascular was flushed by 3 to 5 ml of prechilled Balanced Salt Solution (BSS) (52) into the right ventricle. When the color of the lungs changed to white, the lungs were excised avoieding the peritracheal lymph nodes, then washed twice with BSS (52). Lungs were then removed and cut into 300 mm pieces, and incubated in RPMI 1640 with 5% FCS, 100 U penicillin/streptomycin, 10 mM HEPES, 50 μM 2-ME, 20 mM L-glutamine containing 20 U/ml collagenase (Type Ia) and 1 g/ml DNase (Type I). A volume of 25 ml was used for four to six sets of lungs. After

incubation for 60 min at 37°C on a rotary agitator (approximately 60 rpm) at 37°C, any remaining intact tissue was disrupted by passage through a 21-gauge needle. Tissue fragments and majority of the dead cells were removed by a 250-mm mesh screen, and cells were collected by centrifugation. The cell pellet was suspended in 4 ml of 40% Percoll and layered onto 4 ml of 70% Percoll, then centrifuged (2,400 rpm, 4°C, 30 min) in BSS, and counted. Viability as determined by trypan blue 20 min at 15°C.

There was a relative simplified method to isolate the lung lymphocyets. After the lung was excised, it was cut into small pieces and was treated by mechanical ways as to be homogenated in PBS, and then the suspension was filtered through 75 μm filters to remove the major tissue fragments, and was filtered through 15 μm gilters to get the lymphocytes with a high purity more than 90%.

The alveolar epithelium is composed of two cell types that are morphologically and functionally distinct. Type I alveolar epithelial cells (AECs) are large elongated cells that make up ~95% of the surface area of the lung and, The type II AEC is considered to be the progenitor cell type that gives rise to both type I cells by differentiation and type II cells by division.

To isolate type I/II AECs of mouse, lung was perfused *via* the pulmonary artery with RPMI 1640 medium containing 25 mM HEPES (solution I) at 37°C. The lung was lavaged *via* the trachea six times with $Ca^{2+}Mg^{2+}$-free PBS containing 5 mM each EDTA and EGTA at 37°C and then was instilled with 10 ml of solution II (solution I with 10% dextran) containing elastase at 4.5 U/ml. The enzyme-instilled lung was incubated at 37°C for 10 min; an additional 30 ml of the elastase solution was instilled continuously via the trachea over 30 min. After enzymatic digestion, the trachea and large airways were dissected and discarded. The lung tissue was minced to 1-cubic mm fragments in solution II containing 20% fetal bovine serum and 100 μg of DNase/ml (type IV, 2 mg/ml). The lung fragments were gently agitated by end-over-end rotation for 4 min and filtered once through 150 μm nylon mesh and then twice through 20 μm mesh. The resultant cell suspension was centrifuged for 20 min at 250 g over a discontinuous Percoll gradient consisting (bottom to top) of 10 ml Percoll (density 1.080), 10 ml solution II and 10 ml Percoll (density 1.032). After centrifugation, the band formed at the 1.080/solution II interface was harvested, diluted to 50 ml with solution I at 4°C, and centrifuged for 10 min at 250 g. Then immunoselection with magnetic beads was used to remove macrophages and type II cells and then to select type I cells.

To isolate type II AECs of mice, animal was anesthetized and the abdominal cavity was opened, then mice were exsanguinated by severing the inferior vena cava and the left renal artery. The trachea was isolated and cannulated with a 20-gauge luer stub adapter. The diaphragm was cut, and the chest plate and thymus were removed. With the use of a 21-gauge needle fitted on a 10-ml syringe, lungs were perfused with 10-20 ml 0.9% saline via the pulmonary artery. Dispase (3 ml) was rapidly instilled through the cannula in the trachea followed by 0.5 ml agarose (45°C). Lungs were immediately covered with ice for 2 min to gel the agarose. After this incubation, lungs were removed from the animals and incubated in 1 ml dispase for 45 min (25°C). Lungs were subsequently transferred to a 60-mm culture dish containing 7 ml of HEPES-buffered DMEM and 100 U/ml DNAse I, and lung tissue was gently teased from the bronchi. The cell suspension was filtered through progressively smaller cell strainers (100 μm and 40 μm) and nylon gauze (20 μm). Cells were collected by centrifugation at 130 g for 8 min (4°C) and placed on prewashed 100-mm tissue culture plates. After incubation for 1-2 h at 37°C, type II cells were gently panned from the plate and collected by centrifugation.

To isolate bronchoalveolar lymphocytes (BAL), mice were anaesthetized with urethane and a cannula was inserted into the exposed trachea and three 0.5 ml aliquots of sterile saline were injected into the lungs. From the BAL fluid, an aliquot (50 μl) was added to 50 μl of haemolysis solution (0.1% methylene blue in 1% acetic acid). These cells contain the proportion of neutrophils, eosinophils and monocytes (53).

*Characterization of lung lymphocytes*
Slightly more than 40% of the isolated pulmonary mononuclear population was T cells, as defined by expression of CD3 (Table 1, Figure 1C) as well as TCR. These results are consistent with those of Davies and Parrott (54), who found that approximately 45% of lung lymphocytes bore the surface Thy 1 marker. B cells comprised about 30% and macrophages about 5% of the isolated lung mononuclear cells in the size fraction we have analyzed. Approximately 20% of the cells were not stained by any of the antibodies used, and probably represented contaminating red blood cells, neutrophils, and other nonlymphoid cells. The B/T ratio among these lung cells, which demonstrated only a slight predominance of T cells, as well as the high percentage of plasma cells secreting IgA, indicates that the cells were not simply purified from blood contaminating the tissue specimens, but represented a distinct pulmonary lymphoid population.

The development of methods for the purification of lung lymphocytes would be a crucial step. Because of the complex branching structure of the airways, above methods are not readily transferable to studies of lung lymphocytes. Identification of a unique surface marker (or combination of markers) would certainly facilitate the isolation of these cells, but it is not at all clear that such markers exist. The development of newer techniques, such as laser micro-dissection, could facilitate the isolation of pure populations of lung lymphocytes. Although the yield of such a method would be quite limited, techniques that now exist or are in development should allow for the analysis of lymphocyte activation, cytokine production, cytotoxic effects, gene expression, and other phenomena, even when very few lymphocytes are obtained.

## Isolation of uterine lymphocytes

*Components of uterine lymphocytes*

In humans and rodents, the uterine mucosa contains lymphoid aggregates situated in the decidua basalis which are composed of T cells and a few B cells, as well as regulatory T cells (55, 56). These cells could work in parallel but some of them could also help to restrain or increase the effects of other ones.

Decidual macrophages also produced superoxide radicals and the pro-inflammatory cytokine TNF-$\alpha$ when challenged with bacterial lipopolysaccharides, therefore, they are likely to protect the fetus against intrauterine infections which might otherwise lead to preterm labour (57). *In vivo* DCs express high levels of MHC II products and are able to migrate selectively through tissues. Decidual DCs might be important mediators in the regulation of immunological response of maternal T cells against the fetal tissue. Kammerer et al. (58) identified a subpopulation of CD40$^+$ CD45$^+$CD83$^+$HLA-DR$^{++}$ cells with morphological and functional features of typical mature DCs. Later, they found only decidua harbored a significant population of HLA-DR$^+$ DC-SIGN$^+$ APCs, further phenotyped as CD14$^+$CD4$^+$CD68$^{+/-}$ CD83$^-$CD25$^-$. These cells exhibited a remarkable proliferation rate, and a conspicuous association with a natural killer subset (59). In uterus of DBA/2J-CBA/J model (60), the relative number of CD11c$^+$ cells increased from gestation day (gd) 5.5, reaching a plateau on gd 9.5 until gd 17.5, while a transient peak of systemic CD11c$^+$ cells was found on gd 8.5 and gd 10.5.

Very recently, the role of natural Treg in mediating maternal tolerance to the fetus in normal murine pregnancy models was described. It was observed an expansion of CD4$^+$CD25$^+$ T cells in almost all tissues of pregnant compared to non-pregnant female mice independent of the paternal MHC difference (61, 62). In addition, two very recent human studies also confirmed an expansion of Treg cells in human pregnancy (63-65). Interestingly, CD4$^+$CD25$^+$ Treg cells from normal pregnant mice were able to prevent fetal rejection (62).

NKT cells have been found to have an immuno-modulatory role in infection, cancer and transplantation through the production of cytokines (66). Tsuda et al. and Boyson et al. reported that the percentages of NKT cells were increased in decidua as compared with the peripheral blood (67, 68). The latter group also described the expression of CD1d on extravillous trophoblast, which may form part of the NKT-cell ligand. These results raise the possibility that NKT cells might also have an immunomodulatory role in pregnancy.

T cells account for around 10% of leukocytes in human decidua, and remain at much the same density throughout the luteal phase and during the process of decidualization. In contrast, the NK cells increase dramatically in number in the different physiological phase of women. In humans, around 10% of peripheral blood lymphocytes are NK cells (69), which can be defined phenotypically by the expression of CD56 and the absence of CD3, and NK cells fall into two distinct subsets according to their surface density of CD56 (70). The majority of NK cells in human blood has low CD56

expression (CD56$^{dim}$) and expresses high levels of Fc receptor for immunoglobulin G (IgG; Fc$\gamma$RIII) (71) and CD57 (70). Uterine NK cells account for a large percentage (up to 70%) of leukocytes in the human endometrium (EM) and have similar expression of CD56, CD16, and CD57 as the CD56$^{bright}$ blood NK cell subset, and exit a unique phenotype that is distinct from blood NK cells (9, 72, 73). Dr. Jack Strominger et al. strongly suggested that uterine NK cells are a distinct subset (67). One of the well-known major differences between uterine and blood CD56$^{bright}$ cells is the stronger cytolytic activity of the blood subset. It was observed that uNK cells conjugated but failed to polarize their microtubule organizing centre and lytic machinery efficiently (74). Meanwhile, tissue distribution of uNK cells was quite different for various species including human, rat, mouse and pig. The ability to consistently obtain uterine lymphocytes in high purity has allowed studies of decidual cells to proceed steadily for further research.

*Recommended isolation procedure*

There is no commonly accepted protocol for isolating leucocytes from rodent uterine decidua, at the maternal-fetal interface. Traditionally, the isolation methods consist of two main steps--the disintegration of decidual tissue to yield a single cell suspension, and a subsequent gradient centri-fugation leading to a selective enrichment of cells. Two main approaches are usually applied for the first step--mechanical disruption of the tissue or enzymatic treatment. While mechanical disruption is less effective in the cell yield, it preserves the viability and the surface markers of the isolated leukocytes (75, 76). In contrast, enzymatic digestion gives higher yield of lymphocytes but a variable viability counts and loss of cell surface markers (74).

For enzymatic digestion protocol of isolation of human uterine natural killer cells, tissues were obtained immediately following surgery from patients for a variety of clinical conditions, and be sure these patients were enrolled in the study after informed consent. All obtained tissues should be determined at a gross anatomical level. Dr. White recom-mended a mild enzymatic cocktail containing pancreatin, hyaluronidase and collagenase (PHC), which resulted in the highest yield of cells isolated from the uterine endometrium and provided an efficient method for the isolation of relatively large numbers of leukocytes with associated epithelial and stromal cells, while maintaining certain major leukocyte surface antigens intact. The "PHC" enzyme cocktail contained final concentrations of 3.4 mg/ml pancreatin (100 USP U/mg non-specified protease, 8 USP U/mg lipase, 100 USP U/mg amylase), 0.1 mg/ml hyaluronidase (1365 U/mg), 1.6 mg/ml collagenase (221 U/mg), and 2 mg/ml D-glucose, in 1× HBSS containing 50 U/ml penicillin and 50 mg/ml streptomycin (pen/strep) (77). After carefully mincing tissues and incubating in HBSS in the presence of the enzyme cocktails for 2 h at 37°C, cells were dispersed through mesh screen, washed, and analyzed for cell numbers, viability, marker expression. Any red blood cells present were eliminated from the endometrial cells by treatment with lysis buffer (NH$_4$Cl/Tris-HCl) for 5-10 min at room temperature.

Cell viability was determined by uptake of trypan blue dyes under microscopic examination.

Most studies processed with density gradient centrifugation leading to selective enrichment of viable cells. Various gradient media have been developed for specific applications. There are a number of characteristics of the ideal density gradient material. They should, if possible, not alter the cells or particles to be separated and should provide a useful density range for separation. In the vast majority of leucocyte isolation studies, if a gradient centrifugation step has been performed, the commercially available Ficoll media, originally developed for isolation of mononuclear cells from peripheral blood have been used. The disadvantage of these media is that they easily penetrate into cells and cell organelles, making the buoyant densities unphysiological and affecting the viability of the isolated cells (78). Percoll, in contrast, an iso-osmotic, pH neutral, high density solution, has been shown to be a very suitable medium for separation by density gradient centrifugation as it reduces convection, gives a complete separation of several components in a mixture, according to size and density and does not affect the isolated cells. The major advantage with Percoll is, however, that one can match the osmolarity of cells or particles to be separated. Thus, the use of Percoll as the medium of choice, especially in NK cells separations from decidua, has rapidly grown.

The uterine leukocyte band from the lymphoprep centrifugation and the leukocytes, enriched in the interface between 40% and 80% Percoll, were separately collected, which can be further positively or negatively selected by incubation with immunomagnetic microbeads of conjugated anti-human CD56 mAb to get purified uterine NK cells.

NK cells are conspicuous residents of the uterus of rodents and humans during pregnancy. The ability to consistently obtain NK cells in high purity has allowed studies of NK cells to proceed steadily for rat, mouse and human (Figure 1D). However, the isolation of highly enriched NK cells from the rat or mouse has been more difficult because of the morphologic and biological properties of NK cells (79). The distribution and phenotype of uterine NK cells change from implantation to midgestation. Usually, uterine NK cells peaked at the midgestation days of mice or rats (80, 81), but earlier stage for normal pregnant women (82). Dr. M Soares suggested a immunomagnetic approach to isolate rat uterine NK cells (83). Briefly, once pregnant animal at assigned gestation stage was sacrificed, whole uteri were removed and mesometrial decidua was carefully dissected and minced using razor blades in petri dishes incubating on ice. The dissected region should not contain fetal cells on the gestation days used. Then tissue was dispersed in Dispase (2.4 U/ml) containing deoxyribonuclease I (80 U/ml) for 1 h at 37°C. Dispersed cells were recovered by centrifugation and washed with HBSS to remove residual Dispase. Red blood cells were lysed *via* incubation of the cell suspensions with 10 vol lysis buffer for 5 min at room temperature. Cells were recovered by centrifugation and washed twice with isolation buffer. Magnetic separation was processed based on the detailed approach.

*Characterization of uterine lymphocytes*
Implantation of the developing embryo into the wall of the maternal uterus with subsequent development of the placenta is a critical stage of normal pregnancy. Despite the incompleteness of understanding on pregnancy maintenance and development, a complex network of hormones, cytokines and cells at the maternal-fetal interface such as NK cells, macrophages, DCs and T cells could play a role on embryo development and implantation and on the maternal tolerance towards the fetus (55, 56). The uterus is normally sterile, but uterine B cells can respond to antigenic challenge. Recent finding of Strominger group raised the possibility that NKT cells might also have an immunomodulatory role in pregnancy (67). Dendritic cells (< 1% of total cells) have the phenotype of immature myeloid dendritic cells, but their function was remain unknown (84). Immunohistology indicates that approximately 20%-30% of leukocytes in human endometrium are macrophages. This percentage is relatively invariant throughout the menstrual cycle and also in pregnancy. Though the CD3$^+$ T cells found in the decidua differ phenotypically from resting T cells in blood, they cannot be activated by extravillous trophoblast. Uterine NK cells have been proposed to participate in immunological adjustments to pregnancy and in the remodeling of the spiral arteries which was mainly trigger by IFN-γ (55, 74, 85), as well as chorioallantoic placental development (86). Midgestation uterine NK cells are distinct in their cytokine and bioeffector secretory profile and their relative absence of cytolytic activities (87, 88), which are not required for a successful pregnancy, and there was no clear evidence demonstrating peripheral NK cells related to human pregnancy failure (89). In fact, maternal tolerance of the fetal allograft could be the result of the integration of numerous mechanisms of various origins.

## Concluding remarks

In steady-state conditions, the number and distribution of lymphocyte populations are under homeostatic control. New lymphocytes are continuously produced in primary and secondary lymphoid organs must compete with resident cells for survival (44). The primary goal of the cells of the immune system is not only to ensure their own growth and survival, but to secure the body defense in the local tissues. Usually, the isolation of mononuclear cells from different tissues was achieved by a series of combined method consisting of enzymatic and mechanical dissociation, density gradient centrifugation, and adherence to plastic, followed by FACS or MACS sorting. A fair idea of the general mechanisms that regulate their activation and function, particularly the molecular mechanisms was explored. So it is really important to isolate and exploit lymphocytes in different tissues under normal or abnormal conditions. This is the first step for further cell biological projects and the method should be optimized to obtain a nearly complete recovery of different lymphoid subpopulations.

The possibility of exploiting lymphocyte activities as a

cell-based immunotherapy to treat cancer also need stable isolation methods (90). Not until recently have NK cells stepped out of the shadow of T cells to be considered for cellular therapy of malignant diseases. This evolution has been facilitated by the discovery of specific receptors on regulatory lymphocytes that interact with HLA molecules on target cells but also the discovery of specific activating receptors. Since NK cells, DCs and regulatory T cells represent small percentage of the lymphocyte population in blood, separation and enrichment are important steps if those cells are to be used clinically. This is of particular consideration in the setting of allogeneic cells infusion where contaminating T cells could potentially induce graft versus host disease (91).

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