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Transgene expression in various organs post BM-HSC transplantation

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Abstract Gene therapy mediated by bone marrow-derived hematopoietic stem cells (BM-HSC) has been widely used in treating genetic deficiencies in both pre-clinical and clinical settings. Using mitotically inactive cell-targeting lentivirus with separate promoters for our gene of interest (the murine MHC class II (MHCII) chaperone, invariant chain (Ii)) and a GFP reporter, we monitored the expression and function of introduced Ii in various types of professional antigen presenting cells (B cells, macrophages and DC) from different organs (spleen, pancreatic lymph nodes (PLN), BM and blood). Ii and GFP were detected. Ii levels correlated with GFP levels only in macrophages and monocytes from spleen, monocytes from PLN and macrophage precursors from blood. By cell type, Ii levels in PLN cells were more similar to those in spleen cells than to those in blood or BM cells. Functionally, Ii expressed in PLN or spleen had more effect on MHCII abundance than Ii expressed in BM or blood. The results have implications for analysis of the outcomes of gene therapy when both therapeutic and reporter genes are introduced. The findings also have implications for understanding the development of immune molecule function.
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Abbreviations: AA, amino acid; Ab, antibody; ADL, adrenoleukodystrophy; APC, antigen presenting cells; BM, bone marrow; DC, dendritic cells; EF1a, elongation factor 1a; GFP, green fluorescent protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; HSC, hematopoietic stem cells; Ii, invariant chain; IRES, internal ribosome entry sites; Lin, linage; MFI, mean fluorescence intensities; MHCII, major histocompatibility complex class II; MOI, multiplicity of infection; MPB, mobilized peripheral blood; MSCV, murine stem cell virus; PLN, pancreatic lymph nodes; T2A, Thoseaasigna virus 2A

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Introduction

Hematopoietic stem cells (HSC) are multi-potent cells responsible for the development, maintenance, and regeneration of all formed blood elements, including those of the immune system. Bone marrow (BM) is the main source of HSC for experiments in mice, whereas in humans, HSC also are isolated from mobilized peripheral blood (MPB) or cord blood. Autologous BM or MPB are more useful than allogeneic sources in human gene therapy trials (Woods et al., 2002). The unique self-renewal and differentiation properties of HSC guarantee that the transgenes expressed in a small number of HSC result in long-term correction of much larger numbers of mature progeny, making HSC promising target cells for therapy of genetic anemias, immunodeficiencies and metabolic diseases (Aiuti et al., 2013; Bank, 2003; Barrette et al., 2000; Biffi et al., 2013; Wanisch and Yanez-Munoz, 2009). HSC transplantation is also a possible strategy for treating

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leukemia, non-hematopoietic tumors and autoimmune diseases as well as in induction of tolerance for solid organ transplants (Shizuru et al., 2005).

HIV-based lentivirus transduces post-mitotic and non-dividing cells, including HSC, by active transport through the nucleopore, independent of nuclear envelope breakdown during cell division (Buchschacher and Wong-Staal, 2000; Chicurel, 2000; Frimpong and Spector, 2000). Its other characteristics, including the capacity to integrate into the host chromosome with subsequent long-term transgene expression, large coding capability, relatively high transduction efficiency and low immunogenicity, further support its use as a delivery vector in BM-HSC mediated gene therapy (Hu et al., 2011). Single transgene (GFP reporter or therapeutic exogenous gene) expression in the progeny of lentivirus-transduced HSC has been studied widely. GFP expression was observed in BM and spleen of immunodeficient murine recipients reconstituted with human $CD34^+$ HSC from G-CSF—mobilized peripheral blood or cord blood that were transduced with pan- or cell type-specific promoter containing lentivirus (Cui et al., 2002; Kahn et al., 2004; Verhoeyen et al., 2005). Similarly, expression of either the GFP reporter or the therapeutic gene alone was observed in various organs (BM, thymus, spleen, lymph nodes, blood) in normal mice, immunodeficient mice or (the corresponding) single gene deficient mice, after lentivirus-transduced HSC transplant (Astrakhan et al., 2012; Heckl et al., 2011; Levasseur et al., 2003; Marodon et al., 2003; Mostoslavsky et al., 2006; Perumbeti et al., 2009; Zhang et al., 2007). Sustained GFP expression was detected in blood and BM in nonhuman primates up to 4 y post transplantation with lentivirus-transduced HSC (Kim et al., 2009; Trobridge et al., 2008). In two X-linked adrenoleukodystrophy (ADL) patients who lack ADL protein, this protein was detected in 9–14% of various blood cells and in 17–20% BM $CD34^+$ cells up to 30 m after transplant with the HSC transduced by lentivirus encoding ALD protein. Accordingly, progressive cerebral demyelination was attenuated (Cartier et al., 2009). More recently, effective therapeutic transgene expression mediated by lentivirus-transduced HSC was reported in patients with metachromatic leukodystrophy or Wiskott–Aldrich syndrome (Aiuti et al., 2013; Biffi et al., 2013).

Using a lentiviral vector containing two separate promoters for two transgenes (*i.e.* EF1a for our gene of interest, murine invariant chain (Ii), and MSCV for the reporter gene-GFP), we monitored Ii and GFP levels in various antigen presenting cell (APC) types from different organs in NOD mouse recipients up to 8 m post BM-HSC transplantation. We analyzed the correlation between Ii and GFP levels in different organs (blood, BM, pancreatic lymph nodes (PLN) and spleen) and the correlation between Ii levels in PLN and Ii levels in other organs. We also examined the function of the introduced Ii. The results provide information relevant for analyses of *in vivo* expression of two transgenes introduced together. They also suggest that the expression level and function of certain immune molecules may develop along with maturation of immune cells, such as APC.

Materials and methods

Lentiviral vector and viral packaging

cDNA of 3xflag-tagged WT or mutant Ii (M98A) (Rinderknecht et al., 2010) was cloned into the multiple cloning site driven by an MSCV promoter in a dual-promoter lentiviral vector, containing GFP driven by an EF1a promoter (System Bioscience, Mountain view, CA). The positions of GFP and Ii were then switched so that EF1a and MSCV become promoters for Ii and GFP respectively (Wang et al., 2013).

Lentivirus was produced in 293T cells by calcium phosphate precipitation of the abovementioned dual-promoter vector, envelop plasmid VSV and packaging plasmid PAX2. Culture media were replaced 8 h post-transfection and lentivirus containing supernatants then were harvested 24 h later. Supernatants were filtered, precipitated and concentrated with PEG-it Virus Precipitation Solution (System Bioscience), according to the manufacturer's instructions. Lentiviral titer was determined by measuring % of GFP^+ 293T cells after transduction with time-diluted viruses and confirmed by quantitative real time PCR to determine the vector integration copy number into the host chromosomes (Kutner et al., 2009).

BM-HSC isolation, transduction and transplantation

$ckit^+$ BM cells from 3 to 5 m NOD mice ($CD45.1^+$, 50% of which had high blood glucose, *i.e.* >250 mg/dl) were enriched by CD117 microbeads (Miltenyi Biotec, Auburn, CA) and stained with monoclonal antibodies for linage (Lin) markers (CD3, CD4, CD8, B220, Gr1, Mac1, Ter119) and stem/progenitor cell markers (ckit and Sca-l) then sorted for HSC ($cKit^+Sca1^{hi}Lin^-$) using FACS-Aria (BD bioscience, San Jose, CA) (Rajasekaran et al., 2013). HSC were transduced with lentiviruses encoding wt or mutant Ii at MOI = 80 for 8 h in the presence of 8 μg/ml polybrene (Lu et al., 2011) after pre-activation with 100 ng/ml SCF and 100 ng/ml TPO overnight. 10,000 transduced HSC/mouse were transplanted by tail vein injection into 8–12w NOD recipients ($CD45.2^+$, with normal blood glucose) that had been lethally irradiated at 980 cGy. Chimerism (%CD45.1 *vs.* %CD45.2) and levels of GFP and Ii were detected by FACS up to 8 m post-transplantation. Abs and cytokines were purchased from eBioscience (San Diego, CA) and Peptrotech (Rocky Hill, NJ) respectively. NOD mice were bred and housed in the Stanford Veterinary Service Center under the approval of Administrative Panel for Laboratory Animal Care.

Flow cytometry analyses

For *in vivo* GFP and Ii expression, BM, blood, pancreatic lymph nodes (PLN) and spleens were harvested from lentivirus-transduced HSC recipients. After RBC lysis, 1E6 cells were stained with Abs recognizing key markers of different compartments, *i.e.* $B220^+$ for B cells, $CD11b^+$ for myeloid cells, $CD11b^+F4/80^+$ for macrophages or macrophage precursors (ma-precursors in blood), $CD11b^+F4/80^-$ for monocytes, $CD11c^+$ for dendritic cells (DC). GFP expression was measured in different compartments. 2E6 cells stained first with compartment markers were then fixed, permeabilized and stained with Abs against Flag (*i.e.* biotin-labeled anti-Flag Ab (Sigma-Aldrich, St. Louis, MO) followed by PO-conjugated

streptavidin (Invitrogen, Grand Island, NY)) to measure introduced Ii level. Data from stained cells were collected on a LSR II flow cytometer (BD Bioscience) and analyzed with FlowJo software (Tree Star Inc., Ashland, OR). Live white blood cells were gated by FSC and SSC to remove dead cells, red blood cell fragments and platelets. The threshold of positivity for each cell type from each organ was set based on a sample from the same cell type in the corresponding organ of an untransplanted NOD mouse as the negative control (data not shown). In BM samples, immature progenitor and stem cells (CD117$^+$) were gated out before analysis of Ii and GFP levels in differentiated APC. Abs were purchased from Biolegend (San Diego, CA), unless otherwise mentioned.

Ii functional analysis

To assess the function of introduced Ii, flow cytometry was used to measure the levels (histogram and median MFI) of I-A^{g7}, the MHC class II allele expressed by NOD mice. I-A^{g7} levels were compared between the Flag$^+$ (representing cells expressing exogenous Ii) and Flag$^-$ populations (representing non-expressing, likely non-transduced, cells) in recipients of BM-HSC transduced by WT or mutant Ii containing lentiviruses. The Flag expression levels represented by their median MFI values of those two populations were also analyzed. The ratios of median MFI of I-A^{g7} to median MFI of Flag were compared between WT and M98A recipients.

Statistical analyses

Data from 12 wt and 11 mutant Ii recipients from 3 separate transplantations were combined; GFP and Ii expression levels were not significantly different in wt *vs*. mutant Ii recipients (not shown). Correlations between Ii and GFP levels in each APC type from each organ as well as the association between Ii levels in pancreatic lymph nodes (PLN) and spleen, blood and BM were shown by the regression line and analyzed by the nonparametric Spearman rank correlation test. Comparison of I-A^{g7} enhancement (median MFI) in wt or mutant Ii transduced population (Flag$^+$) *vs*. non-transduced population (Flag$^-$) was calculated by 2-tailed paired *t* test. I-A^{g7} levels of wt and mutant Ii transduced populations were compared using a non-paired *t* test. Median MFI of I-A^{g7} in transduced population was normalized to non-transduced population set to 1. These statistical analyses were also applied for evaluating Flag (introduced Ii) level (relative median MFI, normalized to Flag$^-$ population as 1) in Ii transduced *vs*. non-transduced population. I-A^{g7}/Flag ratios (using relative median MFI) in Flag$^+$ population were compared between wt and mutant Ii recipients using non-paired *t* test. All statistical analyses were done with Prism software (GraphPad Software, Inc., La Jolla, CA). $P < 0.05$ was considered statistically significant.

Results

Ii and GFP are detected in various types of APC from different organs

Using qPCR and FACS, we previously observed expression of GFP and Ii in 293T cells transduced with dual promoter lentiviral constructs containing wt or mutant (M98A) Ii and GFP (Wang et al., 2013). To evaluate the *in vivo* expression level of these transgenes using the same constructs (see Materials), transduced NOD BM-HSC were transplanted into lethally irradiated NOD mice. Blood, BM, spleen and pancreatic lymph nodes (PLN) were harvested up to 8 m post-transplantation. As our gene of interest, murine Ii, a chaperone for the assembly and transport of MHC class II, is mainly expressed and functional in antigen presenting cells (APC), we focused on monitoring Ii levels in professional APC (B cells, macrophages and dendritic cells (DC)). Cell types initially were defined with commonly used markers, *i.e.* B220 for B cells, CD11b for macrophages/monocytes and CD11c for DC. Ii levels in monocytes, the immature or pre-activated form of macrophages, were also monitored. In addition, monocytes can develop into DC (Gordon and Taylor, 2005; Sunderkotter et al., 2004). A small subset of blood monocytes expressing macrophage marker F4/80 (Nikolic et al., 2005) were assessed here, as macrophage-precursors. As shown by one representative mouse for each type of recipient, *i.e.* wt and M98A, in Fig. 1, an intermediate level of GFP and usually a lower level of Ii (represented by the expression of the Flag tag) were observed in all types of APC from all 4 organs. In peripheral organs, such as PLN, spleen and blood, macrophages expressed the highest level of transgene followed by DC/monocytes and B cells. The trend is more obvious in Ii expression than in GFP expression. In BM, however, this macrophage > DC > B cells trend was lost. No significant difference was observed between wt and M98A recipients in either GFP or Ii expression in any APC type from any organ, arguing that the Ii point mutation did not affect either Ii or GFP expression.

GFP and Ii levels correlate in myeloid compartments only

We chose to express GFP as a reporter gene together with Ii for two reasons: 1) GFP is easier than Ii to detect by FACS, as the latter requires cell permeabilization; 2) GFP can be used as a marker for sorting live cells. Among the various lentiviral vectors expressing the two transgenes together, those with a separating sequence (IRES or T2A) under a single promoter either have imbalance in expression levels of the two genes (*i.e.* IRES) (Yu et al., 2003) or the particular promoter did not work effectively in the murine HSC environment; (T2A under the MSCV promoter failed to induce *in vivo* GFP and Ii expression, data not shown). However, interference between the two separate promoters also may affect the expression levels of the corresponding genes (Curtin et al., 2008). To investigate whether GFP, as a reporter, reflects the expression of our gene of interest (Ii), we tested the correlation between the two proteins in various APC types from different organs. As shown in Fig. 2, the correlation between Ii and GFP levels was significant only in myeloid compartments (*i.e.* splenic and PLN monocytes as well as blood macrophage-precursors, $P < 0.05$). The correlation of Ii and GFP in splenic macrophages and BM B cells approached, but did not reach significance, at $P < 0.05$. Thus, only in some subsets of myeloid cells in the periphery, GFP levels paralleled Ii levels.

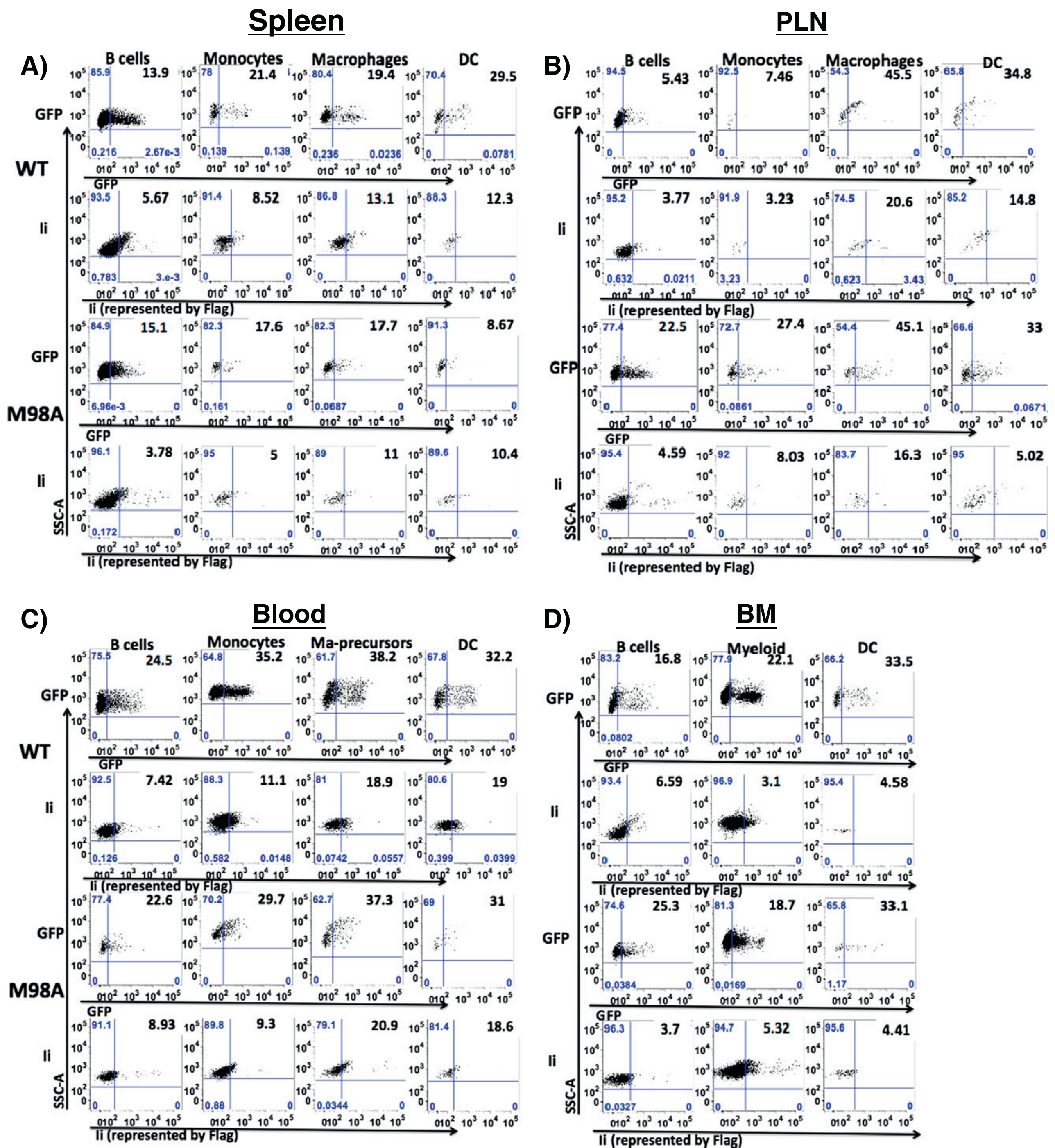


Figure 1 Representative transgene expression in various types of APC from different organs. Organs from Ii wt and M98A Ii recipients were harvested, stained with proper Abs and analyzed by FACS. Panels A, B, C and D show samples from spleens, PLN, blood and BM, respectively, of a representative animal from each group. For each panel, rows 1 and 2 show samples from the wt recipient and rows 3 and 4 show samples from the M98A recipient. Rows 1 and 3 show GFP expression (as % of total donor cells) and rows 2 and 4 show Ii levels (as % of total donor cells). The Ii level is represented by the level of Flag. The threshold of positivity in each cell type from each organ was set using a sample from the corresponding cell type in the corresponding organ of an untransplanted NOD mouse as the negative control (data not shown). Data shown are representative of three independent transplantation experiments.

The correlation of Ii levels in PLN with Ii levels in other organs

Transgene expression level is important for evaluation of the outcome of gene therapy. Blood and BM samples commonly are used for detecting transgene expression in non-human primates and patients (Aiuti et al., 2013; Biffi et al., 2013; Cartier et al., 2009; Kim et al., 2009; Trobridge et al., 2008). As GFP level generally did not reflect Ii level (Fig. 2), we did not compare GFP levels in additional analyses. Rather, to

determine if transgene expression in blood or BM reflected transgene levels in other target organs, we analyzed the correlations between Ii levels in different organs. As PLN are critical sites for mediating the pathogenesis of type I diabetes in NOD mice, we compared Ii levels in PLN to those in blood or BM. Spleen also acts as a secondary immune organ, so we tested the relative expression of Ii in PLN compared to spleen. Ii levels in myeloid cells (monocytes and macrophages) and DC from spleen correlated with those from PLN ($P < 0.05$). Similarly, in blood, Ii levels in macrophage precursors and DC significantly correlated with those in PLN. The correlation between Ii levels of splenic and PLN B cells showed a trend, but was not significant (Fig. 3A). Levels of Ii in circulating B cells and monocytes did not correlate with levels in PLN (Fig. 3B). No correlation with PLN expression of Ii was observed in BM B cells, myeloid cells or DC (Fig. 3C). Together, these data argue that, for Ii as a transgene, expression levels in circulating macrophage-precursors and DC correlate with expression in these cell types in secondary immune organs, but this is not the case for monocytes and B cells.

It was possible that one contributor to the lack of correlation we observed was that the markers we used collected heterogeneous populations that differed in make-up in different organs. To explore this possibility, we analyzed the percentage of APC bearing other markers that are found within the single marker populations we initially analyzed (Fig. 4A, n = 23 mice). B cells represented by $B220^+$ contained very few $CD11b^+$ and $CD11c^+$ cells, *i.e.* <3% in all the organs. DC represented by $CD11c^+$ expressed certain amount of B220 and CD11b, presumably on plasmacytoid DC (pDC) and myeloid DC (mDC) respectively. As it is widely accepted that both subsets belong to DC (Ziegler-Heitbrock et al., 2010), we did not distinguish those two subsets in the DC population. However, a potential concern is the possible overlap between myeloid DC and inflammatory myeloid cells. Macrophages/monocytes can express CD11c under inflammatory conditions without converting into DC (Drutman et al., 2012; Wentworth et al., 2010). T1D in NOD mice results from inflammatory insulitis (Kaminitz et al., 2013), is exacerbated by intestinal inflammation (Alam et al., 2010) and is blocked by anti-inflammatory therapy (Xue et al., 2012), so it was likely that inflammatory myeloid cells are present in these mice. However, we noted that splenic mDC and inflammatory macrophages/monocytes can be distinguished by the differences in the fluorescent intensity of CD11b and CD11c, *i.e.* $CD11c^{high}CD11b^{int}$ (with average MFI of CD11c and CD11b as 4840 and 3079 respectively) for mDC *versus* $CD11b^{high}CD11c^{int}$ (with average MFI of CD11b and CD11c as 7578 and 1089 respectively) for inflammatory macrophages/monocytes (data not shown). Thus, we included only $CD11c^{int}$ cells in the myeloid population and excluded mDC. Of note, inflammatory myeloid cells and myeloid DC can be more accurately separated by Ly6C level (Drutman et al., 2012).

In addition, we found that in $CD11b^+$ cells from spleen and PLN, >5% of cells are also $B220^+$, whereas these double positive cells are present in blood and BM at a negligible frequency. $CD11b^+B220^+$ cells have been found in other autoimmune models, such as experimental allergic encephalomyelitis and MRL-Fas^{lpr} mice (Davoust et al., 2006; Iwata et al., 2010); we speculate that they may play a pro-inflammatory role in those settings. As the function of $CD11b^+B220^+$ cells in diabetes has not been fully elucidated, we used $CD11b^+B220^-$ cells to re-analyze the correlation between GFP and Ii (Flag) expression and the Ii (Flag) expression in different organs (Figs. 4B,C). Compared to our results using total $CD11b^+$ cells (Fig. 2), the correlation between GFP and Flag slightly increased using $CD11b^+$ $B220^-$ cells in spleen and PLN (Fig. 4B). As GFP in splenic and PLN $B220^+$ cells correlated less well with Flag than in macrophages and monocytes (Figs. 2A and B), it is not surprising that analysis of $CD11b^+B220^-$ cells enhances GFP-Flag correlation. Re-analysis of correlations in Ii expression between different organs using $CD11b^+B220^-$ cells shows no statistically significant differences compared to original analysis using $CD11b^+$ cells, except for the correlation between macrophages in PLN and blood; this correlation was substantially reduced, with the P value changing from 0.0176 to 0.0598 (compared Fig. 3 with Fig. 4C).

Introduced Ii was more functional in PLN and spleen than in blood and BM

A key aim of gene therapy is correction of host genetic deficiencies by functional transgene(s). To test the function of introduced Ii, the level of I-A^{g7}, the MHC class II allele expressed by NOD mice, was measured in various APC cell types from different organs. I-A^{g7}, like its human counterpart DQ8, has low affinity for the core class II binding region of Ii (AA 83–107) (Rinderknecht et al., 2010) and is tightly linked to susceptibility to type I diabetes (Hausmann et al., 1999). Point mutation of Ii at M98A significantly increases the affinity between Ii and I-A^{g7}, thereby enhancing the abundance of I-A^{g7} (Rinderknecht et al., 2010).

Similar to our previous *in vitro* data, we saw significant enhancement of I-A^{g7} in all APC cell types expressing introduced M98A Ii genes in PLN (Fig. 5, the third row and Fig. 6B). More remarkable I-A^{g7} elevation by M98A was observed in splenic APC (B cells, monocytes, macrophages and DC, Fig. 5, the first row and Fig. 6A) compared with the same cells in PLN. Specifically, average enhancements of 1.27, 7.3, 6.51, 4.97 fold in median MFI of I-A^{g7} by M98A were observed in transduced-splenic B cells, macrophages, monocytes and DC respectively, compared to untransduced populations set to 1 (Fig. 6A). Although the relative order by cell type was similar, smaller increases in the M98A-induced I-A^{g7} levels were seen in transduced-B cells, macrophages, monocytes and DC from PLN: 0.33, 2.69, 1.04, and 1.26 fold enhancements, respectively (Fig. 6B). We also found that wt Ii increased I-A^{g7} levels in PLN and spleen, generally to a smaller degree than M98A, except in PLN monocytes and DC (Fig. 5 the 2nd and 4th rows as well as Figs. 6A and B). Specifically, in spleen cells, average enhancements of I-A^{g7} median MFI of 0.64, 4.14, 1.47 and 2.66 fold were observed in wt Ii transduced B cells, macrophages, monocytes and DC respectively; in PLN, 0.16, 2.16, 1.86 and 1.57 fold increases in I-A^{g7} median MFI were detected in the corresponding APC cell types (Figs. 6A and B). Although the differences in I-A^{g7} expression driven by M98A compared to wt Ii were only statistically significant in splenic B cells, the trend that M98A enhanced I-A^{g7} to higher level than wt was seen in other cell types (Figs. 6A–B).

To examine the possibility that the enhanced functional effect of M98A compared to wt (Fig. 5 rows 1–4 and Figs. 6A,

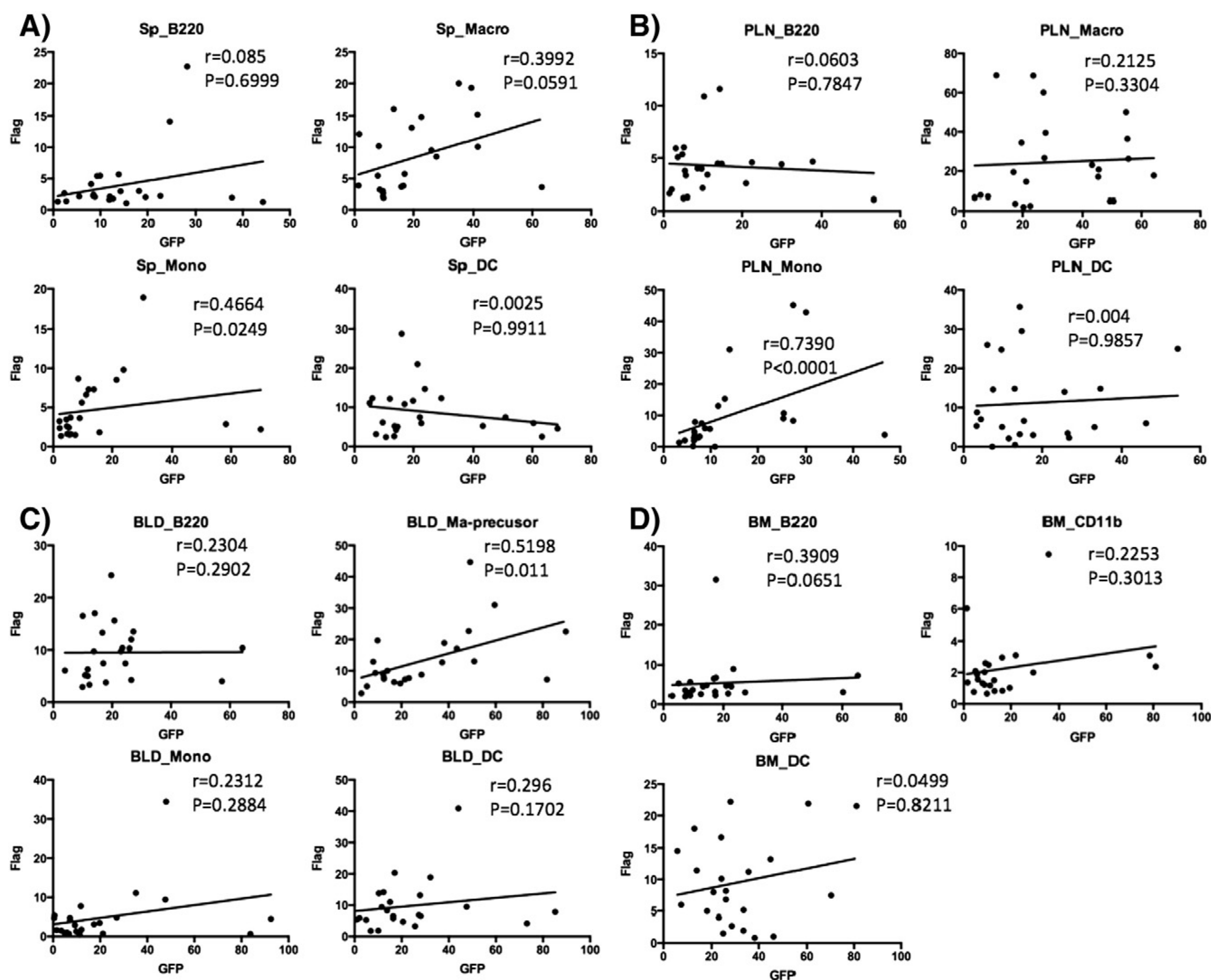


Figure 2 Correlation between GFP and Ii expression in various APC types from different organs. Samples from A. spleen; B. PLN; C. blood; and D. BM demonstrate the relationship between GFP and Ii in B cells, monocytes/macrophages (in blood, macrophages are macrophage precursors whereas in BM, macrophages and precursors are combined into myeloid cells) and DC. The correlations are represented by the regression lines and analyzed by the nonparametric Spearman rank correlation test. r and P levels from the Spearman test are shown for each chart on every panel. Data are from combined wt (n = 12) and M98A (n = 11) Ii recipients from three independent transplantation experiments.

B) is due to the higher expression of M98A Ii *versus* wt Ii, we measured level of introduced Ii, represented by Flag tag expression, in wt *vs.* M98A recipients. As shown in Figs. 6E and F, all transduced populations (*i.e.* Flag$^+$ population, compared to background on Flag$^-$ cells set to 1) expressed significant levels of Flag over background. Flag levels in M98A and wt recipients were very similar, the only difference being between the M98A and wt Flag MFI in splenic macrophages (3.289 ± 0.1692 in M98A *vs.* 2.743 ± 0.1210 in wt; P = 0.0146). When we normalized the change in I-A^{g7} levels for changes in levels of Ii (using median Flag MFI), the effects of M98A compared to wt Ii remained. In splenic B cells, macrophages, monocytes and DC, the average I-A^{g7}/Flag values in M98A were 0.67, 2.6, 2.47 and 1.93 *vs.* 0.46, 2.27, 0.89 and 1.34 in wt. Notably, the difference between M98A and wt effects in splenic B cells remained statistically significant (P = 0.0406). In corresponding cell types in PLN, the average I-A^{g7}/Flag values in M98A were 0.45, 1.34, 0.92 and 0.74 *vs.* 0.39, 0.92, 1.24 and 0.74 in wt. These results demonstrated that the enhanced functional effects of M98A than wt were not due to increased Ii expression (compare Figs. 6I & J *vs.* 6A & B).

The increase in I-A^{g7} was smaller in BM and blood, compared to spleen and PLN. In blood, the enhancing effect of Ii on class II levels could be only seen in the macrophage-precursors (Fig. 5 rows 5 and 6, Fig. 6C). In BM, although statistical significance was reached in more cell types, the increases were small compared to those in PLN and spleen (Fig. 5 rows 7 and 8, Fig. 6D). For example, the average enhancement of median MFI of I-A^{g7} in BM B cells, myeloid cells and DC was 0.09, 0.35 and 0.53 in M98A recipients and 0.07, 0.28 and 0.57 in wt recipients (Fig. 6D). These results suggest that the function of transgenically encoded Ii is influenced by the maturation or activation state of the expressing cells in different locations. No differences of Flag level between wt *vs.* M98A recipients were observed in any APC cell types from blood or BM (Figs. 6G and H). Even after normalization for Flag level, no significant

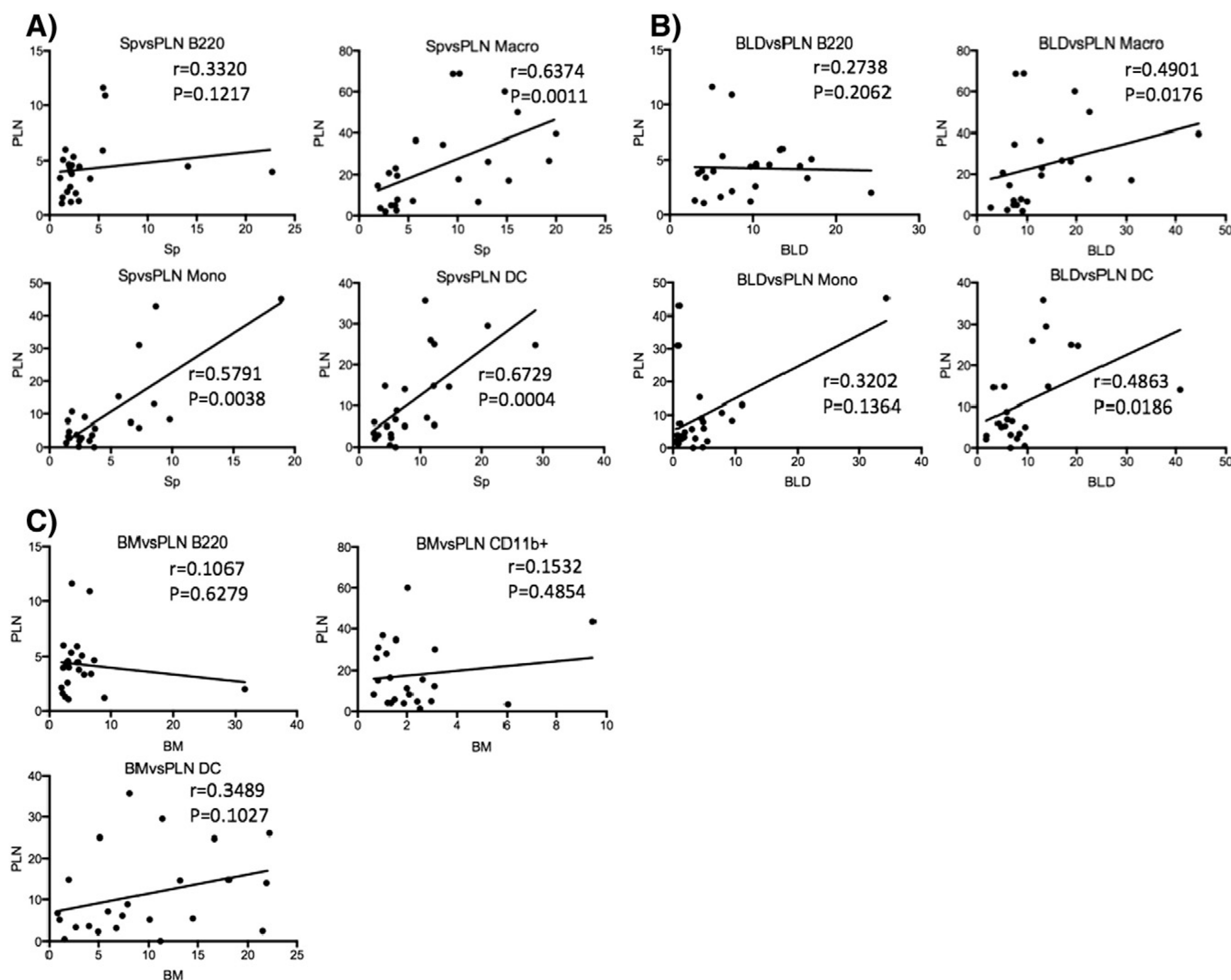


Figure 3 Correlation between Ii level in PLN and its levels in other organs. Ii expression in different APC cell types was compared between PLN and spleen (panel A); PLN and blood (panel B); and PLN and BM (panel C, PLN monocytes and macrophages were combined into myeloid cells). The regression lines were drawn, and the associations were tested by the Spearman test, as described in Fig. 2. Data are from combined wt (n = 12) and M98A (n = 11) Ii recipients from three independent transplantation experiments.

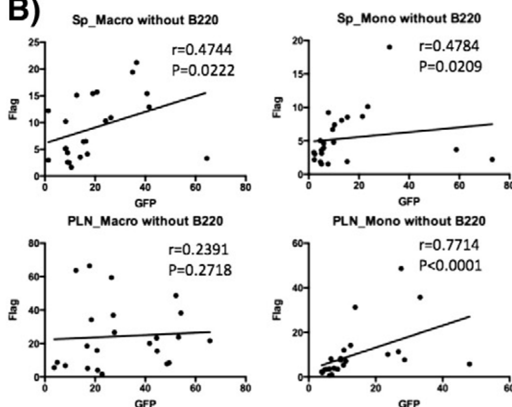
difference between wt *vs.* M98A recipients was observed in I-A^{g7} levels in all APC types from both blood and BM (Figs. 6K–L).

Discussion

Using a dual-promoter lentivirus-transduced BM-HSC transplantation model, we report here that both transgenes (*i.e.* murine invariant chain (Ii) and the GFP reporter) can be detected in various APC cell types from different organs (Fig. 1). Thus, this lentiviral vector using two ubiquitous promoters – the housekeeping Elongation Factor 1a (EF1a) for Ii and Murine Stem Cell Virus (MSCV) for GFP – is efficient in expressing the two transgenes together. However, imbalanced expression of Ii and GFP was observed, with higher expression of GFP than Ii in general and with correlation of GFP and Ii only in monocytes/macrophages (Figs. 1 and 2). This imbalance may be due to vector rearrangements and/or deletions, epigenetic modification(s) or competition between promoters or enhancers for factors or competition for ribosomal association (Curtin et al., 2008). Although the MSCV promoter is functional in human HSC (Choi et al., 2001) and has been optimized in murine embryonic stem cells with additional CPG mutations at LTR region (Swindle et al., 2004), we found that it may be specifically inhibited in the murine HSC environment both *in vivo* and *in vitro* (Wang et al., 2013). On the other hand, EF1a induced the highest transgene expression in human HSC (*in vitro*) and their progeny cells (*in vivo*) among tested promoters, including MSCV (Sirven et al., 2001; Woods et al., 2002). Even with the better performing promoter EF1a to drive Ii expression, we still observed lower levels of Ii than of GFP encoded by the commercially optimized codons (Figs. 1 and 2). Thus, further improvement of expression of the gene of interest by optimization of the vector and/or gene codons (Ellis, 2005; Moreno-Carranza et al., 2009) or by using a cell-specific promoter (Cui et al., 2002) likely is warranted. As we only observed the correlation between GFP and Ii in monocytes/macrophages and there are technical difficulties detecting the GFP$^+$Ii$^+$ population (*i.e.* Ii detection requires permeabilization, whereas this procedure bleaches the fluorescence of GFP), the use of GFP as a pan-representative for Ii should be done with caution in this setting.

A)

Organ	Cell type	Major marker used	% of cells bearing the other two APC markers	
Spleen	B cells	B220$^+$	CD11b$^+$: 2.403 ±1.323	CD11c$^+$: 1.781±1.070
	Macrophages/monocytes	CD11b$^+$	B220$^+$: 6.414±5.163	CD11c$^+$: 13.161±9.704
	DC	CD11c$^+$	B220$^+$: 15.989±11.455	CD11b$^+$: 44.257±22.777
PLN	B cells	B220$^+$	CD11b$^+$: 2.547±0.865	CD11c$^+$: 2.024±0.937
	Macrophages/monocytes	CD11b$^+$	B220$^+$: 16.266±9.896	CD11c$^+$: 10.249±6.916
	DC	CD11c$^+$	B220$^+$: 18.101±10.810	CD11b$^+$: 21.876±15.088
BLD	B cells	B220$^+$	CD11b$^+$: 2.403±1.548	CD11c$^+$: 2.058±1.186
	Macrophages/monocytes	CD11b$^+$	B220$^+$: 1.363±0.789	CD11c$^+$: 8.682±5.397
	DC	CD11c$^+$	B220$^+$: 12.536±9.980	CD11b$^+$: 65.517±12.765
BM	B cells	B220$^+$	CD11b$^+$: 1.630±1.507	CD11c$^+$: 2.482±1.280
	Macrophages/monocytes	CD11b$^+$	B220$^+$: 1.436±1.243	CD11c$^+$: 2.310±1.956
	DC	CD11c$^+$	B220$^+$: 32.921±12.649	CD11b$^+$: 48.791±10.731



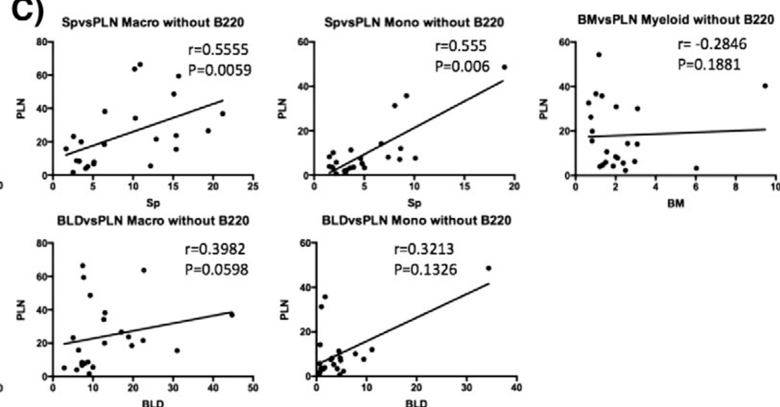
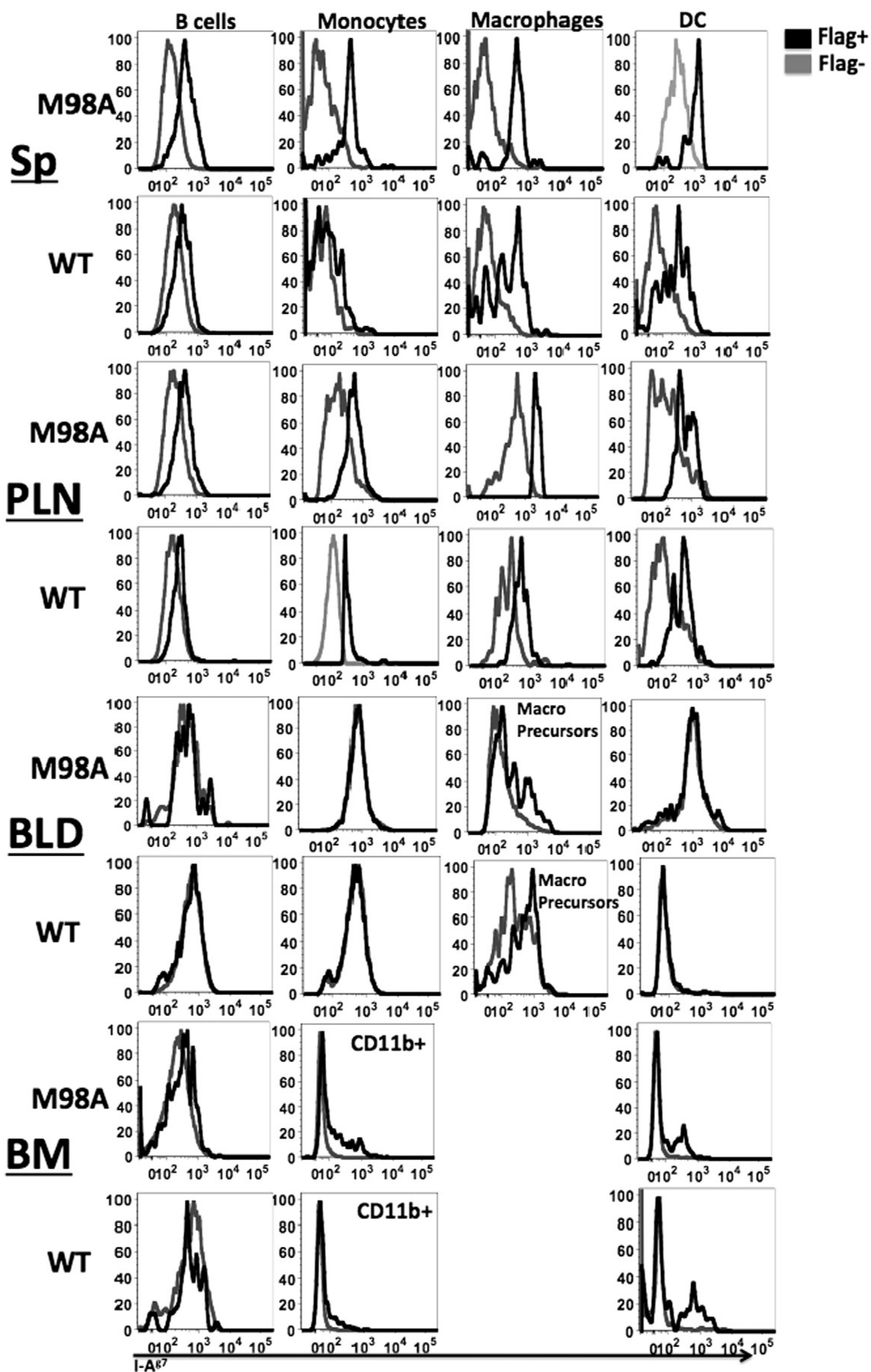


Figure 4 Analysis of CD11b$^+$B220$^-$ cells. A. Percentage of cells bearing other APC markers. Data summarized from all 23 mice and shown as mean ± SD. B. GFP-Flag correlation analysis in spleen and PLN macrophages and monocytes after removal of B220$^+$ subset from CD11b$^+$ population. C. The association of Flag expression in PLN and other organs in CD11b$^+$B220$^-$ population. No statistical difference between wt and M98A recipients for any sub-population from any cell type was observed (data not shown), so the two were combined. Correlations were tested by the Spearman test, as described in Fig. 2.

We also observed the highest levels of Ii and GFP expression in macrophages, intermediate levels in DC, and lowest expression in B cells from blood, spleen and PLN (Fig. 1). The trend is not observed in BM. The higher level of transgene expression in myeloid compared to lymphoid compartments has been reported (Amsellem et al., 2002; Mostoslavsky et al., 2005). The same trend was observed in progeny derived from HSC transduced with oncoretrovirus (Klug et al., 2000). Possible mechanisms underlying lower transgene expression in B cells include chromatin remodeling accompanying B cell commitment, the absence of positively acting transcription factors or the presence of silencing factors, and/or higher levels of DNMT3 activity in lymphoid cells compared to myeloid cells (Klug et al., 2000). These factors all contribute to silencing of transgene expression at the transcriptional level. It also is possible that the EF1a promoter works better in myeloid than in lymphoid compartments. As DC include both myeloid and lymphoid subsets (Martin et al., 2000), the transgene level is expected to be intermediate among the three cell types.

Blood and BM are the most common samples collected for detecting transgene expression in mice, nonhuman primates and humans after administration of HSC-mediated gene therapy. This is useful for some therapeutic transgenes carried by blood cells (Aiuti et al., 2013; Biffi et al., 2013; Cartier et al., 2009; Kim et al., 2009; Sirven et al., 2001; Trobridge et al., 2008). However, the expression of therapeutic genes can be substantially lower in target organs, such as the lung, compared to blood (Aguilar et al., 2009). In addition, the expression of certain immune molecules, like MHC class I molecules, derived from lentivirus-transduced HSC were only transiently detected cells at the protein level in peripheral blood, although their mRNAs were consistently expressed (Zhang et al., 2005). Lower levels of GFP driven by EF1a or an APC-specific promoter were observed in spleen compared to BM (Cui et al., 2002). Here, we did not observe any correlation between Ii expression levels in BM and PLN in B cells, myeloid cells and DC. However, a confounding variable is that the markers we used allow inclusion of cells at different development stages. For example,

Figure 5 Functional analysis of introduced Ii. I-A^{g7} levels in exogenous Ii-expressing (represented by Flag expression, black line) *vs.* non-expressing populations (gray line) were compared in spleen (rows 1 and 2); PLN (rows 3 and 4); blood (BLD, rows 5 and 6) and BM (rows 7 and 8) from one wt Ii containing lentivirus-transduced mouse (rows with even numbers) and one M98A-transduced mouse (rows with odd numbers) 5 m post-transplantation. Data shown are representative of three independent transplantation experiments.


B cells
Monocytes
Macrophages
DC
Flag+
Flag-
Sp
M98A
WT
PLN
BLD
BM
Macro Precursors
CD11b+
I-A^{g7}

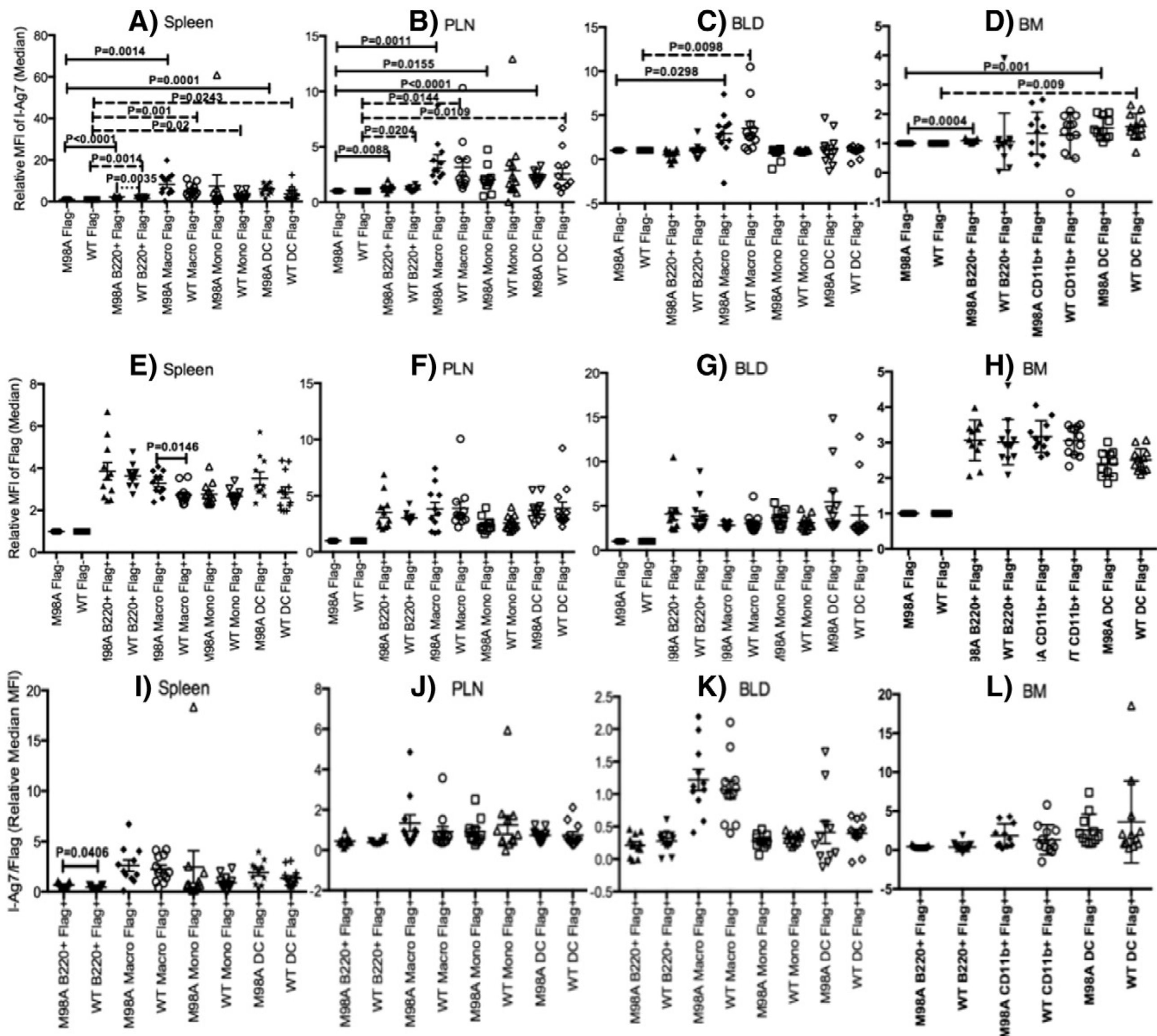


Figure 6 Relative median MFI of I-A^{g7}, Flag and I-A^{g7}/Flag in exogenous Ii-transduced *vs.* non-transduced populations. Relative median MFI of I-A^{g7} in Ii-transduced *vs.* non-transduced populations from various APC cell types were compared in spleen (A); PLN (B); blood (C); and BM (D). Panels A-D: Median MFI of transduced cells was normalized to that of non-transduced cells set to 1. Comparisons between relative I-A^{g7} median MFI in transduced population and non-transduced population in HSC recipients transduced with each lentivirus (*i.e.* wt or M98A) were analyzed by the paired *t* test. Comparisons between I-A^{g7} level of wt and M98A derived from same cell (marker-defined) population and organ were analyzed by the non-paired *t* test due to different numbers of wt *vs.* M98A recipients. Only those differences that reached $P < 0.05$ are shown. The differences between transduced *vs.* non-transduced populations in M98A and wt recipients are represented by bold lines and dashed lines, respectively. The difference between wt and M98A recipients from same source is shown by the dotted line. Panels E–H, relative median MFI of Flag in non-transduced population (Flag$^-$, set to 1) and transduced population (Flag$^+$, normalized to Flag$^-$) from different organs (E, F, G, H for spleen, PLN, blood and BM respectively) harvested from wt or M98A recipients were compared, using paired or non-paired *t* test as in panels A–D. Panels I–L, the ratios of relative median MFI of I-A^{g7} to relative median MFI of Flag in the transduced population (Flag$^+$, normalized to Flag$^-$ which was set to 1) from different organs (I, J, K, L for spleen, PLN, blood and BM respectively) of wt or M98A recipients were compared using non-paired *t* test. Data combined wt (n = 12) and M98A (n = 11) Ii recipients from three independent transplantation experiments.

B220$^+$ cells in BM contain 4 stages of premature subsets before mature B cells (Nagasawa, 2006), whereas B220$^+$ cells in PLN and spleen are mostly mature B cells. This issue needs to be considered, especially when analyzing BM. Blood Ii level correlated with PLN Ii level only in DC (Figs. 3 and 4). Thus, blood can be used as an indicator of Ii transgene expression in certain, but not all, cell compartments.

For some genes, the difference in transgene expression in BM/blood and other target organs can be both quantitative and qualitative. For example, the activities of introduced

acid α-glucosidase, which is deficient in a lysosomal glycogen storage disorder, Pompe disease, were different in blood, BM and spleen 8 m post lentivirus-transduced HSC transplant (van Til et al., 2010). The activities of lentivirus-encoded α-L-iduronidase, which is deficient in another lysosomal storage disorder, Type I mucopolysaccharidosis, were also different in PBMC compared to other target organs, including the brain (Visigalli et al., 2010). Accordingly, in a recent trial of human gene therapy mediated by lentivirus, the introduced gene function was detected directly in the target organ using cerebrospinal fluid, in addition to blood (Biffi et al., 2013). In our studies, introduced Ii had more enhancing effect on I-A^{g7} levels in secondary immune organs, such as spleen and PLN than it did in BM and blood (Figs. 5 and 6). The professional APC (*i.e.* B cells, macrophages and DC) we studied all originate in BM and are released into circulation, followed by maturation in secondary immune organs, such as spleen and lymph nodes (Gordon and Taylor, 2005; LeBien and Tedder, 2008). Ii is required for B cell maturation (Matza et al., 2002), and Ii level increases during the development of B cells (data not shown, https://gexc.stanford.edu/ (Seita et al., 2012)). GM-CSF (granulocyte-macrophage colony-stimulating factor), the main cytokine promoting the transition of monocytes to mature macrophages, increases Ii and not class II expression in monocytes, but not in macrophages (Klagge et al., 1997). Ii deficiency affects the maturation of DC carrying certain murine class II alleles, *i.e.* $H2^b$ (Rovere et al., 1998). As Ii is involved in the maturation of all three professional APC cell types, it is perhaps not surprising that we observed Ii function at sites of mature cells (*i.e.* secondary immune organs), compared to BM and blood. The incomplete function of introduced Ii in APC from BM and blood may be due to higher levels of cellular cystatin C than in APC from spleen or PLN. Cystatin C is an inhibitor of cathepsin S, the main protease in charge of Ii processing in tissues other than thymus, and is expressed at higher levels in immature *vs.* mature DC (Pierre and Mellman, 1998). Our results suggest that during the maturation of APC, Ii function is enhanced, in addition to the reported increase in its synthesis (Engering et al., 1998). The phenomenon we observed likely is not broadly generalizable, but may extend to other immune molecules that, like Ii, interact with other developmentally regulated proteins.

When normalized for expression differences, M98A mutant Ii had a modestly increased ability to enhance I-A^{g7} levels compared to wt Ii, confirming our *in vitro* data (Rinderknecht et al., 2010). However, in these *in vivo* experiments, overcoming the reduced affinity between Ii and I-A^{g7} by increased wt expression alone is also an efficient way to stabilize I-A^{g7} and may have therapeutic utility.

Conclusions

We report that two transgenes (invariant chain and GFP as a reporter gene) were both expressed using a dual-promoter lentivirus, with the EF1a promotor driving Ii expression. After transplantation of transduced hematopoietic stem cells (HSC), GFP expression correlated with Ii expression in certain myeloid cells, but not other HSC-derived cells. Ii levels in APC in bone marrow did not reflect Ii expression in APC in secondary immune organs, specifically spleen and pancreatic lymph nodes (PLN); a contributor to this result may be differences in the composition of $B220^+$ cells in these locations. Blood Ii levels paralleled Ii levels in PLN only in DC. Interestingly, Ii expression had more robust functional consequences in the more mature immune environments, such as spleen and PLN, compared to BM and blood cells, potentially due to other interacting proteins expressed in these cells. Our results highlight the phenomena of importance for the analysis of gene expression after transplantation of genetically-modified HSC, particularly evaluation of transgene expression in relevant target organs.

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