Interleukin-6 receptor-alpha signaling drives anti-RBC alloantibody production and T-follicular helper cell differentiation in a murine model of red blood cell alloimmunization

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MATERIALS AND METHODS

Mice

C57BL/6J, IL-6KO(B6.129S2-*Il6*^{tm1Kopf}/J), FVB, RosaTdtomato(B6.Cg*Gt(ROSA) 26Sor*$^{tm14(CAG-tdTomato)Hze}$/J), CD4-CRE(Tg(Cd4cre)1Cwi/BfluJ), CD45.2^{+}OT-II(B6.Cg-Tg(TcraTcrb425cbn), and CD45.1^{+}B6(B6.SJL) mice were purchased from Jackson Laboratory. FVB.HOD mice were generated as previously described[1]. IL-6Rα knock-out (IL-6Rα^{KO}) and IL-6Rα-flox mice were a kind gift from Dr. Angela Drew (University of Cincinnati) and were generated as described previously[2]. To generate IL-6Rα^{TKO} mice, IL-6Rα-flox mice were first crossed with RosaTdTomato mice for eventual determination of cell-specific CRE activity. IL-6Rα-flox/Rosa26TdTomato mice were then crossed with CD4-CRE mice for T cell specific deletion of IL-6Rα. CD45.1^{+} OT-II mice were generated through crossing CD45.2^{+} OT-II mice with CD45.1^{+} B6.SJL mice. IL-6Rα^{KO} OT-II mice were generated by crossing IL-6Rα^{KO} mice with CD45.2^{+} OTII mice. CD45.1^{+}CD45.2^{+} heterozygous mice were generated by crossing C57BL/6J (CD45.2^{+}) mice with CD45.1^{+} B6.SJL mice. All mouse protocols were approved by the Institutional Animal Care and Use Committees of Harvard Medical School and University of Virginia, Charlottesville.

Both IL-6Rα^{KO} and IL-6Rα^{TKO} mice show no significant differences from WT C57BL6/J mice in baseline serum IgG levels and B and T lymphocyte frequencies. Additionally, these mice do not appear to have other recognizable gross abnormalities when housed under specific pathogen free conditions.

Murine blood collection, storage, and transfusion

Collection, processing, and storage of FVB.HOD blood was performed as described previously[3]. Briefly, blood from FVB.HOD mice was collected aseptically directly into the anticoagulant citrate phosphate dextrose adenine solution (CPDA-1, Boston Bioproducts) through cardiac puncture. The final CPDA-1 concentration was kept at 14% (v/v). Collected HOD blood was leukoreduced using a neonatal leukoreduction filter (Purecell Neo, Pall Corporation, Port Washington, NY). Leukoreduced blood was centrifuged at 1200 X *g*, adjusted to a final hematocrit level of 75%, and aliquoted in 500µl units in Eppendorf tubes. Some of the aliquots were stored at 4°C for 7-10 days (stored HOD RBCs) and the rest were kept at room temperature and used for transfusions within a few hours (fresh HOD RBCs).

Serum cytokine measurements

Serum from recipient mice was collected 90 minutes and 14 days post-transfusion for cytokines and anti-HOD antibody measurements respectively. Cytokine levels were measured using the 32-plex mouse cytokine panel (EMD Millipore) and analyzed via Luminex MAGPIX (Luminex).

Serum antibody measurements

Measurement of anti-HOD alloantibody production via ELISA

Antibody responses to transfused HOD RBCs are directed against the HEL antigen[1], and anti-HEL antibody levels were measured via limiting dilution titers on high binding elisa plates in order to maximize the minimal detectable signal and overall dynamic range of our assay. This allowed reproducible detection of anti-RBC antibodies generated in response to fresh HOD transfusion that were clearly above untransfused controls. Serum from transfused mice was collected 14 days post-immunization. To determine anti-HOD IgG titers, ELISA (High Binding, Corning) plates were coated overnight at 4°C with 10μg/ml Hen Egg Lysozyme (Sigma-Aldrich) in PBS. Plates were then washed and incubated with blocking buffer (2% BSA and 0.05% Tween-20 in PBS) for 1h at 37˚C. Serum samples were serially diluted (starting at 1:200 for stored blood and 1:50 for fresh blood and diluted by a factor of 4 each time for a total of 12 dilutions) in blocking buffer and incubated in the coated plates for 2h at room temperature. Horseradish peroxidase-conjugated goat anti-mouse IgG Fcγ specific antibody (Jackson ImmunoResearch) was then used as a secondary stain at a 1:5000 dilution. Wells were developed using TMB substrate and quenched with 2N H_2SO_4 after 10min. Optical densities were measured at 450nm and end-point titers were calculated using GraphPad Prism through interpolation of the cutoff value from the fit of the optical density vs. (1/serum dilution) curve for each sample using the "plateau followed by one-phase exponential decay" model. The cutoff value was defined as the average plus three standard deviations of signals from background wells (i.e. signal values from wells incubated with blocking buffer alone).

Anti-HOD flow cytometry crossmatch

Serum collected two weeks post-transfusion was diluted 1:5 with FACS buffer (PBS supplemented with 0.5% BSA, 2% fetal calf serum, and 0.1% sodium azide) and incubated with target HOD RBCs or control, antigen-negative, wild-type FVB RBCs for 30 minutes at room temperature. Freshly isolated HOD and FVB RBCs were used at a concentration of 1% (v/v) in FACS buffer. After primary incubation, RBCs were stained with goat anti-mouse IgG secondary antibody conjugated to APC (BD Biosciences) at a dilution of 1:100 for 30 minutes at room temperature. Samples were analyzed using the LSR II flow cytometer (BD Biosciences) and data were analyzed using FlowJo software (Tree Star, Ashton, OR) and GraphPad Prism. Median fluorescence intensity (MFI) of control FVB RBCs crossmatched with serum from transfused mice was used to define the background MFI for each serum sample. The difference between MFIs of control FVB RBCs and target HOD RBCs, defined as adjusted median fluorescence intensity, was used as a measure of relative anti-HOD antibody levels.

Adoptive transfer and flow cytometry

Adoptive Transfer

Single splenocyte cell suspensions from 8-10 weeks old donor OT-II mice were prepared using gentle mechanical disruption followed by RBC lysis with AcK buffer (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA). Naive $CD4^+$ T cells were isolated through negative selection using the mouse naïve $CD4^+$ T cell isolation kit (Miltenyi Biotec). Purified OT-II cells were suspended in PBS and 5000 cells/mouse were injected into recipient mice through the retro-orbital sinus. For the WT and IL-6Rα^{KO} OT-II mix experiment, both WT and IL-6Rα^{KO} OT-II cells were resuspended independently in PBS at a concentration of $5X10^4$ cells/ml and then mixed 1:1. Recipient WT $CD45.1^+CD45.2^+$ heterozygous C57BL/6J were injected with 100µl of the mixed OT-II cells through retro-orbital injections resulting in the transfer of 2500 cells/mouse each of WT and IL-6RαKO OT-II cells (5000 total OT-II cells/mouse).

T follicular helper cell staining

Recipient mice were transfused with HOD RBCs 48 hours following adoptive transfer. Spleens were harvested 4 days post-transfusion and splenocytes prepared as described above. TFH stains were performed as previously described[4]. Briefly, to determine the number of TFH cells defined as $CD4^+CXCR5^{hi}PD\text{-}1^{hi}$, splenocytes were first stained with biotinylated rabbit anti-mouse CXCR5 antibody (1:50), followed by staining with biotinylated goat anti-rabbit IgG (2µg/ml), and finally cells were stained with streptavidin brilliant violet (BV) 421 (1:100) along with anti-CD4 BV510 (1:100), anti-CD8 PerCP-Cy5.5 (1:200), anti-B220 PerCP-Cy5.5 (1:200), anti-PD-1 APC (1:50), anti-CD44 PE-Cy7 (1:200), anti-CD45.1 FITC (1:100), and anti-CD45.2 PE (1:100). For determination of TFH cells defined as $CD4^+CXCR5^{hi}Bcl6^{hi}$, splenocyte surface staining was performed as described above, except for the exclusion of anti-PD-1 from the final antibody cocktail. The stained cells were then fixed, permeabilized, and stained intracellularly with either Alexa-647 conjugated anti-Bcl6 antibody (1:50) or Alexa-647 mouse IgG1κ isotype control (1:50) using the Foxp3 nuclear staining kit (eBioscience) according to manufacturer's protocol. Cells were analyzed using either the LSR II flow cytometer (BD Biosciences) or FACS Canto II cytometer (BD Biosciences) and data were analyzed using FlowJo and GraphPad Prism.

Surface IL-6Rα and intracellular pSTAT3 staining

For surface IL-6Rα staining of $CD4^+$ T and B lymphocytes, peripheral blood from C57BL/6J, IL-6Rα^{KO}, and IL-6Rflox/CD4CRE (IL-6Rα^{TKO}) mice was collected via sub-mandibular bleeding and RBCs were lysed using AcK lysis buffer. Leukocytes were stained with a combination of anti-CD4 Alexa-488 (Biolegend), anti-B220 BV421 (Biolegend), and mouse anti-IL-6Rα APC conjugated antibody (R&D Systems). For intracellular pSTAT3 staining in response to IL-6 stimulation, single splenocyte suspensions from C57BL/6J, IL-6R^{KO}, and IL-6Rα^{TKO} were prepared as described above. Splenocytes were re-suspended at a concentration of $2X10^6$ cells/ml in RPMI-1640

supplemented with 5% FBS and stimulated with a saturating dose (200ng/ml) of murine IL-6 (Peprotech) for 15 minutes at 37˚C. Splenocytes were then fixed in 1.5% paraformaldehyde for 10 minutes at room temperature, followed by permeabilization with Phosflow Perm Buffer III (BD Biosciesnces) for 30 minutes on ice. Fixed cells were stained with anti-CD4 Alexa-488, anti-B220 BV421, and either Alexa-647 conjugated mouse anti-pSTAT3 (pY705) (BD Biosciences) or Alexa-647 conjugated mouse IgG2aκ isotype control (BD Biosciences) for 30 minutes at room temperature. Stained cells were subsequently analyzed via flow cytometry as described above.

Statistical Analyses

Data were compared using Mann-Whitney U test with P values less than 0.05 considered as significant. For comparisons of more than one group, one-way Kruskal-Wallis analysis of variance test was initially performed followed by Mann-Whitney U test for post-hoc comparisons. All statistical tests were performed using GraphPad Prism software.

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SUPPLEMENTARY FIGURE LEGENDS

Supplementary figure 1. Flow cytometry gating strategies and staining controls for characterization of IL-6Rα levels and downstream signaling in WT mice. Figures (A) and (B) depict gating strategies for determination of cell specific (A) surface IL-6Rα levels and (B) intracellular pSTAT3 levels by first gating on lymphocytes via forward and side scatter properties, followed by gating on single cells via comparison of forward scatter width and area, and finally gating on $CD4^+$ T cells and $B220^+$ B cells through surface staining of CD4 and B220 levels respectively. (C) Representative flow cytometry histograms demonstrating the specificity of intracellular anti-pSTAT3 staining in T cells (top panel) and B cells (bottom panel). WT C57BL/6J mouse splenocytes were stimulated with 200ng/ml murine IL-6 for 15min at 37˚C. Splenocytes were then fixed, permeabilized, and stained with either an anti-pSTAT3 or the corresponding Isotype control antibody. Shaded histograms indicate IL-6 stimulated cells stained with the indicated antibody, and dashed histograms indicate the corresponding unstimulated cell population stained with the anti-pSTAT3 antibody.

Supplementary figure 2. Characterization of cell-specific IL-6Rα levels and downstream signaling in IL-6Rα mutant mice. Surface IL-6Rα levels and intracellular pSTAT3 levels in response to IL-6 stimulation in $CD4^+$ T cells and $B220^+$ B cells from WT, $IL\text{-}6R\alpha^{KO}$ (KO), and $IL\text{-}6R\alpha^{TKO}$ (TKO) mice were measured through flow cytometry. Representative flow cytometry histograms depicting surface IL-6Rα staining (top panel) and intracellular pSTAT3 staining (bottom panel) in (A) $CD4^+$ T cells and (C) $B220^+$ B cells from the indicated strains. Shaded and dashed histograms represent anti-IL-6Rα stained and the corresponding unstained populations respectively for IL-6Rα staining, and anti-pSTAT3 stained IL-6 stimulated and the corresponding unstimulated cells respectively for intracellular pSTAT3 staining. Median fluorescence intensity (MFI) of surface IL-6Rα levels on (B) $CD4^+$ T cells and (D) $B220^+$ B cells from the indicated strains along with the MFI from corresponding unstained (Un) cell populations.

Supplementary figure 3. Generation of anti-HOD alloantibody levels in wild-type and IL-6Rα mutant mice through flow cytometry crossmatch. Wild-type, IL-6RαKO (KO), and IL-6RαTKO (TKO) mice were transfused with fresh or stored HOD RBCs, and serum collected 14 days post-transfusion. Anti-HOD alloantibody levels were determined through flow cytometric crossmatch using 1:5 sera dilution. Figure shows combined results from 3 independent experiments. Bar on scatter plots represents median value. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

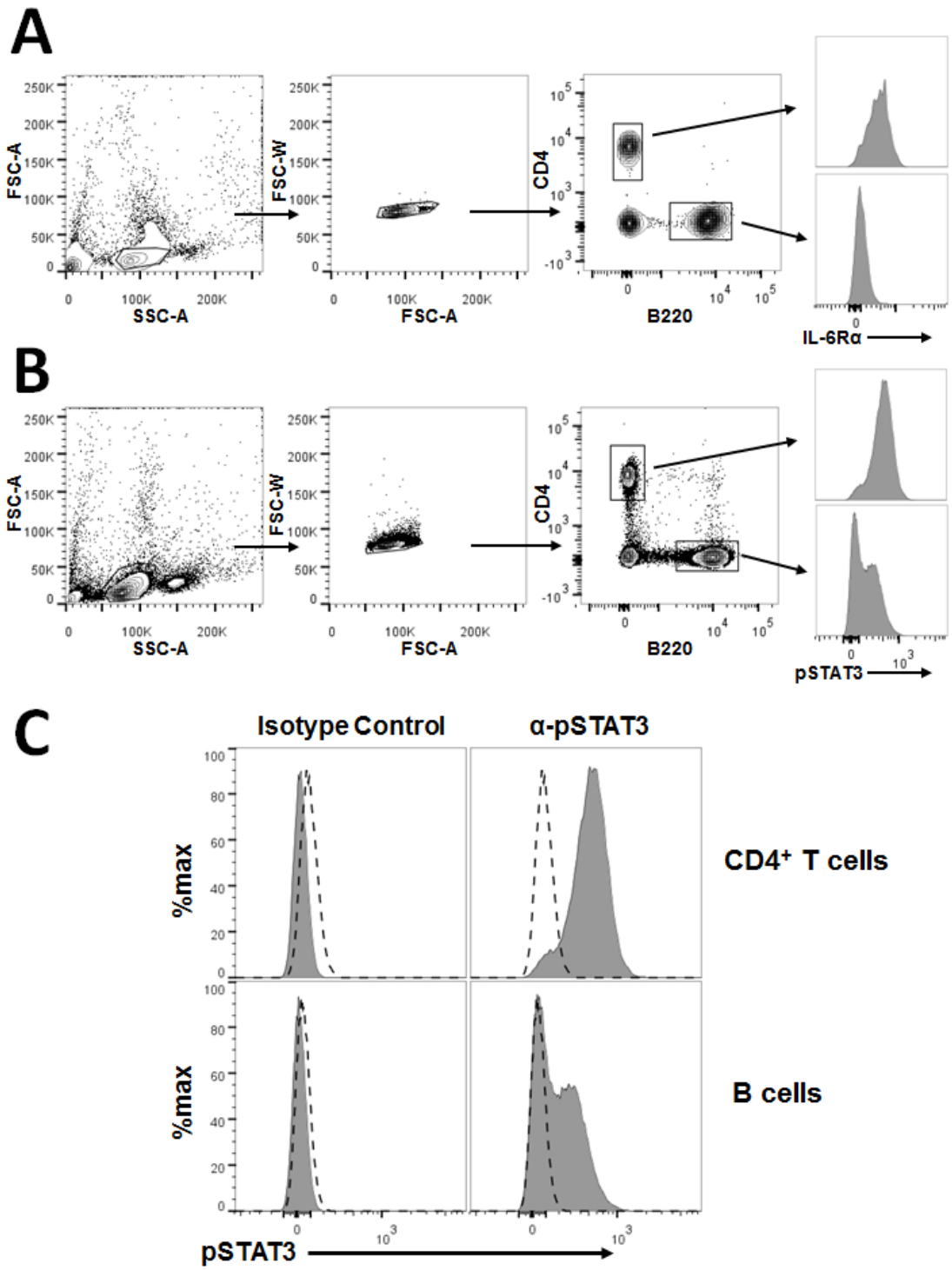
Supplementary figure 4. Serum pro-inflammatory cytokines that were either unchanged or undetectable in response to stored RBC transfusion in recipient mice. 20 cytokines (from a total of 32 cytokines measured via Luminex) whose expression either remain unchanged in response to transfusion with stored HOD RBCs or was undetectable in the assay are depicted. Individual plots show serum levels of inflammatory cytokines (as labeled) in WT and IL-6KO (6KO) C57BL/6J mice (aged 10-12 wks) 90min post-transfusion with stored HOD RBCs compared to untransfused (Un) WT and 6KO mice. Table depicts a list of cytokines that were included in the 32-plex Luminex assay but were undetectable in serum across all experimental conditions, with

the exception of LIX, which was present at saturating concentration outside the detection limit of the assay for all experimental conditions. Figure shows combined data from 3 independent experiments. Bars on scatter plots represent median values. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns = P > 0.05.

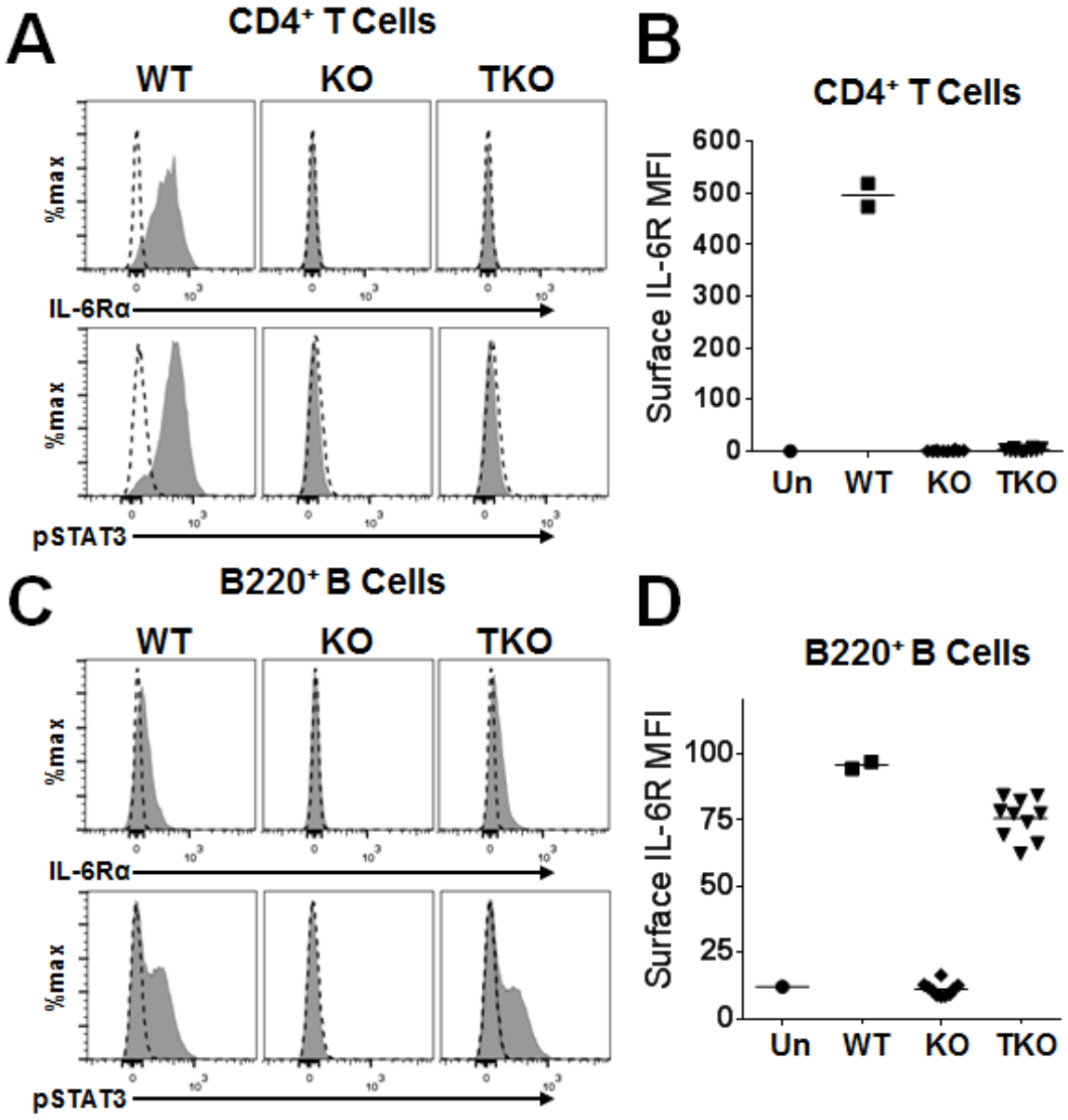
Supplementary figure 5. Representative flow cytometry figures and gating strategy for relative development of TFH cells in WT or IL-6Rα^{KO} OT-II cells in response to HOD RBC transfusion. (A) Gating strategy for determination of relative proliferation and TFH development in the adoptively transferred mixture of WT and IL-6R^{KO} OT-II cells in response to HOD RBC transfusions. Splenic lymphocytes were initially gated on through forward and side scatter properties, followed by gating on single cells using forward scatter width and area information to exclude doublets. Next, CD4^{+} T cells were gated on by selecting cells staining positive for CD4, and excluding cells staining positive for B220 and CD8. WT and IL-6RαKO OT-II cells were selected by gating on cells singly positive for CD45.1 and CD45.2 respectively while simultaneously excluding the endogenous CD4^{+} T cells double positive for CD45.1 and CD45.2. Figures (B) and (C) show representative flow cytometry contour plots depicting levels of (B) CXCR5^{hi}PD-1^{hi} TFH cells and (C) CXCR5^{hi}Bcl-6^{hi} TFH cells within the WT and IL-6R^{KO} (KO) OT-II cells in mice that were untransfused, transfused with fresh HOD RBCs, or transfused with stored HOD RBCs. (D) Representative flow cytometry contour plots showing WT OT-II cells stained with either anti-Bcl6 or the appropriate isotype control antibody from mice transfused with stored HOD RBCs. Results from isotype control staining were used to confirm the specificity of intracellular anti-Bcl6 staining in mouse splenocytes and further used to determine appropriate gating for CXCR5^{hi}Bcl-6^{hi} TFH cells within the OT-II cells.

SUPPLEMENTARY FIGURES

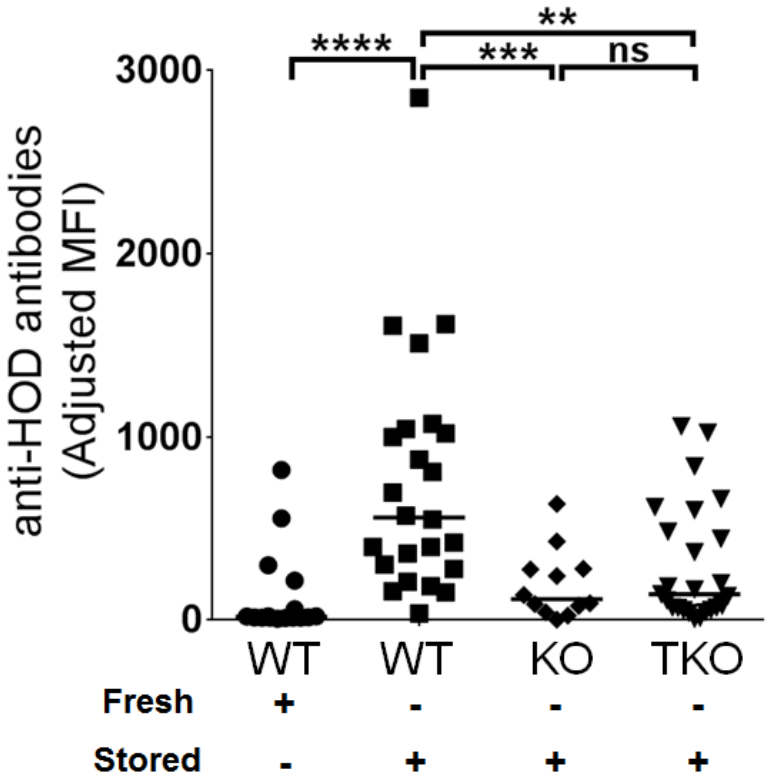
Supplementary Figure 1



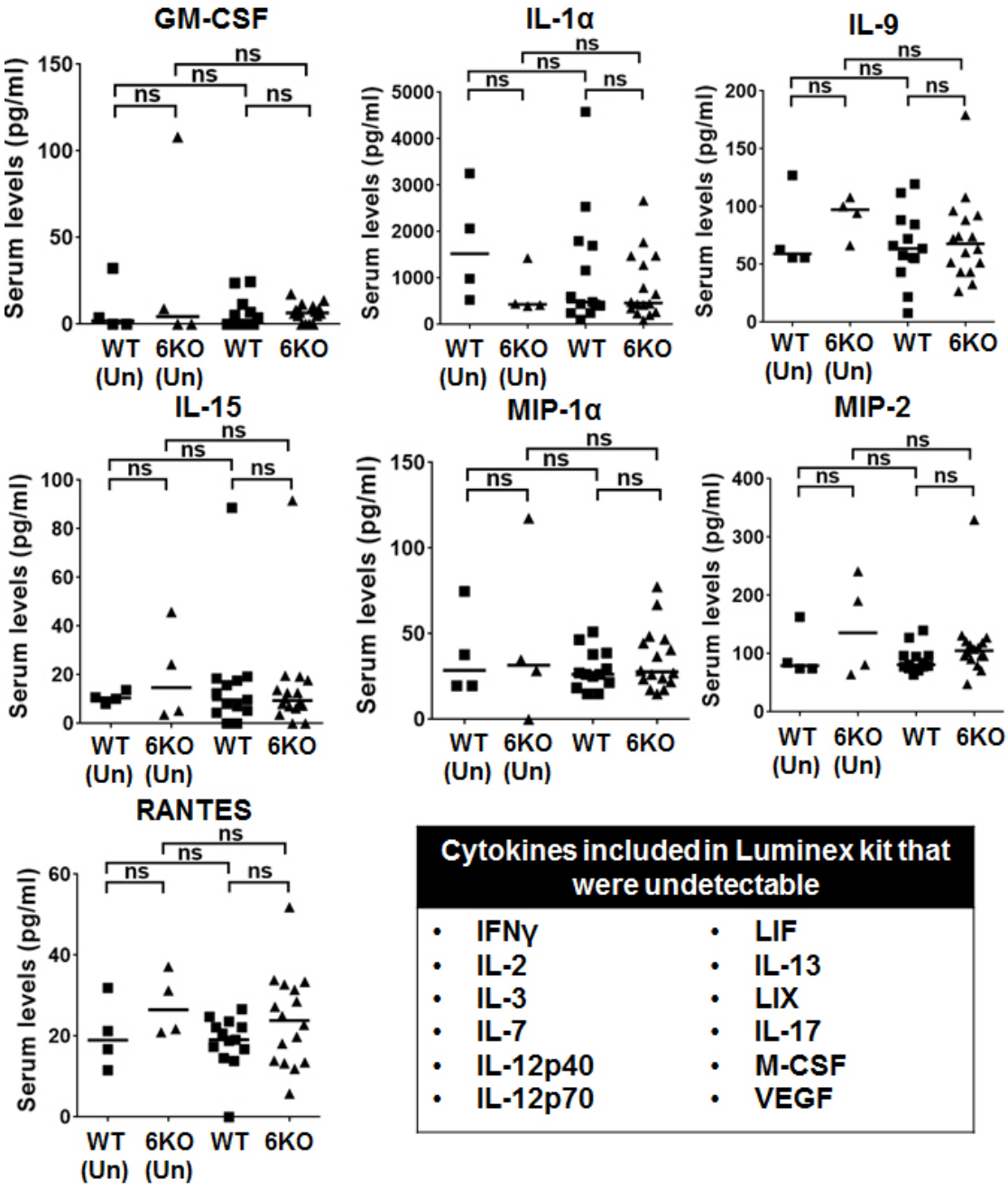
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5