Potential of membranes surrounding the fetus as immunoprotective cell-carriers for allogeneic transplantations

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Materials and methods:

10F7MN cell culture: 10F7MN hybridomas are formed from the fusion of Balb splenocytes with Sp2/0-Ag14 myeloma cells. 10F7MN cells were purchased from American Type Culture Collection (Manassas) and maintained in DMEM/F12 medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) at 37°C with 5% CO2 in a humidified incubator. Cell were cultured at a density of 1 x 10^6 cells/ml and sub-cultured thrice per week.

Isolation of human erythrocytes from total blood: Blood was collected from a healthy donor and was layered on top of LymphoprepTM (Stem Cell Technologies), and was subjected to density gradient centrifugation at 500 x g for 20 mins at room temperature. Erythrocytes depleted of light-density leukocytes were collected from the pelleted cells and were washed to remove any residual LymphoprepTM and blood constituents.

Mouse alloantibody screening assay: Splenocytes from 10-weeks-old Balb mice were collected and incubated with an anti-FcR blocking antibody (Biolegend). Subsequently cells were incubated with serum collected from syngeneic (Balb) and allogeneic (B6) mice transplanted with 10F7MN cells. Serum samples collected 3 weeks after 10F7MN cell-transplantation were used in this assay. After washing cells were stained with a biotin-conjugated anti-Igk antibody (BD Pharmingen) to detect allo-antibody binding. Cells were then washed and stained with streptavidin-allophycocyanin (BD Pharmingen). Cells were additionally stained with B220 and TCRβ antibodies (BD Pharmingen) to identify B and T cell populations, respectively. Stained samples were analyzed by flow cytometry and MFIs of Igκ staining on the TCRβ+B220- population was calculated as described above.

In vitro Mixed Lymphocyte Reaction (MLR) assay: MLR assay was performed with 1 x 10^5 B6 responder and Balb stimulator cells in a U-bottom 96-well plate. Cells were plated in duplicate and incubated at 37°C in 5% CO_2 for 4 days in medium consisting of RPMI 1640 medium containing 10% heat-inactivated FBS, 1% penicillin/streptomycin, 50 μM β-mercaptoethanol and 1% L-glutamine (all purchased from Gibco). Cells were stained with CD4 and CD8 antibodies

(Biolegend) and were analyzed by flow cytometry to determine the proliferation of responder T-cells.

In vitro Mixed Lymphocyte Reaction (MLR) assay: Splenocytes harvested from 8-10 weeks old B6 and Balb mice were used as responder and stimulator cells, respectively. To determine the proliferation of the T-cells, the responder B6 splenocytes were labeled with 15 μM carboxyfluorescein succinimidyl ester (CFSE) (Affymetrix) as described previously15. As a positive control to determine the T-cell proliferative response, Dynabeads™ Mouse T-Activator CD3/CD28 (Thermo Fisher Scientific) were used to stimulate the responder B6 splenocytes. To determine the immunosuppressive activity of membranes, the aforementioned experimental conditions were tested both in the presence and absence of membrane-derived cells. Membrane derived cells were plated at the equal and twice in number corresponding to the responder and stimulator cells. Cells were incubated at 37°C in 5% CO2 for 4 days. Details of the MLR assay has been described in Supplementary methods. Cells were stained with CD4 and CD8 antibodies (Biolegend) and were run on a flow cytometer. MLR assay was performed three times and samples were run in duplicate.

Cytokine detection assay: B6 and Balb splenocytes each at density of 1 x 10^5 cells were cultured in the presence and absence of syngeneic B6 membrane derived cells at 37°C in 5% CO_2 for 4 days in medium consisting of RPMI 1640 medium containing 10% heat-inactivated FBS, 1% penicillin/streptomycin, 50 μM β-mercaptoethanol and 1% L-glutamine (all purchased from Gibco). Membrane derived cells were also tested at 1 x 10^5 and 2 x 10^5 (2X) cells/well concentrations corresponding to the 1 x 10^5 responders and stimulators. After 96 hours, culture supernatants were collected and assayed using the Th1/Th2/Th9/Th17/Th22/Treg Cytokine 17-Plex Mouse ProcartaPlex™ Panel (Invitrogen) for targets IFNγ, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-5, IL-6, TNFα, GM-CSF, IL-18, IL-10, IL-17A, IL-22, IL-23, IL-27 and IL-9 cytokines following the manufacturer's protocol. Cytokines were analyzed and measured with the Luminex 200 platform (Luminex, Austin, TX) with BioManager Software (BioRad). The Luminex assay was performed three times, and standards and samples were run in duplicate.

Statistics: Data are presented as mean ± SEM. GraphPad Prism version 6.00 (GraphPad software, La Jolla, CA, USA) was used for all the statistical analyses. Nonparametric Mann-Whitney t-test was used for unpaired comparisons. The effect of membranes on the cytokine response was tested using one-way ANOVA with Dunnett's post test to compare all groups to B6 splenocyte+Balb splenocyte and B6 splenocyte+CD3/CD28 beads as control groups. Differences in the antibody responses among the various groups described in the alloantibody detection assay section was tested using two-way ANOVA using Tukey's multiple comparison test. $P \leq 0.05$ was considered significant.

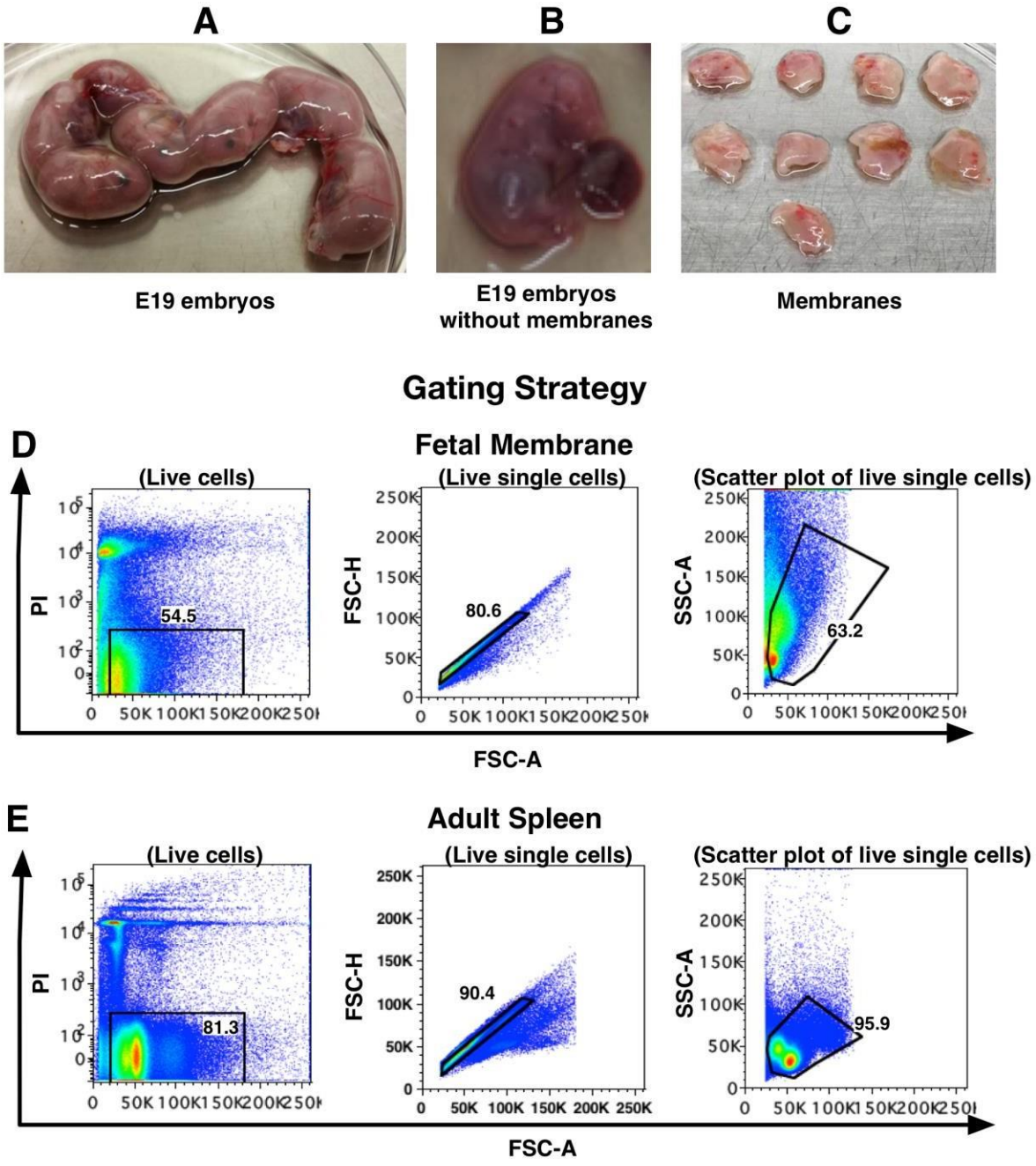

Figure S1: (A) E19 embryos with membranes; (B) E19 embryos without membranes; (C) Harvested membranes surrounding the embryos; (D and E) Gating strategy opted for flow cytometry analysis, showing live and single cells from membranes and adult mice spleen were selected for the subsequent downstream surface marker expression analysis.

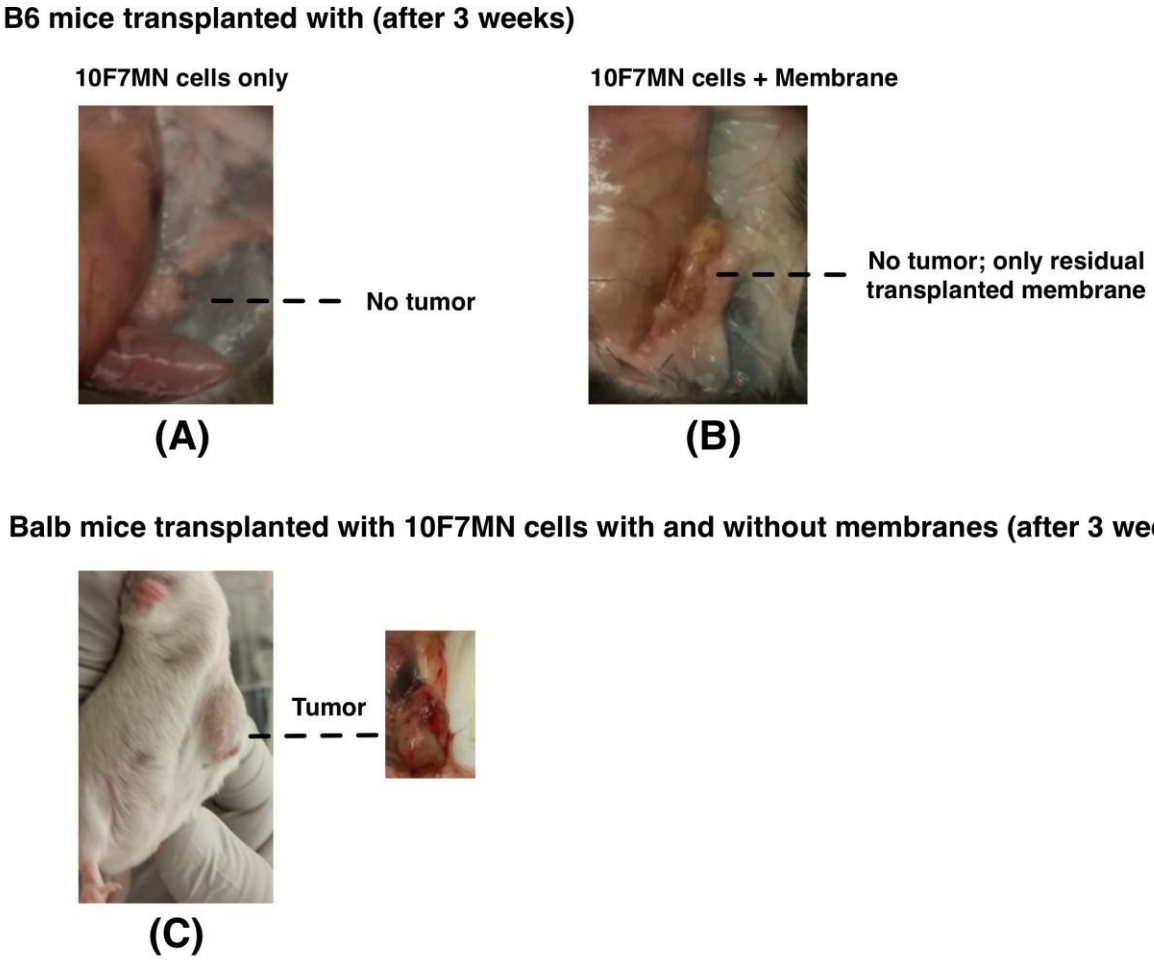

Figure S2: Demonstration of the phenotypic outcome of the 10F7MN hybridoma cells in Balb (syngeneic) and B6 (allogeneic) mice after 3 weeks of transplantation. Upper panel (A) 10F7MN cells in B6 mice formed no tumors (B) Co-transplantation of 10F7MN cells with membranes derived from B6 mice did not form tumors (C) 10F7MN cells in Balb mice formed tumors

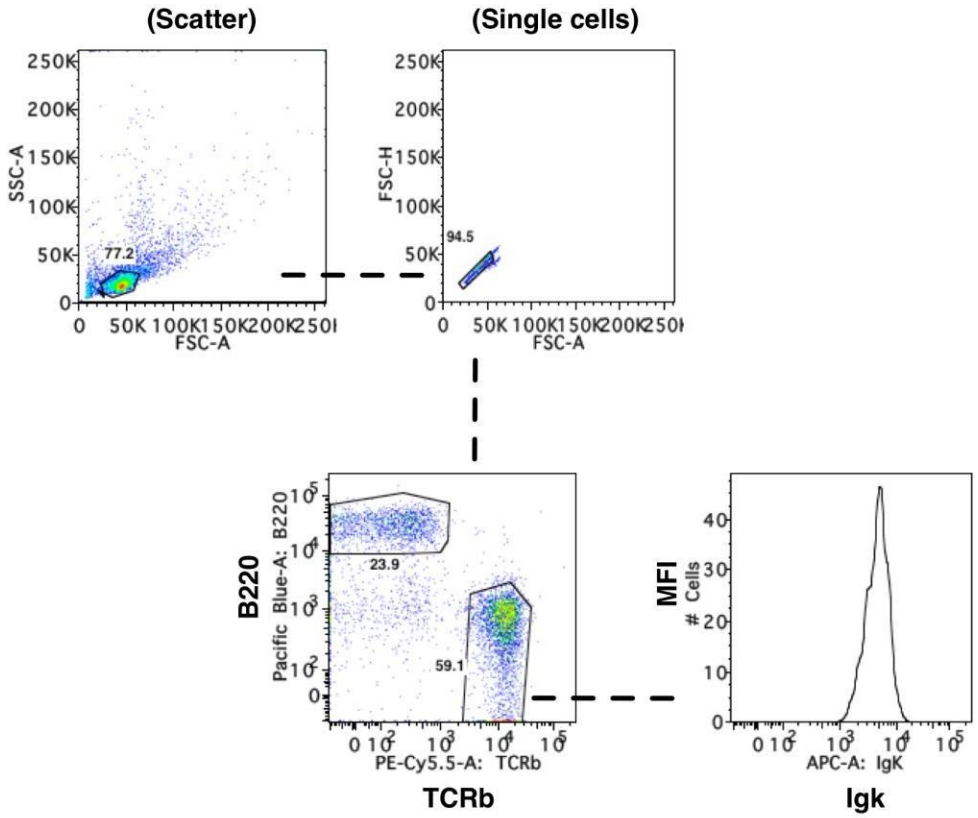

Figure S3A: Schematic representation of strategy employed to determine the median fluorescence intensity of IgK staining on single TCRb+ cells of Balb splenocytes

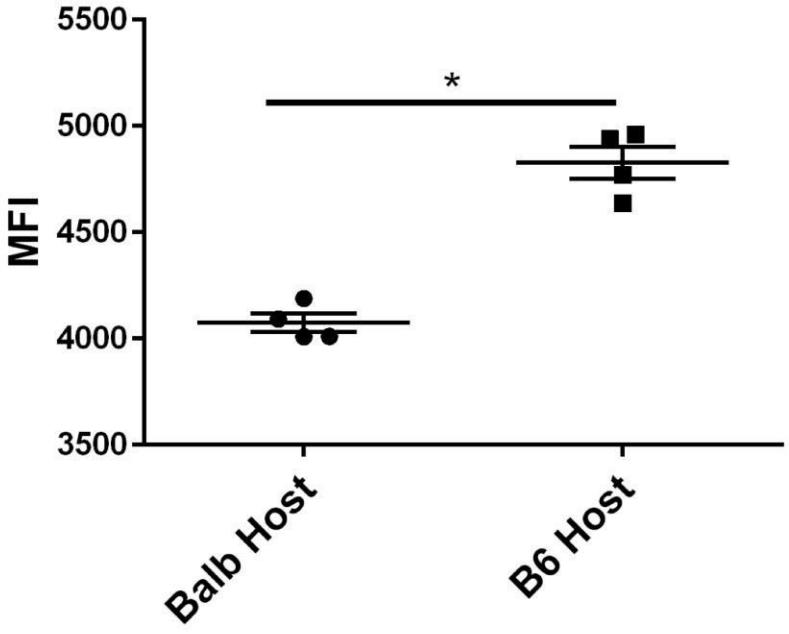

Figure S3B: Comparison of antibody responses against 10F7MN cells in Balb and B6 hosts

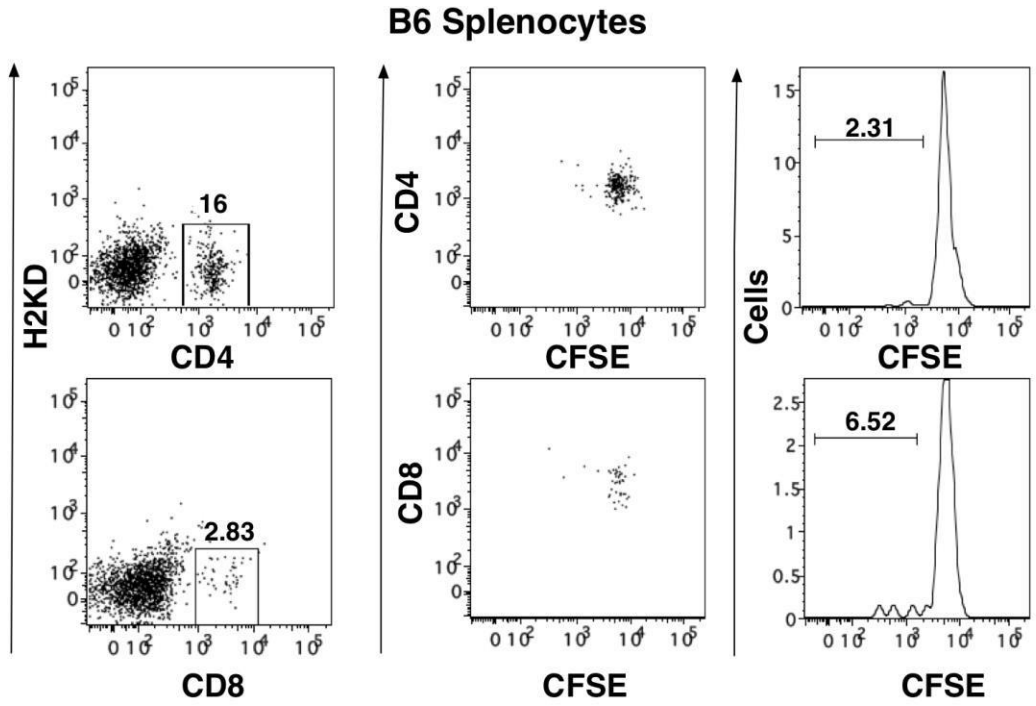

Figure S4: Representation of negative control for the mixed lymphocyte reaction (MLR) assay, showing responder B6 CD4 and CD8 T-cell proliferation rate in the absence of allogeneic stimulus

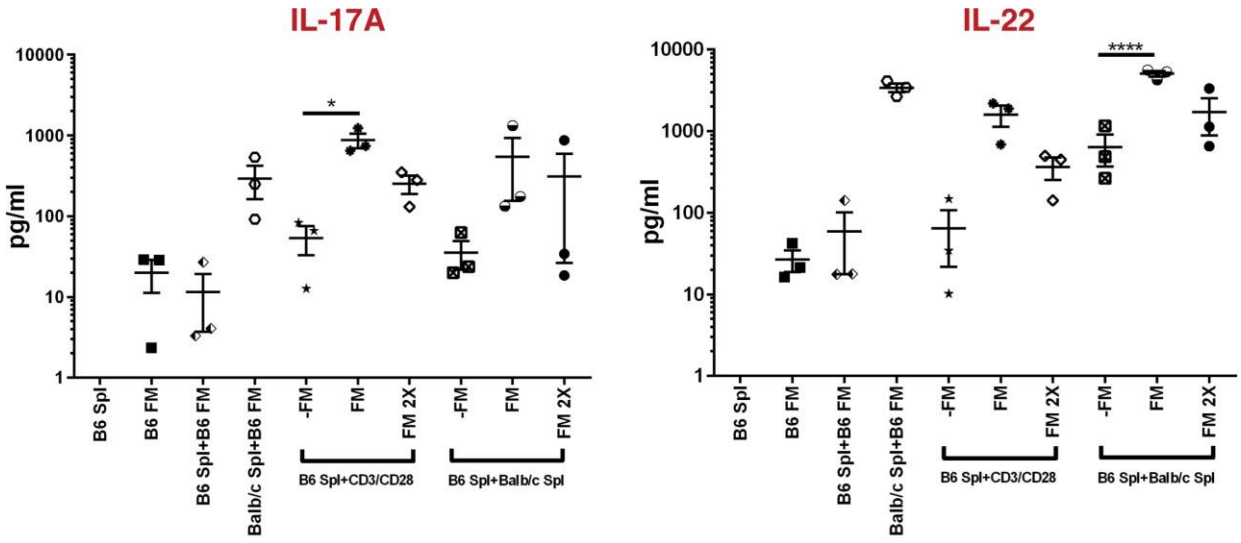

Figure S5A: Analysis of levels of IL-17A and IL-22 in the supernatants collected from mixed lymphocyte reaction assay

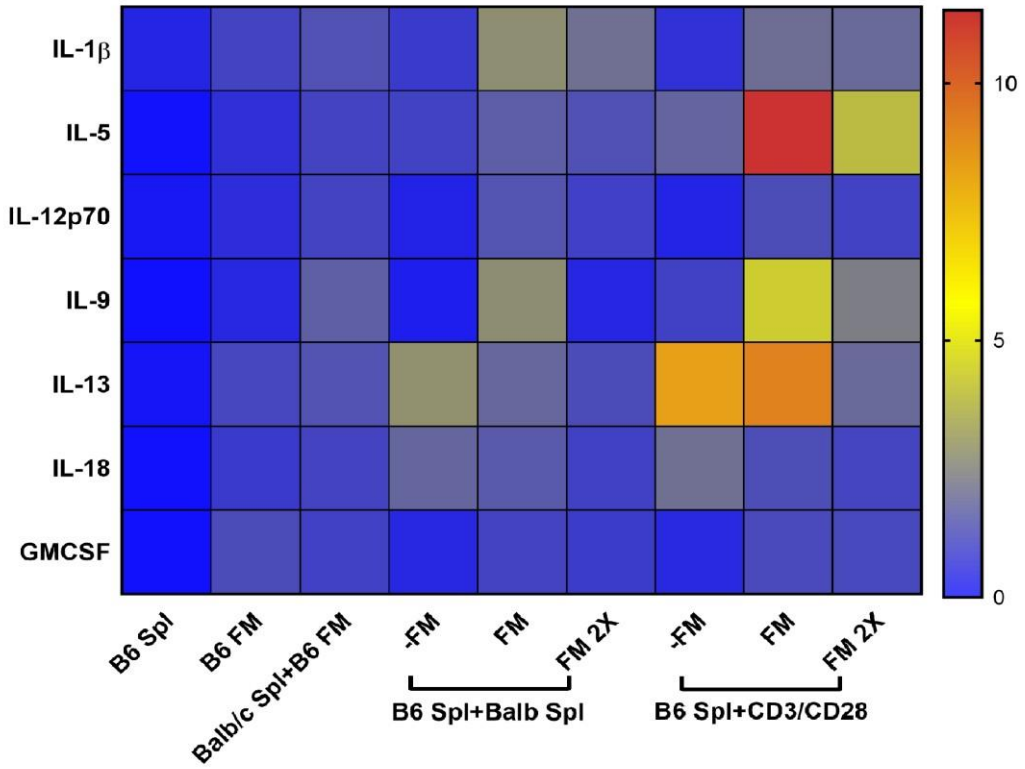

Figure S5B: Heat map showing cytokine levels in the supernatants collected from mixed lymphocyte reaction assay