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Supplementary Materials for

Inhibition of LTi Cell Development by CD25 Blockade Is Associated with Decreased Intrathecal Inflammation in Multiple Sclerosis

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The PDF file includes:

Supplementary Materials

Materials and Methods

Subjects

Five cohorts of patients and controls were utilized in the current study: 1. For the Influenza vaccination study, 6 daclizumab treated patients and 8 age/gender matched controls provided 10cc of blood EDTA prior to vaccination, were immediately vaccinated (NDC 19515-885-07, FluLaval^{TM} 2008/2009 formula, viral antigen specificity: A/Brisbane/59/2007 IVR-148, A/Uruguay/716/2007 NYMC X-175C, B/Florida/4/2006), and then provided a second 10cc blood EDTA ~7 days after vaccination. 2. The daclizumab trial cryopreserved peripheral blood mononuclear cells (PBMC) cohort consisted of 17 MS patients who participated in 2 NIH daclizumab clinical trials (clinical trial identifiers NCT00071838 and NCT00001934) and finished at least 12 months of daclizumab dosing. Lymphocytapharesis was collected and cryopreserved at baseline (before administration of daclizumab) and after 3 and 8 months of treatment with daclizumab. 3. Fresh PBMCs of patients who participated in DAC HYP trial Cohort B (NCT01143441) were collected prior to and 6 months after initiation of DAC HYP (Fig. 1C). 4. To assess functional changes in ILCs, 10cc of blood EDTA was obtained from 7 MS patients on long-term (>1 year) daclizumab therapy (now enrolled in DAC HYP trial Cohort A; NCT01143441) and 7 age/gender matched untreated MS patients. 5. Fresh PBMCs from 6 healthy donors, 17 untreated MS patients and 13 daclizumab-treated MS patients (DAC HYP Cohorts A & B) were used to investigate RORγt^{+}/c-kit^{+} LTi cell populations.

Antibodies

The following surface and intracellular flourochrome-conjugated antibodies from BD Bioscience, eBioscience, or R&D systems were used: CD3 (UCHT1), CD4 (RPA-T4), CD7 (eBio124-1D1), CD11c (B-Iy6), CD14 (M5E2), CD19 (HIB19), CD25 (7G7), CD34 (4H11), CD56 (B159), CD122 (Mik-β1), CD123 (7G3), CD127 (hIL7R-M21), CD161 (DX12), Granzyme A/B (CB9), IL-22 (142928), LTα (359-81-11), STAT5 (pY694, 47), TNFα (MAb11), RORγt(600380), c-kit(104D2), OX40L(11C3.1) and CD30L(116614).

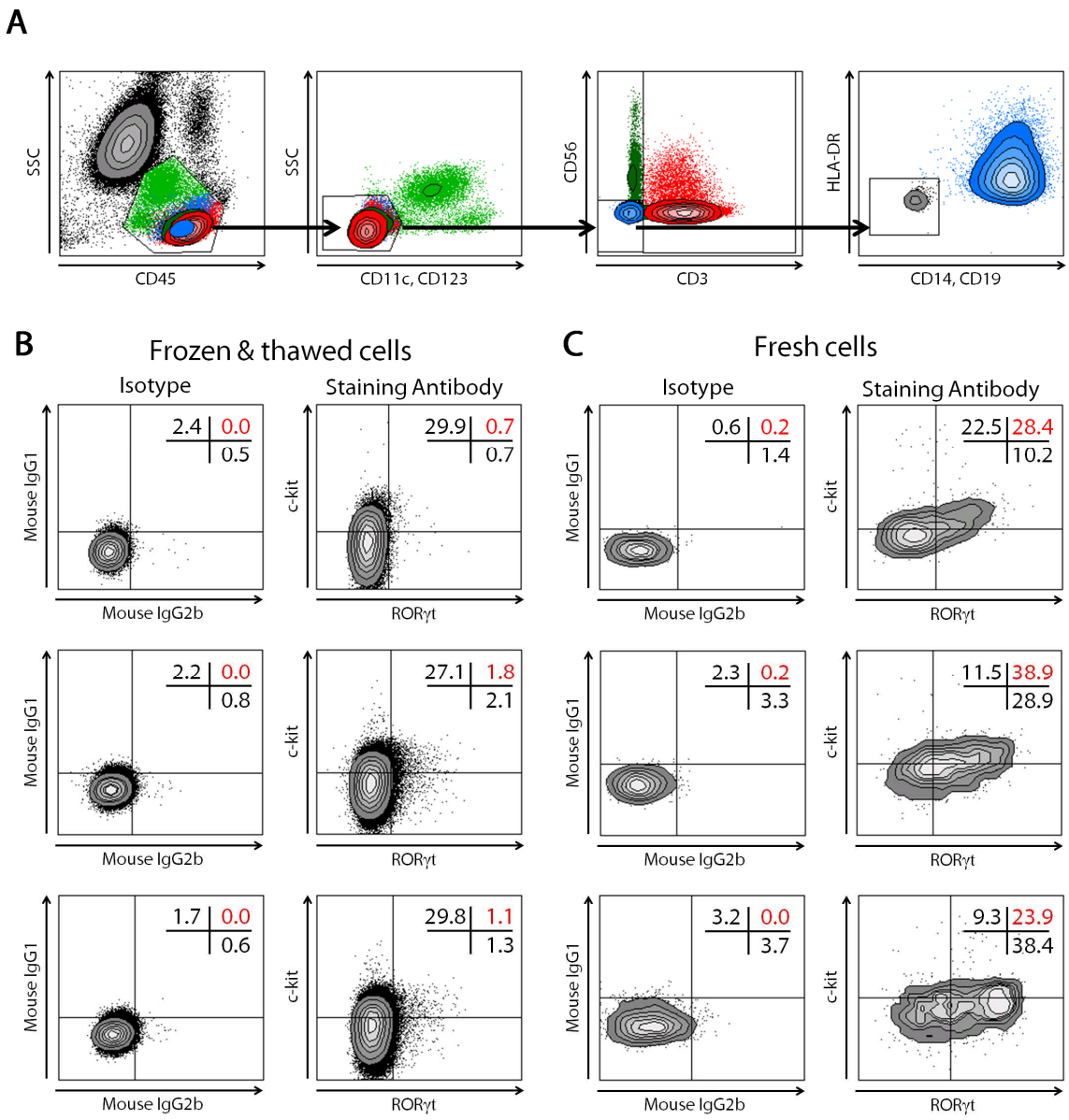

Fig. S1. Intracellular expression of RORγt on CD45$^+$ c-kit$^+$ Lin$^-$ ILCs from fixed/permeabilized cryopreserved PBMCs and fresh whole blood cells

(A) Flow cytometry gating strategy for CD45$^+$ Lin- ILCs

(B) Representative raw contour plots for expression of c-kit and intracellular RORγt on CD45$^+$ Lin- ILCs stained from cryopreserved PBMCs of 3 MS patients

(C) Representative raw contour plots for expression of c-kit and intracellular RORγt on CD45$^+$ Lin- ILCs stained from fresh whole blood cells of 3 MS patients

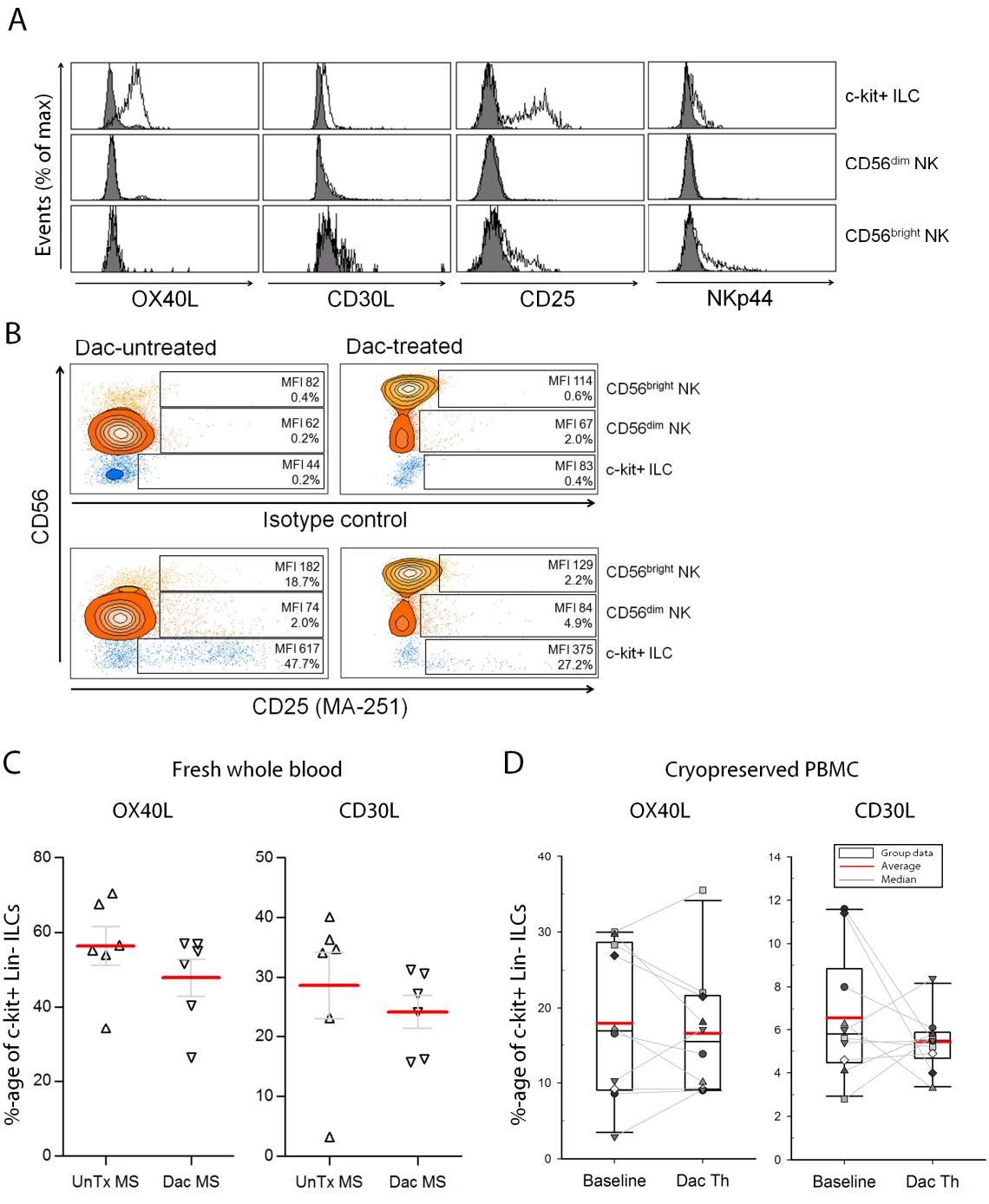
A
Events (% of max)
c-kit+ ILC
CD56dim NK
CD56bright NK
OX40L
CD30L
CD25
NKp44
B
Dac-untreated
Dac-treated
MFI 82
0.4%
MFI 62
0.2%
MFI 44
0.2%
MFI 114
0.6%
MFI 67
2.0%
MFI 83
0.4%
CD56bright NK
CD56dim NK
c-kit+ ILC
CD56
Isotype control
MFI 182
18.7%
MFI 74
2.0%
MFI 617
47.7%
MFI 129
2.2%
MFI 84
4.9%
MFI 375
27.2%
CD25 (MA-251)
C
Fresh whole blood
OX40L
CD30L
%-age of c-kit+ Lin- ILCs
UnTx MS
Dac MS
D
Cryopreserved PBMC
Group data
Average
Median
Baseline
Dac Th

Fig. S2. Surface expression of OX40L, CD30L, CD25, and NKp44 on c-kit^{+} ILCs, CD56^{dim} NK cells, and CD56^{bright} NK cells and change of OX40L and CD30L phenotype on CD45^{+} c-kit^{+} ILCs by daclizumab

(A) Representative raw flow cytometry histograms for expression of OX40L, CD30L, NKp44 and CD25 on c-kit^{+} ILCs, CD56^{dim} NK cells and CD56^{bright} NK cells are shown. Fresh uncoagulated peripheral blood samples were used. (Raw data of Figure 1E)

(B) Representative raw flow cytometry plots for expression of CD25 on c-kit^{+} ILCs, CD56^{dim} NK cells and CD56^{bright} NK cells are shown. Representative comparison between the daclizumab-untreated and the daclizumab-treated patient is presented.

(C) Percentage of OX40L or CD30L-expressing CD45^{+} c-kit^{+} ILCs in fresh blood derived from daclizumab-untreated (UnTx MS, n=6) and daclizumab-treated patient groups (Dac MS, n=6)

(D) Percentage of CD45^{+} c-kit^{+} ILCs which express LTi markers, OX40L or CD30L was determined by flow cytometry. Samples are from the cryopreserved PBMCs of 10 patients enrolled in the DAC HYP clinical trial at the baseline and 6 months treatment (Dac Th). Because we observed that cryopreservation and thawing process significantly decreased expression of OX40L and CD30L in comparison to fresh cells, we consider the data obtained from cryopreserved samples unreliable. The horizontal bars represent the mean of each of the groups.

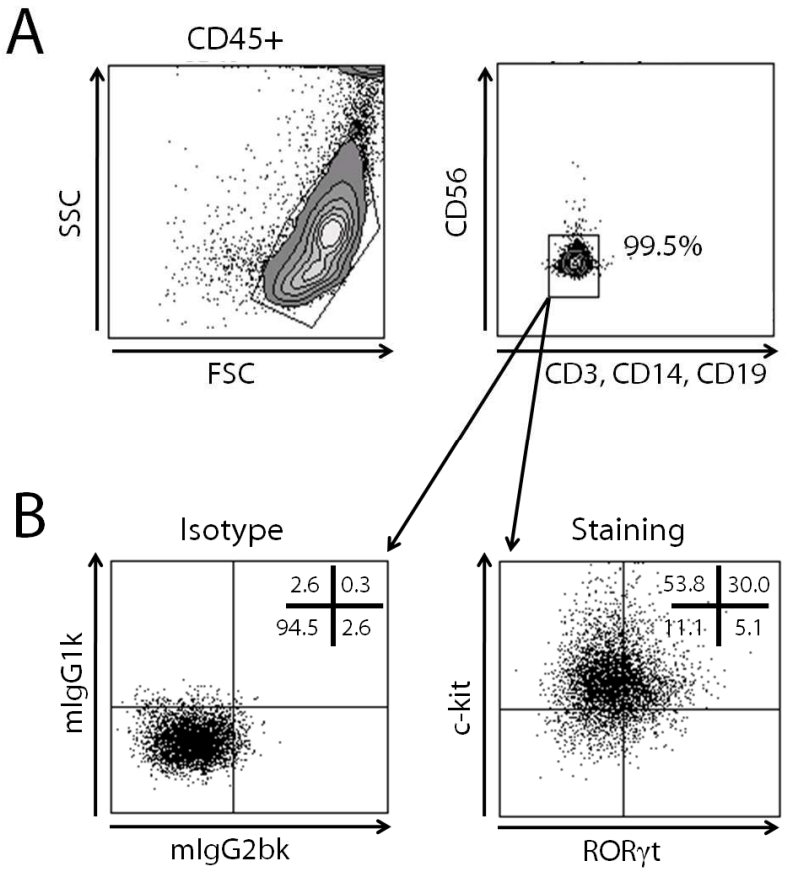

Fig. S3. Purity of isolated c-kit$^+$ ILCs
(A) Purity check of isolated c-kit$^+$ ILCs determined by flow cytometry after using the lineage cell depletion kit and anti-CD34 depletion kit.
(B) c-kit/RORγt phenotype of isolated c-kit$^+$ ILCs.

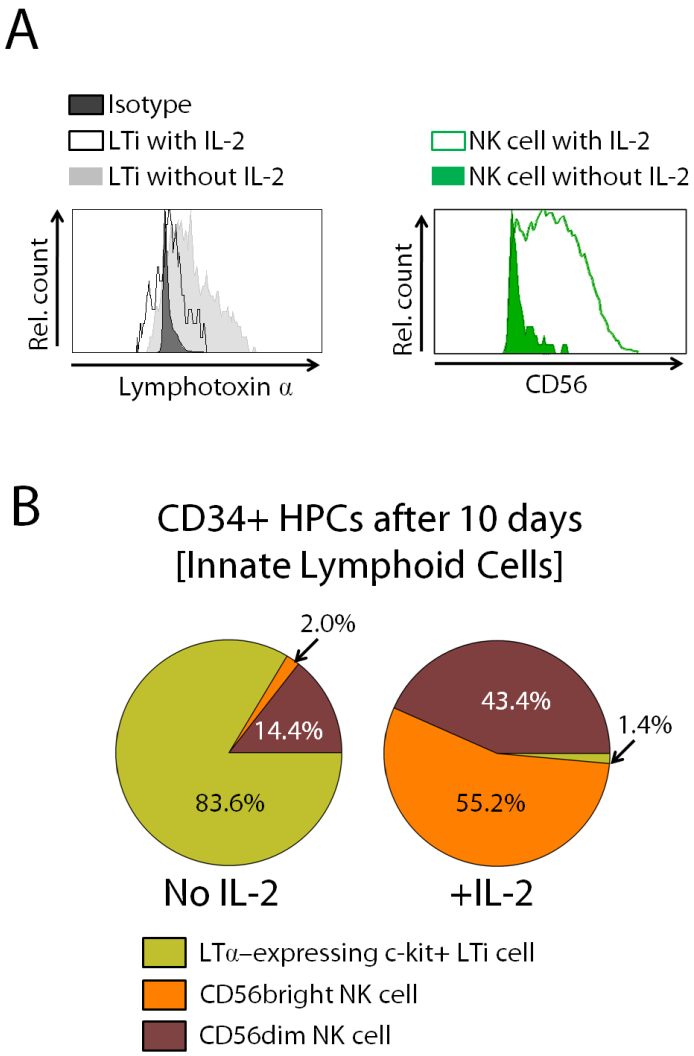

Fig. S4. In vitro differentiation of CD34^{+} HPCs into LTi versus CD56^{bright} NK cells
(A) Histogram of LTα expressed on differentiated LTi cells and histogram of CD56 expressed on differentiated NK cells from isolated CD34^{+} HPCs with or without IL-2 for 10 days. (Histogram of Figure 4B)
(B) The group data depicting the proportion of CD56dim NK, CD56bright NK and LTα-expressing c-kit^{+} LTi cells.