Supplementary Materials

Detailed methods

Patients

All patients in this study were enrolled at Beijing Anzhen Hospital, Capital Medical University. Among fifty patients, 15 of 50 patients were used for monocyte subsets analysis and Ki-67 staining by flow cytometry; 5 of 50 patients for morphology of monocyte subsets; 10 of 50 for surface makers analysis of different monocyte subset; 10 of 50 for *In vitro* differentiation assay; and 10 of 50 for TNF-α mRNA and protein detection. All patients gave informed consent according to the protocol approved by the local Research Ethics Committee of Beijing Anzhen Hospital, Capital Medical University. Patients with infection, tumors, pregnancy, or autoimmune disorders were excluded. The primary diseases of the patients undergoing cardiac surgery with CPB were rheumatic valvular heart disease. The procedures included isolated cardiac valve replacement (mitral valve replacement or aortic valve replacement, 30 patients), double valve replacement (16 patients), and valve replacement with coronary artery bypass grafting(4 patients). Eight patients received atrial fibrillation ablation. The range of perfusion times was 61–190 minutes (average: 106.1 ± 39.2 minutes). All patients were ultimately discharged.

CPB protocol and management of patients

The CPB circuit consisted of a Type S III roller pump (Stoeckert, sorin, Müchen, Germany), a Quadrox oxygenator with Safeline coating, an arterial filter (Maquet, Jostra Medizintechnik AG, Hirrlingen, Germany), and a standard tube with no coating (Kewei, Guangdong, China). The patients undergoing CPB were cannulated and a bypass was established. To protect the myocardium, a cold blood-high potassium cardioplegic solution was perfused anterogradely through the root of the ascending aorta.

For all operation, general anesthesia was performed with intubation and inhaled sevoflurane. Anesthesia was maintained with Attacurium, propofol and sufentanil. After tracheal intubation, patients were supported with adequate oxygen. Routine antibiotic prophylaxis was administered. Patients did not receive corticosteroids before, during, or after the operation. Open heart surgery was performed under mild hypothermia (32ºC), and hypothermia was maintained by a heat exchanger in the circuit. Protamine was used to counteract heparin after the CPB operation. All patients were postoperatively treated in an intensive care unit. Blood samples were drawn after onset of anesthesia (pre), and postoperatively after CPB at 0 days, 1 day, 3 days, 5 days, and 7 days.

Animal surgical preparation and cardiopulmonary bypass

Adult male Sprague-Dawley rats (350-400 g, specific-pathogen-free) were obtained from Vital River Laboratories (Beijing, China) and housed under standard conditions. All procedures performed on animals were approved by the Animal Care Research Ethics Committee of the Capital Medical University of China. Rat were anaesthetize with intraperineal injection of chloral hydrate and inhaled isoflurane. After fixed fixed on an operation table (37 ºC), rats were intubated with 14 gauge cannula. Continuous mechanical ventilation was set at appropriate parameteters

(tidal volume, 8 mL/kg; respiratory rate, 55 cycles/minute; I:E = 1:1; maximal airway pressure, 5-10 cm/H2O; oxygen flow rate, 0.01-0.02 L/minute. Rats were intubated with 14 gauge cannula. Continuous mechanical ventilation was set at appropriate parameters (tidal volume, 8 mL/kg). Anesthesia was maintained with isoflurane during the subsequent procedure. After surgical level anesthesia was achieved, the left femoral vein was cannulated with a heparinized 22 gauge cannula for drug administration (2, 3). Heparin (500 IU/kg body weight) was administered after placement of the first cannula. The homolateral femoral artery was cannulated with another heparinized 22 gauge cannula to monitor systemic arterial pressure and collect arterial blood samples for blood gas analysis. The right femoral artery was cannulated with a 22 gauge cannula connected to the perfusion line. A 16 gauge cannula (with multiple orifices on the tail) was inserted into the right external jugular vein and then advanced into the right atrium to serve as a venous outflow line.

All of the CPB circuits were individually used, and consisted of a 10 mL syringe used as a venous reservoir, a roller pump (with silicone tube with an inner diameter of 4 mm), and a small animal-designed membrane oxygenator (5 mL prime volume; membrane area for gas exchange was 558 cm^2). The entire circuit was primed with 12 mL of fluid, composed of 6 mL autoblood, 4 mL polygeline, 0.5 mL 5% sodium bicarbonate, 0.5 mL 20% mannitol, and 1 mL heparin. The venous outflow line and the perfusion line were connected after discharging residual bubbles in the membrane oxygenator throughout the intrinsic cycle. A 0.2 mL arterial blood sample was collected through the left femoral artery, and activated clotting time (ACT) and blood gas were tested. When ACT exceeded 480 seconds, parallel circulation was started. The CPB outflow was progressively increased to 100 mL/kg/minute, and the ventilation rate was lowered to 30 cycles/minute. Within 15 minutes of CPB, the temperature was decreased to 28-30^{o}C to simulate the clinical medium low temperature, then the rats received CPB for 60 minutes. During the entire experiment, mean arterial blood pressure was maintained at 75-90 mmHg. Arterial blood pressure, mean arterial blood pressure, and rectal temperature were monitored during the entire period of CPB. Blood samples were collected to perform blood gas analysis every 20 minutes.

Splenectomy model of healthy rats

During isoflurane anesthesia, a left lateral incision was made and the abdominal cavity of the rat was opened. Ligatures were placed around the blood vessels and the spleen was carefully removed and placed in cold phosphate-buffered saline solution (4). The abdominal cavity was washed with physiological saline to ensure there was no bleeding, and the incision was sutured to close the abdominal cavity. After 2 days, the CPB model was used.

Specimen collection

Rats were sacrificed by decapitation at the indicated times, and arterial blood was collected with a heparinized syringe at the indicated times. For BALF, the trachea was exposed to allow insertion of a catheter, through which the lung was filled and washed 3 or 3 or 5 times with 1 mL of PBS containing 5 mM EDTA without Ca^{2+}or Mg^{2+} (5). Whole blood and BALF were placed on ice for

analysis by flow cytometry.

Evaluation of acute lung injury

For histology of lungs, rat lungs were instilled with 10% formaldehyde solution and embedded in paraffin at the indicated times. Subsequently, paraffin sections of 5 μm were stained with hematoxylin-eosin. Lung wet/dry (W/D) weight was calculated as an indicator of edema. For the wet/dry lung weight ratios, the lungs of rat were weighed at pre-CPB, 0 hour, 3 hours, 12 hours, and 24 hours post-CPB to determine the final wet lung weight. Then the lungs were dried in an oven at 60ºC for 48 hours and weighed again to determine the dry weight. The wet/dry weight ratio was then calculated (6). The total protein concentration in BALF from rats at the indicated times was measured using the Pierce BCA Protein Assay Kit (Life Technology, Grand Island, NY, USA).

Immunologic markers and flow cytometry analysis

Antibodies used for flow cytometry analysis included fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, Peridinin chlorophyll (PerCP)-, peridinin chlorophyll protein complex with cyanin-5.5 (PerCP-Cy5.5)-, or Allophyxoxyanin (APC)-conjugated anti-human or anti-rat monoclonal antibodies and the corresponding isotypes. Human monocytes were stained with anti-human CD14, CD16, CD45, HLA-DR, CD86, CD64, or Ki-67 antibodies (BD Biosciences and BD Pharmingen, Palo Alto, CA, USA). Monocytes from fresh peripheral blood were gated on $CD45^+$ nucleated cells and excluded of dead cells. Human peripheral blood monocytes were divided into four subsets according to the expression of CD14 and CD16: $CD14^{low}CD16^-$ immature monocytes (Mo0), $CD14^{high}CD16^-$ classic momocytes (Mo1), $CD14^{high}CD16^+$ intermediate monocytes (Mo2) and $CD14^{low}CD16^{+/high}$ nonclassical monocytes (Mo3). The subsets were defined based on isotype controls. Carboxyfluorescein-diacetate succinimidyl ester (CFSE) (Invitrogen, Portland, OR, USA) was used for intracellular staining of human $CD4^+$ T cells. Rat monocytes were identified with CD11b, CD43, CD45 (Biolegend, San Diego, CA, USA), and CD172a (AbD Serotec, Oxford, UK). Rat macrophages were stained with CD68 (AbD Serotec). Data acquisition was performed on a FACSCalibur flow cytometer (BD, Palo Alto, CA, USA) using CellQuest software, and data analysis was performed using FlowJo Software (Ashland, OR, USA).

Cell sorting

For cell sorting, peripheral blood mononuclear cells (PBMCs) from patients or rats were isolated by Ficoll (human; GE, Pittsburgh, USA) or Percoll (rat; Sigma, St Louis, MO, USA), density gradient centrifugation, followed by incubation with indicated antibodies. Different human or rat monocytes were sorted by the FACS Aria II (BD) with >98% purity.

Morphological analysis

Morphology of sorted cells was determined using Wright-Giemsa staining. The images were taken at 1000-fold magnification using a light microscope (BX51; Olympus, Tokyo, Japan), plus

Camedia 30 camera and DP software (both from Olympus).

CSFE staining and cell proliferation assay

Allogeneic human $CD4^+$ T cells from healthy donors for the proliferation assay were separated using Magnetic-activated cell sorting (MACS) magnetic CD4 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). T cell proliferation was determined by measuring changes in fluorescence using CFSE labeling. Freshly isolated $CD4^+$ T cells from healthy donors were incubated with 1M CFSE (Invitrogen, Portland, OR, USA), and 1×10^5 CFSE-labeled cells were cultured alone or together in 96-well plates with 1×10^5 sorted monocyte subsets from patients who received CPB. The cells were co-cultured in anti-human CD3-coated (5 μg/mL, OKT3 mAb; eBioscience, San Diego, CA, USA) plates in the presence of soluble anti-human CD28 (5 μg/mL; eBioscience). After 72 to 96 hours of culture, analysis of CFSE fluorescence was performed using FACSCalibur flow cytometer (BD, Palo Alto, CA, USA). The percentage suppression was determined based on the percentage of dividing CFSE-labeled cells as compared with the percentage of dividing CFSE-labeled cells when cultured alone.

Real-time PCR for TNF-α RNA detection

Total RNA was isolated from 1×10^4 to 1×10^5 sorted human blood monocytes subsets from patients undergoing CPB one day postoperatively or rat monocyte subsets sorted from CPB rat blood, lung, and BALF using the RNeasy Micro Kit (Qiagen, Hilden, Germany). Complementary DNA was generated using a High Capacity RNA-to-cDNA Kit (Invitrogen). Quantitative real-time PCR for human or rat TNF-α was performed using TaqMan (Invitrogen). GAPDH was used to normalize gene expression, and the levels of gene expression were expressed as fold changes or relative mRNA levels.

ELISA or CBA for TNF-α protein detection

For the detection of TNF-α protein expression by human monocyte subsets, 2×10^4 $CD14^{low}CD16^-$ (Mo0), $CD14^{high}CD16^-$ (Mo1), and $CD14^{high}CD16^+$ (Mo2) monocytes in the peripheral blood from the patients at 1d post-CPB were sorted with FACS Aria II, cultured with LPS (1pg/mL) in the medium (1640 RPIM with 10%FBS) for 3 hours, and the supernatant was collected and detected by CBA human inflammatory cytokines kit (BD, USA). For the secretion of TNF-α by rat monocyte subsets, 2×10^4 $CD43^{low}$ and $CD43^{high}$ monocytes in the peripheral blood, and lung tissues from model rat at 3 hours post-CPB were sorted with FACS Aria II, cultured with LPS (1pg/mL) in the medium (1640 RPIM with 10%FBS) for 3 hours, and the supernatant was collected and detected by BD OptEIA Rat TNF ELISA Kit (BD, USA).

Supplemental Figures Legends

Supplementary Figure 1. The kinetic change of the numbers of total monocytes in the 10 individual patients with CPB and each spot represents the cell count of an individual patient.

Supplementary Figure 2. The expression of HLA-DR, TLR-4, CD86, and CD64 in four monocyte subsets after 1 day from patients receiving CPB was analyzed by cytometry and presented as % of different monocyte subsets (n = 15). Mean ± SEM values are indicated. ANOVA was applied to analyze the expression difference of surface and functional molecules of different monocyte subsets.

Supplementary Figure 3. The whole peripheral blood samples from patients before CPB were cultured for indicated times using an *in vitro* differentiation assay. Representative scatter diagrams of flow cytometry (A) and kinetic change of different monocyte subsets (B) are shown from 10 independent experiments. The repeated measurement data analysis of variance methodology was applied and followed by *post hoc* Bonferroni analysis.

Supplementary Figure 4. The kinetic change of the percentage (A) and the numbers (B) of different monocyte subsets in the 10 individual patients with CPB and each spot represents an individual patient.

Supplementary Figure 5. The Dynamic changes of Mean Fluorescence Intensity (MFI) and percentage of HLA-DR, TLR-4, CD86, and CD64 in four monocyte subsets from patients receiving CPB was analyzed by flow cytometry. Mean ± SEM values are indicated (n = 10). The repeated measurement data analysis of variance methodology was applied and followed by *post hoc* Bonferroni analysis. Repeated-measure analysis of variance showed that the main effect of the percentage of different monocyte subsets on the kinetic change of monocyte subsets was significant ($P < 0.001$).

Supplementary Figure 6. Dynamic changes of $CD43^{low}$ and $CD43^{high}$ monocyte numbers. Dynamic changes of $CD43^{low}$ and $CD43^{high}$ monocyte numbers the lungs, and the BALF of rats receiving CPB at the indicated times (n=3).

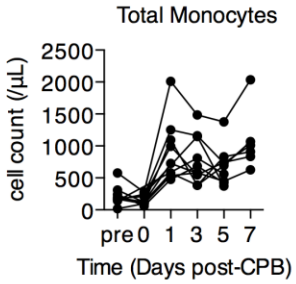
Supplementary Figure 7. The $CD43^{high}$ monocytes were selectively labeled by fluorescent latex microspheres. Latex positive monocytes in the peripheral blood and lung tissues analyzed by flow cytometry at 24 hours after injection of microspheres, and 7 days post-CPB.

Supplementary Figure 8. $LX^{+}CD68^{+}$ macrophages in BALF. Representative flow cytometric profiles (A) and the percentages (B) of latex positive macrophages in the peripheral blood and lungs at 3 hours and 24 hours post-CPB.

Supplementary Figure 9. Monocyte subsets from patients receiving CPB did not inhibit T cell

proliferation. **A**. CFSE-labeled $CD4^+$ T cells from healthy controls with anti-CD3/CD28 stimulation were co-cultured alone or with MDSCs from septic patients at a ratio of 1:1 for 3-4 days. The percentages of non-proliferating cells are indicated. Data are representative of three independent experiments. **B**. CFSE-labeled $CD4^+$ T cells from healthy donors were co-cultured alone with sorted Mo0, Mo1, and Mo2 monocytes from patients receiving CPB at the indicated times at a ratio of 1:1 for 3 to 4 days. The percentages of non-proliferating cells were analyzed by flow cytometry. Data are representative of 5 independent experiments.

Supplementary figures



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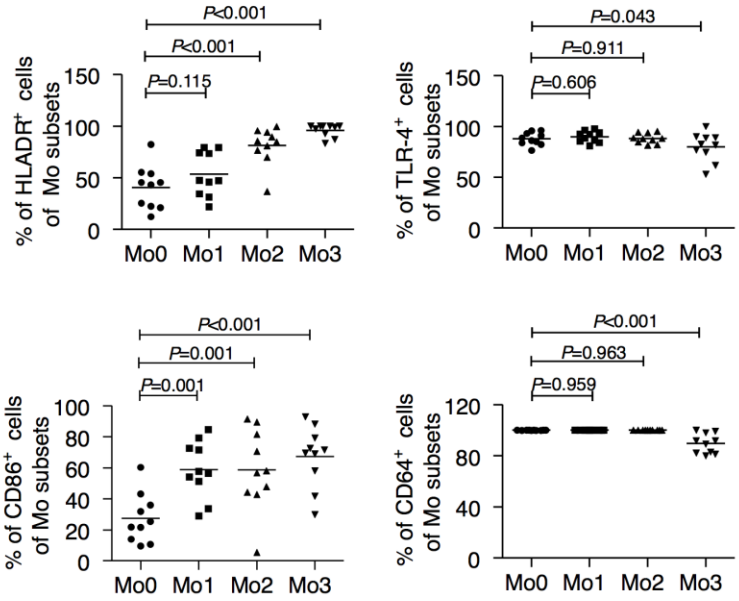
Supplementary Figure 1



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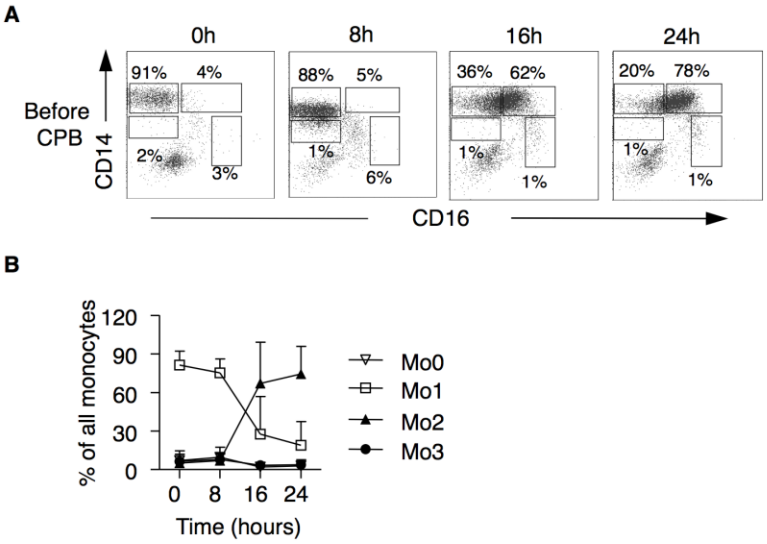

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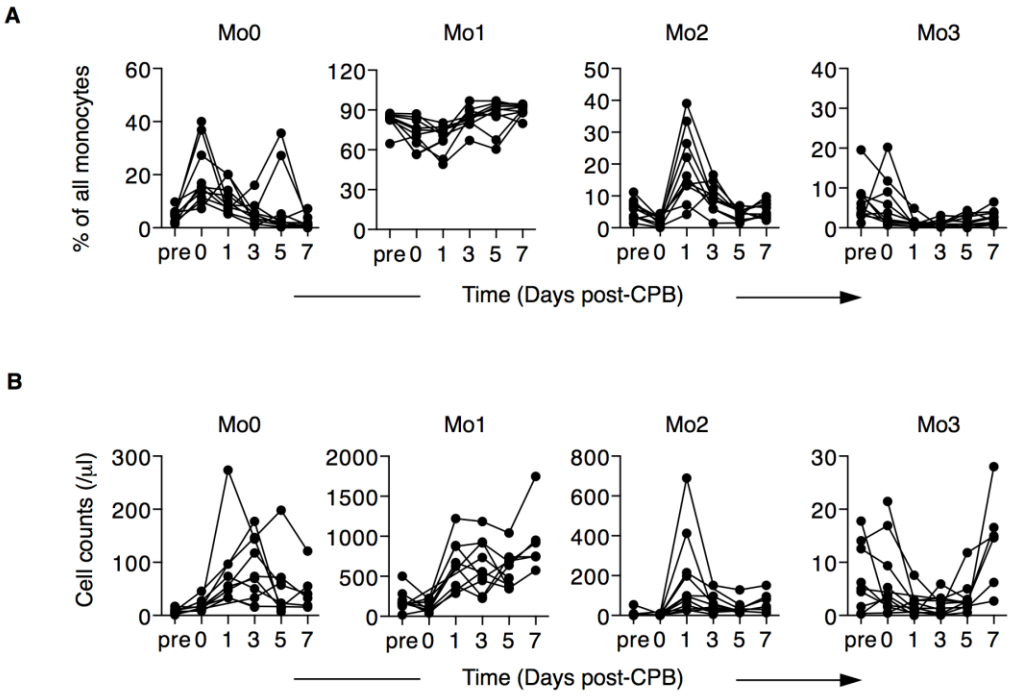

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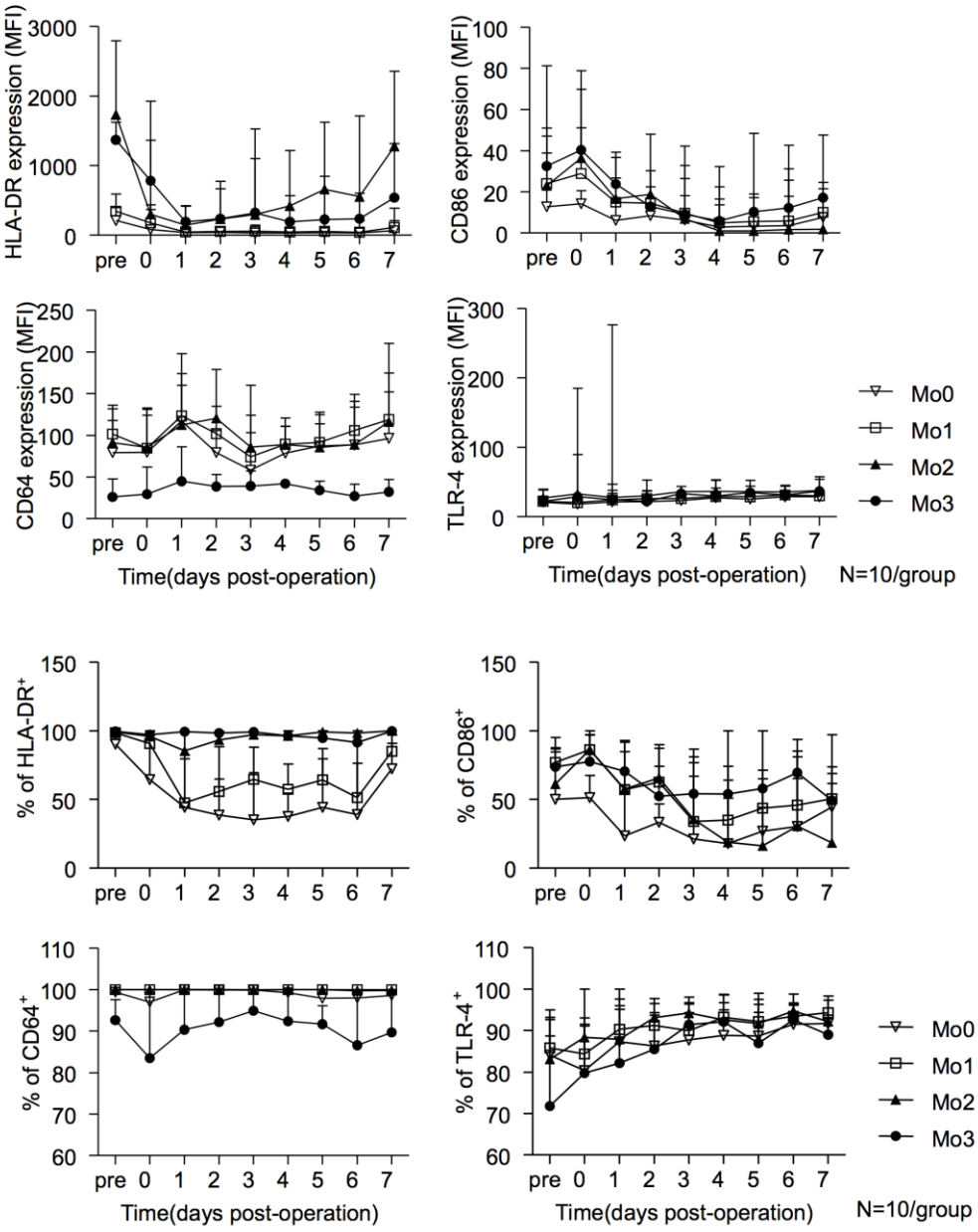

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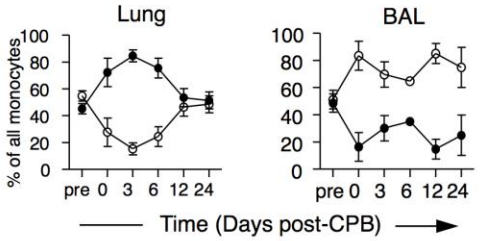

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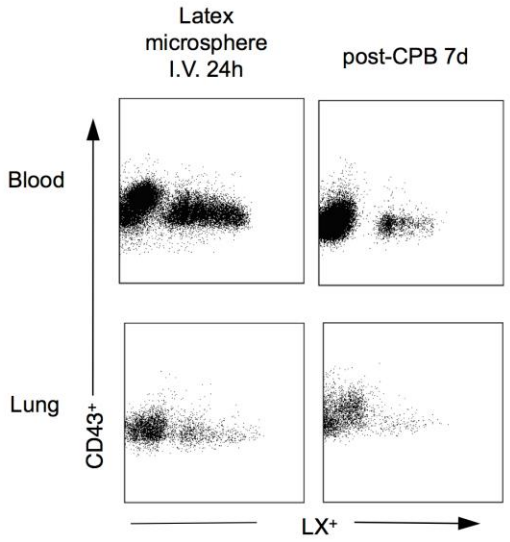

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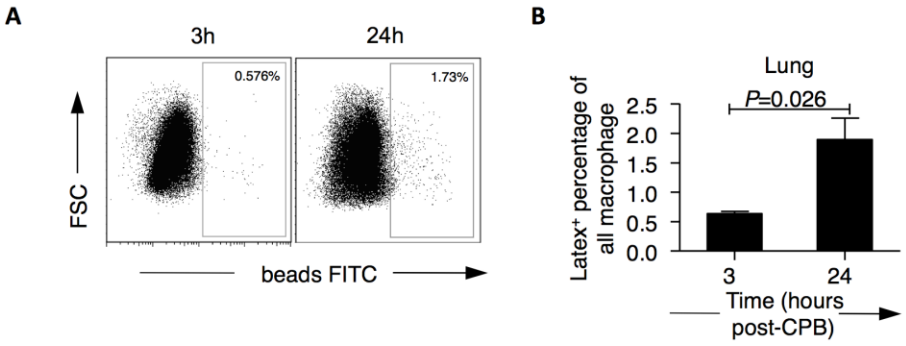

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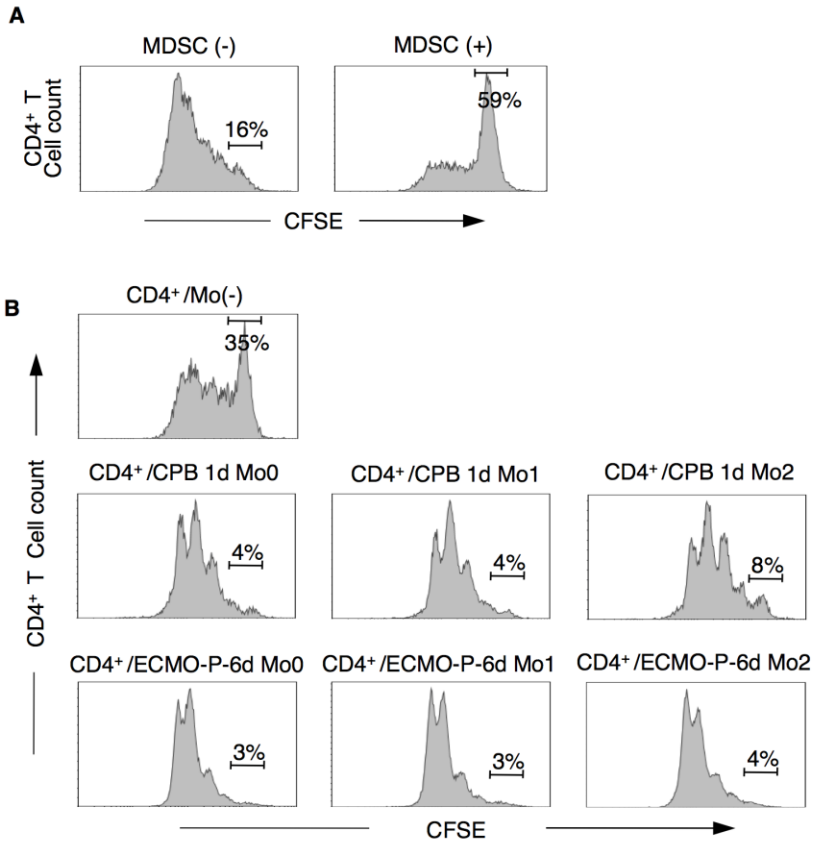

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Supplementary Figure 9