

## ***In vivo* effects of horse and rabbit antithymocyte globulin in patients with severe aplastic anemia**

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Manuscript received on March 4, 2014. Manuscript accepted on May 30, 2014.

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## **Supplementary Materials**

**Cytokine Analysis:** The levels of platelet-derived growth factor (PDGF), interleukin 1 receptor antagonist (IL-1ra), IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, basic fibroblast growth factor (FGFb), eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon  $\gamma$  (IFN $\gamma$ ), CCL2, CCL3, CCL4, CCL5, Interferon  $\gamma$ -induced protein 10 (IP-10), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), and vascular endothelial growth factor (VEGF) in the plasma at different time points were measured using magnetic multiplex assays simultaneously according to the manufacturer's instructions (Bio-rad Laboratories, Hercules, CA).

**Enzyme-linked immunosorbent assay (ELISA) for detection of ATG concentrations in the plasma:** ELISA plates (Nunc, Roskilde, Denmark) were coated with rabbit anti-horse IgG (Fab'2) (LifeSpan BioSciences, Seattle, WA) or chicken anti-rabbit IgG (10  $\mu$ g/mL, Genetex, Irvine, CA) over night at 4 $^{\circ}$ C. To prevent non-specific binding, the plates were blocked with 10% fetal calf serum/phosphate buffered solution (FCS/PBS) for 1 hour at room temperature. Fifty  $\mu$ L plasma samples diluted 1:1000 with 1% bovine serum albumin (BSA)/PBS were added to each well in duplicate and incubated at room temperature for 1 hour. Series diluted hATG or rATG were used as standards. Plates were washed 5 times with PBS containing 0.05% tween-20 (PBST), and then 100  $\mu$ L of diluted biotin-conjugated goat anti-horse IgG (Fab'2) antibody or biotin-

conjugated goat anti-rabbit IgG (1:18,000. LifeSpan BioSciences) were added to all wells and incubated for 1 hour. One hundred  $\mu\text{l}$ /well 1:250 diluted avidin-horseradish peroxidase. (eBioscience, San Diego, CA) were added and incubated for 30 minutes. Plates were washed for 7 times followed by addition of 100  $\mu\text{l}$  Tetramethylbenzidine (TMB, eBioscience) Substrate Solution and incubated until color development. One hundred  $\mu\text{l}$  2N  $\text{H}_2\text{SO}_4$  were added to stop the reaction and the optical density (O.D.) at 450 nm measured with a microtiter plate reader (Victor 3, PerkinElmer, Waltham, MA).

**ELISA for detection of anti-ATG antibody titers in the plasma:** ELISA plates were coated with r-ATG or h-ATG (10  $\mu\text{g}/\text{mL}$ ) over night at 4°C. After blocking, 50  $\mu\text{L}$  plasma samples diluted at 1:200 with 1% BSA/PBS were added to each well in duplicate and incubated at room temperature for 1 hour. One hundred  $\mu\text{L}$  of diluted peroxidase-conjugated goat anti-human IgG antibody (1:20,000. Calbiochem, La Jolla, CA) were added to all wells and incubated for 1 hour. Alternatively, peroxidase-conjugated goat anti-human IgA- or IgM-specific antibodies were used to detect isotype specificity. TMB substrate solution was used for color development.

**Western blotting:** One  $\mu\text{l}$  of plasma obtained from patients at different time points was electrophoresised, transferred onto polyvinylidene difluoride membrane. Membranes were blocked in 3% milk/TBS, and incubated with peroxidase-conjugated anti-horse IgG or anti-rabbit IgG (1:1000. LifeSpan BioSciences). After washing, membranes were reacted with Super-Signal chemiluminescent reagent (Pierce, Rockford, IL) and then exposure to X-ray film.

**Flow cytometry analysis:** To detect affinity of ATGs in the plasma of ATG-treated patients to lymphocytes, peripheral blood mononuclear cells (PBMCs) from healthy controls were stained with allophycocyanin (APC)-conjugated anti-human CD3 antibody or other surface makers first.

Then 10  $\mu$ L of patients' plasma obtained at different time points were incubated with PBMCs at room temperature for 30 min. After washing, PBMCs were stained with fluorescein isothiocyanate (FITC) -conjugated goat anti-horse IgG (Fab'2) antibody or anti-rabbit IgG (LifeSpan BioSciences). Finally, samples were subjected to flow cytometry.

To quantify the recovery of different immune cells during and after ATG treatment, PBMCs of available patients at different time points were thawed, washed, counted, and re-suspended in phosphate buffered saline (PBS). Viability staining was performed for 30 min in presence of LIVE/DEAD Aqua fixable viability dye (Invitrogen, Carlsbad, CA) followed by washing in staining buffer (PBS supplemented with 1% normal mouse serum, 1% goat serum and 0.02% sodium azide; Gemini Bioproducts, West Sacramento, CA). Cell membranes were then stained at room temperature for 30 minutes with the following directly conjugated monoclonal antibodies: anti-CD45 quantum dot (QD) 800 (Invitrogen), anti-CD3 APC-H7 (BD Biosciences), anti-CD4 v450 (BD Biosciences), anti-CD8 QD 605 (Invitrogen), anti-CD 38 Pacific Blue (BD Biosciences), and anti-CD19 QD 655 (Invitrogen). Cells were then washed three times with FACS staining buffer and were acquired the same day. Data were acquired on a Becton Dickinson LSR II. Data were acquired using DIVA 6.1.2 software (BD, San Jose, CA) and the analysis was performed using Flowjo 9.3 (Treestar Inc., San Carlos, CA). For the analysis, lymphocytes were identified based on their forward and side scatter properties. Subsequently, dead cells were excluded through the use of an aqua viability dye. T cells were identified by expression of CD3 and CD45 among previously selected viable lymphocytes.