Early Th1 immunity promotes immune tolerance and may impair graft-*versus*-leukemia effect after allogeneic hematopoietic cell transplantation

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Supplemental methods

Flow cytometric phenotype analysis of T cells.

After obtaining informed consent, peripheral blood mononuclear cells (PBMCs) were collected from healthy volunteers or from HCT recipients at the time of neutrophil engraftment (defined as the first of 3 consecutive days with an absolute neutrophil count ≥ 0.5 x 10^9/L) and at day+30 using Ficoll-Hypaque, as per an IRB-approved protocol. After thawing, cells were cultured with Leukocyte Activation Cocktail (Beckton Dickinson or BD; PMA, ionomycin, and brefeldin A) for 5 hours at 37°C in 5% CO_2. Surface staining was performed using amine viability dye (Invitrogen) and a mixture of antibodies with indicated fluorophore to the following targets: CD8 [PacOrange], CD4 [Alexa 700], cutaneous lymphocyte antigen (CLA) [FITC; BD], CD3 [Brilliant Violet 650; BioLegend], CD14 [PE-TR; Invitrogen], and α4β7 [PE; kind gift from Millennium Pharmaceuticals, Inc., which we conjugated]. Cells were fixed and permeabilized with Cytofix/Cytoperm (BD) followed by intracellular staining with antibodies to IFN-γ [Horizon V450], IL-17A [Alexa 647] or Foxp3 [Alexa 647], all from BD. Data were acquired using a LSRII flow cytometer (BD) and analyzed with FlowJo software version 10.0 (Tree Star) by a scientist (M.T.R.) blinded to the clinical information. Circulating Th1 cells (CD4+ IFN-γ+) and Th17 cells (CD4+IL-17+) were quantified. Circulating skin-homing (CLA+), gut-homing (α4β7+), and conventional (CLA-α4β7-) Th1 or Th17 subsets were identified and expressed as a percentage of the total Th1 or Th17 population, respectively (Supplemental Figure 1). Absolute cell numbers were calculated for patient samples only using percentages of cells from flow

cytometric analysis coupled with simultaneously acquired complete blood count data.

For PD1 and PD-L1 analyses, unstimulated PBMC's were surface stained for 30 minutes at room temperature with titrated antibodies to CD14 [PE-TR; Invitrogen], PD-L1 (CD274) [BV421; Biolegend], CD3 [PerCP-Cy5.5], CD4 [Alexa 700], and PD1 (CD279) [PE; BD]. The frequency of PD1 (CD279) expression on CD4+ T cells and PD-L1 (CD274) on CD14+ monocytes was determined using methods similar to those described for the Th1 / Th17 analyses (Supplemental Figure 2).

Soluble suppression of tumorigenicity 2 (sST2) analysis.

Serum samples were collected at the same time-points as PBMC isolation (engraftment and day+30). After centrifugation, serum samples were aliquoted and cryopreserved at -80°C until analysis. Serum sST2 levels were measured by an enzyme-linked immunosorbent assay (ELISA) protocol using a quantikine kit (R&D Systems).

Statistical analysis.

Differences were compared with the Mann-Whitney U test, Wilcoxon signed-rank test, or χ^2 for continuous / independent, continuous / dependent, or categorical variables, respectively. The relationship between 2 continuous variables was determined using Spearman's rank correlation coefficient. Cumulative incidence

curves were created for non-relapse mortality (NRM) (censored for relapse),

aGVHD (censored for death and relapse), and malignancy relapse (censored for

death in remission). Overall survival was estimated by the Kaplan-Meier Method.

Groups were compared using Cox Proportional Hazard Regression. A 2-tailed *P*-

value <0.05 was considered significant [IBM SPSS Statistics, Version 22 (IBM

Corp, Armonk, NY)].

Supplemental Figure 1. Identification of CLA+ (skin-homing) and α4β7+ (gut-homing) Th1 or Th17 cells using 8-color multiparametric flow cytometry. Peripheral blood mononuclear cells were stimulated for 5 hours with PMA/ionomycin in the presence of brefeldin A. Cells were surface-stained with an assay containing: CD14, CD3, CD4, CD8, CLA, α4β7, and amine viability dye, fixed and permeabilized and then intracellularly stained with IFN-γ and IL-17. Viable CD3+CD4+ T cells were identified (not shown) and quadrants were created using two-parameter comparisons assessing (A) IFN-γ vs. IL-17 and (B) CLA vs. $\alpha 4\beta 7$. IFN-γ+ Th1 cells or IL-17+ Th17 cells were isolated from the *total* CD4+ population. The gating strategy generated from panel B was then applied to the Th1 or Th17 populations to determine the percentages of CLA+ (skin-homing), α4β7+ (gut-homing), or CLA- α4β7- (conventional) subsets (panel C).
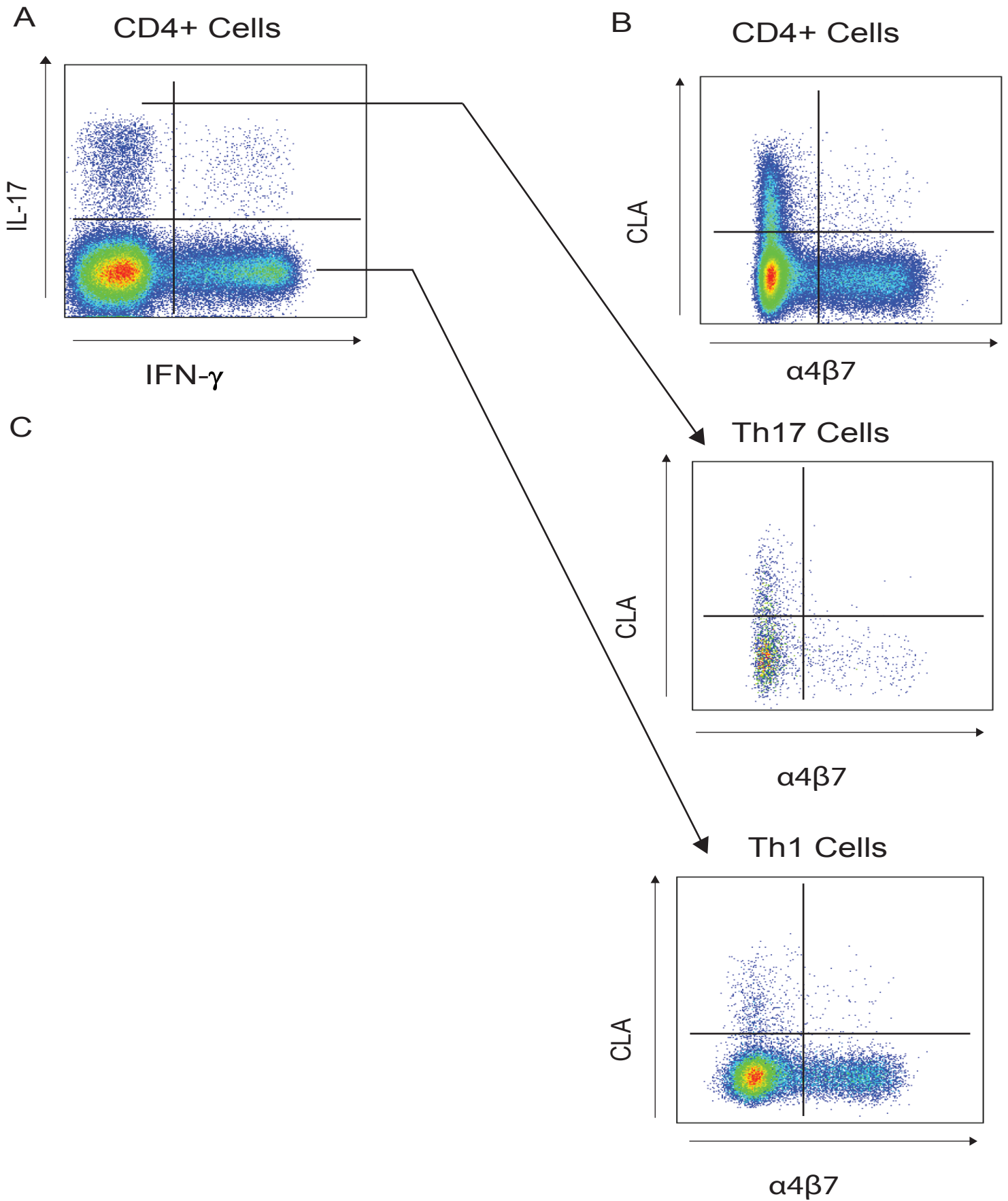
Supplemental Figure 2. Gating strategy for determining PD1 and PDL1 expression by CD4+ T helper cells and CD14+ monocytes, respectively. Fluorescence minus one (FMO) controls were created by staining peripheral blood mononuclear cells (PBMCs) with all reagents accept the antibody to PD1 (CD279). CD3+CD14- T cells were identified followed by gating on CD4+ cells (data not shown). A two-parameter comparison of CD4 vs. PD1 was then applied to the CD4+ population from the FMO control. Gates were created to clearly demarcate the PD1 negative population **(a)**. The gating strategy from panel **(a)** was then applied to the sample of interest to quantify the frequency of PD1 expression in the *total* CD4+ population [panel **(b)**]. Another FMO control was

created by staining PBMCs with all reagents accept the antibody to PDL1 (CD274). CD14+CD3- monocytes were identified (data not shown). A two-parameter comparison of CD14 vs. PDL1 then was applied to the CD14+ population from the FMO control. Gates were created to clearly demarcate the PDL1 negative population **(c)**. The gating strategy from panel **(c)** then was applied to the sample of interest to quantify the frequency of PDL1 expression in the *total* CD14+ population [panel **(d)**].

Supplemental Figure 3. Th1 cells do not express Foxp3. Peripheral blood mononuclear cells were cultured for 5 hours with PMA, ionomycin, and brefeldin A. Cells were stained with: CD14, CD3, CD4, and an amine viability dye. After fixation and permeabilization, cells were stained intracellularly with IFN-γ and Foxp3. Cells expressing the viability dye or CD14 were excluded and live CD3+CD4+ T cells were isolated (not shown). IFN-γ and Foxp3 expression were compared in the *total* CD4+ population.
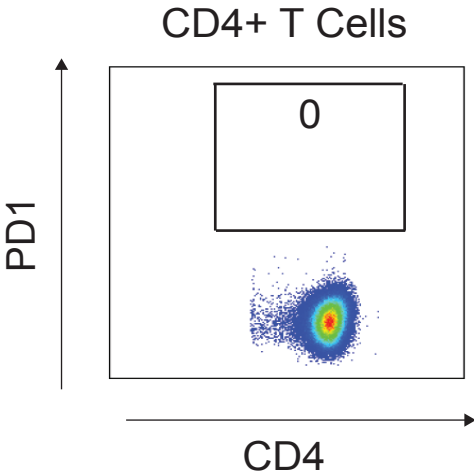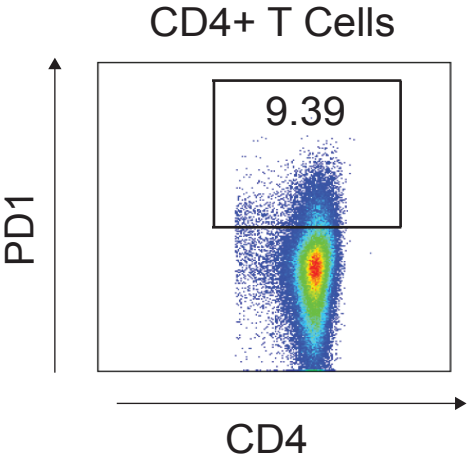
Supplemental Figure 1

A   CD4+ Cells

IL-17

IFN-γ

B   CD4+ Cells

CLA

α4β7

C

Th17 Cells

CLA

α4β7

Th1 Cells

CLA

α4β7

Supplemental Figure 2

A   Fluorescence Minus One
         Controls

CD4+ T Cells

PD1

0

CD4

B   Patient Samples

CD4+ T Cells

PD1

9.39

CD4

C

CD14+ Monocytes

PD-L1

0.06

CD14

D

CD14+ Monocytes

PD-L1

1.10

CD14

Supplemental Figure 3

A

CD4+ Cells

Foxp3

IFN-γ