SUPPLEMENTAL DATA

METHODS

Patient eligibility. Patients with MM ≥18 yrs of age who were candidates for SCT with no morphologic evidence of myelodysplasia on their pretreatment bone marrows were eligible. No more than two prior treatment regimens, concurrent immunotherapy, radiotherapy, chemotherapy or anti-myeloma therapy were allowed. Patients who received non-chemotherapy induction were eligible for the protocol. Patients could be leukapheresed while on thalidomide or lenalidomide but no sooner than 2 weeks after the last steroid pulse dose. Eligibility included: ECOG performance status 0-2, absolute lymphocyte count > 500/mm³, platelet count ≥ 75,000/mm³**,** bilirubin ≤ 2.0 mg/dL**,** AST and ALT ≤ 3 times upper limits of normal, creatinine ≤ 2.0 mg/dL, left ventricular ejection fraction ≥ 45%, pulmonary function parameters (diffusion capacity, FEV_1 and FVC) ≥ 50%, no uncontrolled infections including human immunodeficiency virus (HIV) infection, severe metabolic disorders, pregnancy or nursing mother, fertile patients needed to use effective contraception, and no other active malignancy (except non-melanoma skin cancer) that required myelosuppressive chemotherapy or radiation therapy. The exclusion criteria were: patient with a prior SCT, more than 4 cycles of lenalidomide in combination with other agents or greater than 1 year of lenalidomide as a single agent.

Leukapheresis and T cell expansion. A separate leukapheresis was performed to obtain PBMC for ATC expansion prior to G-CSF mobilization of peripheral blood stem cells. PBMC were activated with muromonab-CD3 and expanded in IL-2 to generate ATC. The cells were grown in breathable flasks (FEP Bag Type 750-C1, American Fluoroseal Corporation, Gaithersburg, MD) in RPMI 1640 medium (Lonza Inc., Walkersville, MD) supplemented with 2% pooled heat inactivated human serum (Valley Biomedical, Winchester, VA). ATC were split every 2-3 days based on cell counts. After 14 days of culture, ATC were harvested and armed with a pre-titrated dose of 50 ng of CD20Bi/million ATC, washed, and cryopreserved in multiple

aliquots. Aliquots were sent for bacterial and fungal cultures (Detroit Medical Center Microbiology Laboratory), endotoxin testing (Lonza), and mycoplasma testing (Bionique Testing Laboratories, Inc., Saranac Lake, NY). Aliquots were also retained for quality control testing for cytotoxicity directed at Daudi lymphoma cell targets and phenotyped [17, 21, 22] for $CD3^+$, $CD4^+$, $CD8^+$, $CD56^+$, $CD19^+$, and $CD20^+$ cells.

Criteria for removal from the study. Patients were removed from protocol for: 1) grade 4 non-hematological treatment-related toxicity; 2) initiation of other therapy; 3) patient decision to stop; 4) when it was deemed in the best medical interest of the patient by the physician; or 5) unexplained delay in the delivery of treatment.

Phenotyping of bone marrow mononuclear cells (BMMC). BM aspirates were collected prior to IT and 2-weeks post IT from the patient's undergoing SCT. BMMC were phenotyped for CMPC using multicolor flow cytometry panel including CD19, CD20, CD34, CD38, CD27 and CD138.

Phenotyping of PBMC. PBMC from the patients who received IT and undergoing SCT were sequentially evaluated to assess changes in cellular phenotype. PBMC and the cell product were stained for CD3, CD4, CD8, CD25, CD19, CD20, CD34, CD38, CD27, CD45RO, CD45RA, CD56, and CD138.

Specific cytotoxicity, IFN-γ EliSpots, and serum cytokines. Functional assays were performed before the 1^{st} infusion, 3 weeks after the 2^{nd} infusion and at multiple time points after SCT. IFN-γ Elispots were used to measure both CD8 mediated memory CTL activity and CD4 mediated helper responses. Spontaneous and Daudi, RPMI 8226, and K562 stimulated IFN-γ EliSpots produced by PBMC were assessed at baseline and the designated time points. IFN-γ EliSpots were assessed after 18 hour exposure with an effector to tumor ratio of 10:1 as described [16, 17] [23]. Elispots were scored positive if the EliSpots/10^6 PBMC plated was ≥ 30

spots; a positive increase was defined as ≥ 2 fold increase in EliSpots over the pre-immunotherapy (pre IT) baseline.

Cytokines were measured in the serum samples at selected time points using a 25-plex human cytokine Luminex Array (Invitrogen) using the Bio-Plex system (Bio-Rad Lab., Hercules, CA). The multiplex panel included IL-1β, IL-1 receptor antagonist (RA), IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-13, IL-17, tumor necrosis factor (TNF-α), interferon-alpha (IFN-α), IFN-γ, GM-CSF, macrophage inhibitory protein (MIP-1α), MIP-1β, interferon-inducible protein (IP-10), MIG, Eotaxin, Regulated on Activation Normal T Cell Expressed and Secreted (RANTES) and monocyte chemotactic protein (MCP)-1. The limit of detection for these assays is <10 pg/mL.

Quantitation of anti-SOX-2 IgG. Anti-SOX-2 levels were quantitated by ELISAs using goat anti-human IgG (Invitrogen, Carlsbad, CA). For specific SOX-2 ELISA, a total of 50 ng of SOX-2 human recombinant protein (R&D Systems) was used to coat 96 well plates in 100 μl of PBS. The amount of anti-SOX-2 was calibrated against a known amount of anti-SOX-2 antibodies as standard (R&D Systems). Serum samples were tested at serial dilutions against the anti-SOX-2 standard and anti-SOX-2 levels were reported as ng/ml.

RESULTS

ATC characteristics. ATC were expanded up to 52.0 $x10^9$ cells. In the harvest product, the median viability was 90.15% (CI, 88.6, 91.9%) and the mean percent of CD3, CD4, and CD8 cells were 93.6, 66.2, and 39.1%, respectively (**Table S1**). ATC derived from 12 MM patients armed with CD20Bi exhibited a median 14.4% (CI, 10.4, 18.9%) specific cytotoxicity directed at CD20+ Daudi cells in ^{51}Cr release cytotoxicity assay at an effector target ratio (E:T) of 25:1. aATC derived from 14 normal subjects exhibited a mean of 31.9% (range: 14.4 to 48.4%) specific cytotoxicity directed at CD20+ Daudi targets.

Infusion support and toxicity monitoring. The side effect profile was consistent with grade 1 and 2 side effects of mild cytokine storm. There were no persistent grade 3 side effects. No patients required hospitalization. One patient developed a subclavian vein thrombosis secondary to a clot in a peripheral vein during a cell infusion. The side effects included fever, chills, malaise, nausea/vomiting, tachycardia, hypotension, headache, transient hypoxia, hypertension, and dyspnea (**Table 1B**). The patients continued oral hydration on the evening of the infusions. No patients were removed from the study or required dose-modifications for toxicities.

Engraftment of lymphocytes, neutrophils and platelets. All twelve patients engrafted with a median day of engraftment (500/mm^3) for neutrophils (ANC) of 17 days (CI, 15.0, 19.2 days) following SCT. The median day to a platelet count of 20 x 10^3/mm^3 was 18 days (CI, 16.5, 23.8 days). There were no delays in platelet recovery or need for extended transfusion support. The median day to lymphocyte engraftment of 500/mm^3 was 14 days for 11 patients (CI, 11,19 days). Data were not available on one patient for lymphocyte engraftment. **Figure 1B** summarizes the mean absolute lymphocyte count (n=11), ANC, and platelets for the patients. All patients engrafted without any complications. Eight patients received G-CSF to accelerate engraftment. The effect of G-CSF administration could not be evaluated due to small numbers in each group.

Lymphocyte recovery. Phenotyping was performed at pre IT, 1, 2, and 3 weeks after immunotherapy, and 3, 6, and 12 months after SCT. The mean percent CD3+, CD4+, and CD8+ cells are shown at the indicated time points in **Figure S1**. The mean (n = 12) proportion of CD3+, CD4+ and CD8+ cells was 27.7, 8.8, and 16.5% at 1 month after SCT, respectively. By 3 months after SCT, the mean proportions of CD3+, CD4+, and CD8+ cells reached 29.9%, 9.9%, and 23.0%, respectively. The CD4/CD8 ratio by 3 months had increased to >1. The mean proportions of CD20+ cells were 5.0% during first 1-3 months after SCT and the median

proportion of CD19+ cells during first 3 months was 8.05%. The proportion of CD8/CD45RO+ cells increased to >15% at 3 months after SCT and stayed above 12% up to 1 year.

Infectious disease complications. There were no viral, bacterial, or fungal infections during the first 3 months after SCT.

Quantitative IgG and anti-TT IgG serum levels. Serum IgG levels were obtained in 12 patients to determine whether targeting CD20+ B cells early post SCT would impair anti-TT B cell reconstitution. Anti-TT levels were evaluated to determine the amount of anti-TT immunity that was transferred in the autograft. **Figures S3A and S3B show** IgG and anti-TT levels at pre SCT and 3 months after SCT. The mean serum levels of IgG before and after SCT were 6.5±4.9 mg/ml and 10.3±4.9 mg/ml, respectively. The mean serum levels of anti-TT before and after SCT were 8.4±10.7 μg/ml and 28.3±26.5 μg/ml, respectively. These data show that targeting $CD20^+$ B cell prior to SCT did not impair recovery of $CD20^+$ cells nor did it impair the development or function of IgG and anti-TT secreting B cells during the first 100 days after SCT.

Discussion

The pre-transplant vaccinate and boost after transplant approach is supported by the transfer of immunity in studies SCT for MM that show immunity to pneumococcal conjugate, human telomerase reverse transcriptase, anti-apoptotic protein survivin, and influenza in lymphopenic individuals could more rapidly be restored by adoptive transfer of anti-CD3/anti-CD28 coactivated T cells followed by booster immunizations [31-33]. This study shows that immunization via targeted T cells induced the development of endogenous anti-MM specific immunity that not only was transferred in the stem cell graft but also could be boosted by a single infusion of targeted ATC in 3 of 4 patients.

Pretargeting MM CMPC with BATs may release intracellular and intranuclear proteins that were processed and presented by endogenous APC to the immune system to induce both Th_1 and antibody responses. It is clear that SOX-2 specific memory B cell clones were transferred in the immune stem cell product and persisted for months. It is not clear whether the anti-SOX-2 levels are clinically protective. This pilot cohort suggests that humoral, cellular, and NK responses may provide enough anti-CMPC or anti-MM effect to decrease or help prevent relapse and progression. The presence of anti-SOX-2 and anti-MM helper and cytotoxic T cells that could be boosted with a single BATs infusion suggest such responses could sustained.

Similarly, SCT would transfer pre-existing anti-TT immunity and serve as an irrelevant recall antigen and specificity control. In order to have normal levels of IgG and anti-TT at 3 months after SCT without supplemental intravenous gamma globulin or booster immunizations to TT, polyclonal B cells and antigen-specific B cells in the stem cell graft would have had to undergo reconstitution without any impediments. Alternatively, the T cell infusions provided helper activity for anti-TT memory B cell reconstitution so that the differentially depleted CD20+ populations did not contain the memory B cells or there was a compensatory expansion of the anti-TT secreting B cells after SCT. The increased levels of IgG and anti-TT are likely due to helper activity provided by BATs. The mechanism(s) responsible for such an effect would be the subject of another investigation.

Table S1. T Cell Characteristics in Harvest Product

Harvest Product	Mean (%)	Range (%)
CD3+	**94.6**	**84.4-98.3**
CD3/4+	**66.2**	**24.8-81.1**
CD3/8+	**39.1**	**10.2-71.3**
CD4/25+	**56.2**	**8.8-75.9**
CD8/25+	**33.3**	**0.4-63.0**
CD3+(16/56)+	**0.8**	**0.1-26.2**
CD3-(16/56)+	**11.6**	**0.35-63.7**
CD19+	**2.4**	**0.3-5.3**
CD20+	**0.5**	**0.1-4**
CD4+(45RA-/45RO+)	**85.1**	**17.1-97.3**
CD4+(45RA+/45RO-)	**0.8**	**0.2-24.3**
CD8+(45RA-/45RO+)	**79.3**	**2.7-94.0**
CD8+(45RA+/45RO-)	**1.7**	**0.5-57.9**
Ratio of CD4/CD8	**1.4**	**0.4-4.3**

Table S2. EliSpot scores and fold change in patients who relapsed versus who remained in remission relative to pre BATs EliSpots.

EliSpot response Against RPMI-8226							
Patient ID	**Clinical Response**	**PostIT**		**2wk-3M PostSCT**		**6-12M PostSCT**	
20024	REL	14.0		2.0		2.0	
20030	REL	2.2	+	0.8		2.2	+
20032	REL	7.5	+++	2.7	+	2.7	+
20035	REL	2.5	+	1.3		8.7	+++
20048	REL	10.1	+++	1.4		3.3	++
20027	uCR	0.9		0.4		0.7	
20036	uCR	3.8	++	4.0	++	6.6	+++
20037	uCR	2.3		4.1	++	12.8	+++
20039	uCR	2.1	+	2.1	+	12.5	+++
20040	uCR	0.9		1.8		8.1	+++
20047	uCR	2.4	+	0.4		0.3	
20050	uCR	3.2	++	7.5	+++	7.8	+++
EliSpot response Against Daudi							
Patient ID	**Clinical Response**	**PostIT**		**2wk-3M PostSCT**		**6-12M PostSCT**	
20024	REL	2.3	+	0.7		0.7	
20030	REL	8.3	+++	5.5	+++	17.5	+++
20032	REL	3.6	++	2.7	++	3.1	++
20035	REL	1.7		1.3		3.7	++
20048	REL	4.5	++	0.4		1.3	
20027	uCR	0.4		0.6	+	0.9	
20036	uCR	3.5	++	2.2	+	1.8	
20037	uCR	1.0		3.4	+	13.6	+++
20039	uCR	1.5		0.5		9.0	+++
20040	uCR	1.0		2.5	+	7.1	+++
20047	uCR	1.5		0.2		0.3	
20050	uCR	0.6		0.9		1.9	+/-
EliSpot response Against K562							
Patient ID	**Clinical Response**	**PostIT**		**2wk-3M PostSCT**		**6-12M PostSCT**	
20024	REL	2.1	+	0.1		0.1	
20030	REL	14.3	+++	1.8		8.0	+++
20032	REL	9.2	+++	2.9	++	2.9	+
20035	REL	4.4	++	1.7		12.5	+++
20048	REL	4.6	+++	0.2		3.3	++
20027	uCR	1.0	+++	1.0		0.4	
20036	uCR	2.9	+	1.9		2.6	+
20037	uCR	1.8		1.3		46.7	+++
20039	uCR	4.8	+++	0.2		8.8	+++
20040	uCR	9.5	+++	15.3	+++	42.3	+++
20047	uCR	0.3		0.1		1.6	
20050	uCR	0.9		1.3		10.5	+++

Elispots were scored positive if the EliSpots/10^6 PBMC plated were ≥ 30 spots; a positive increase was defined as ≥2 fold (+), 3-5 fold (++), >5 fold (+++) increase in EliSpots over the pre-immunotherapy (pre BATs) baseline. PostIT=Post BATs

Figure Legends

Figure S1. Flow cytometry performed on PBMC at the indicated time points after BATs and SCT show mean proportions of CD3+, CD4+, and CD8+ T cells after SCT (Left panel); CD4 to CD8 ratio after BATs and SCT (Right panel); proportions of CD4/CD45RO+ and CD8/CD45RO+ (Left lower panel) and proportions of CD19 and CD20+ cells.

Figure S2. Enhanced IFN-γ EliSpots by PBMC directed at RPMI 8226, Daudi and K562 post BATs and post SCT for each of 12 patients at pre BATs, post BATs, 0.5-3M and 6-12M post SCT.

Figure S3. Transfer and reconstitution of polyclonal IgG and anti-TT IgG. The results for individual patients are presented. **A)** Total IgG was tested before and at 3 months after SCT, IgG levels increased in 5 out of 12 MM patients. **B)** Anti-TT IgG levels before and at 3 months after SCT were analyzed to determine whether TT specific memory B cells were transferred in the stem cell product. Anti-TT specific antibody levels were elevated in 8 out of 12 MM patients.

Figure S1

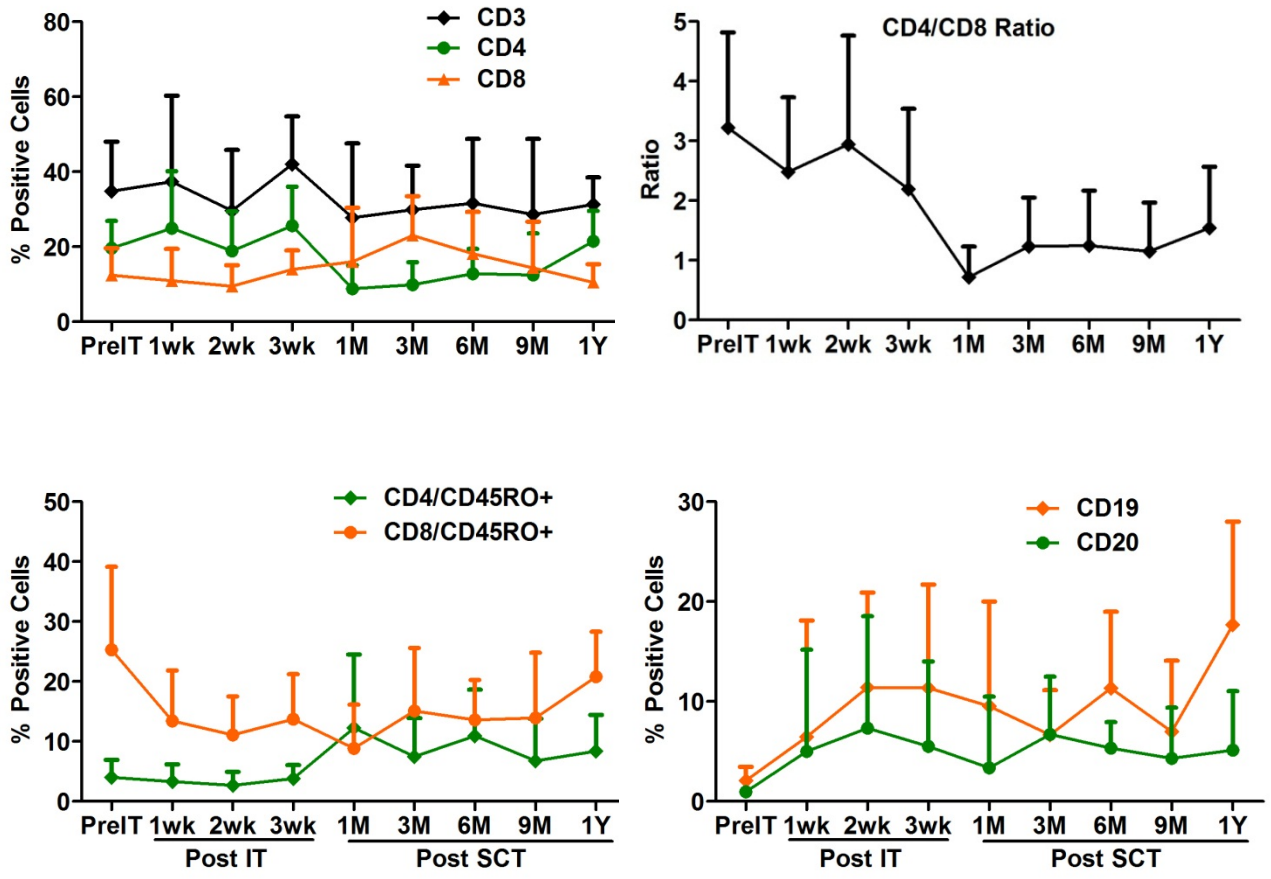

CD3
CD4
CD8
% Positive Cells
80
60
40
20
0
PreIT 1wk 2wk 3wk 1M 3M 6M 9M 1Y
CD4/CD8 Ratio
Ratio
5
4
3
2
1
0
PreIT 1wk 2wk 3wk 1M 3M 6M 9M 1Y
CD4/CD45RO+
CD8/CD45RO+
% Positive Cells
50
40
30
20
10
0
PreIT 1wk 2wk 3wk 1M 3M 6M 9M 1Y
Post IT
Post SCT
CD19
CD20
% Positive Cells
30
20
10
0
PreIT 1wk 2wk 3wk 1M 3M 6M 9M 1Y
Post IT
Post SCT

Figure S2

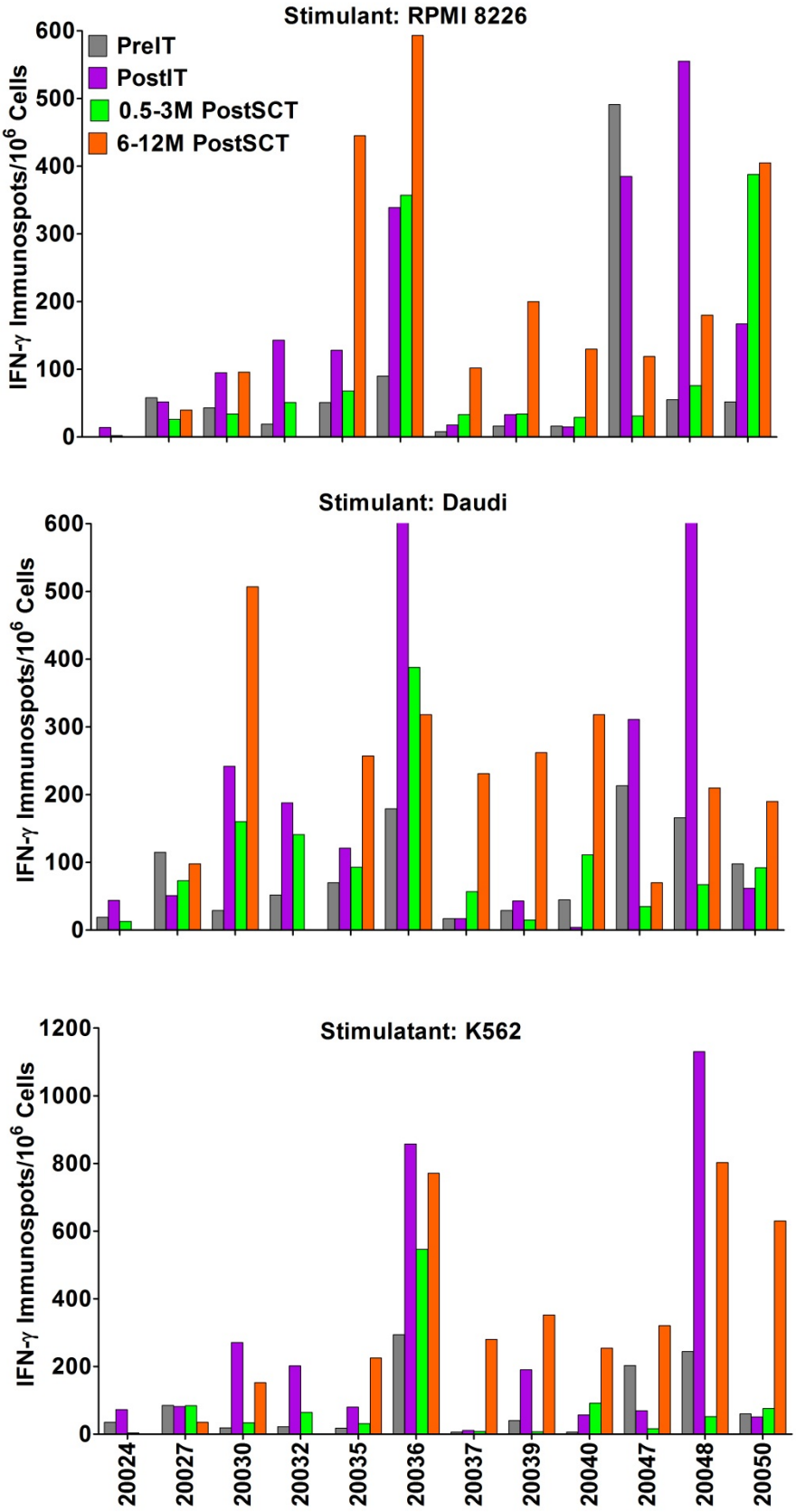
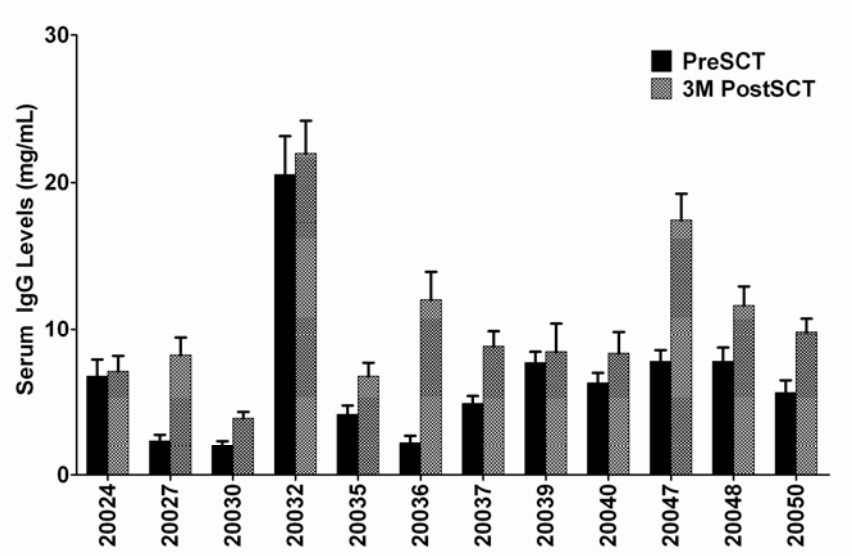

Stimulant: RPMI 8226
PreIT
PostIT
0.5-3M PostSCT
6-12M PostSCT
IFN-γ Immunospots/10^6 Cells
0
100
200
300
400
500
600
Stimulant: Daudi
IFN-γ Immunospots/10^6 Cells
0
100
200
300
400
500
600
Stimulatant: K562
IFN-γ Immunospots/10^6 Cells
0
200
400
600
800
1000
1200
20024
20027
20030
20032
20035
20036
20037
20039
20040
20047
20048
20050

Figure S3 A



S3 B