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## Fucosylation with Fucosyltransferase (FT)-VI or FT-VII Improves Cord Blood Engraftment

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### Abstract

**Background**—Advantages associated with the use of cord blood (CB) transplantation include the availability of cryopreserved units, ethnic diversity and lower incidence of graft-*versus*-host disease when compared to bone marrow or mobilized peripheral blood. However, poor engraftment remains a major obstacle. We and others have found that *ex vivo* fucosylation can enhance engraftment in murine models and thus *ex vivo* treatment of CB with fucosyltransferase (FT)-VI prior to transplantation is under clinical evaluation (NCT01471067). However, FT-VII appears to be more relevant to hematopoietic cells and may alter acceptor substrate diversity. In this study, we compare the ability of FT-VI and FT-VII to improve the rapidity, magnitude, multi-lineage and multi-tissue engraftment of human CB hematopoietic stem and progenitor cells (HSPC) *in vivo*.

**Methods**—CD34-selected CB HSPC were treated with recombinant FT-VI, FT-VI or mock control, then injected into immunodeficient mice and monitored for multi-lineage and multi-tissue engraftment.

**Results**—Both FT-VI and FT-VII fucosylated CB CD34<sup>+</sup> cells *in vitro*, and both led to enhanced rates and magnitudes of engraftment when compared to untreated CB CD34<sup>+</sup> cells *in vivo*. Engraftment following treatment with either fucosyltransferase was robust at multiple timepoints, and in multiple tissues, with similar multi-lineage potential. In contrast, only FT-VII was able to fucosylate T- and B-lymphocytes.

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#### Conflicts of Interest

All authors, with the exception of Dr. Leonard Miller (please see details below), declare no conflict of interest exist. Leonard Miller, Ph.D. is Vice President of Research and co-founder of America Stem Cell, Inc. America Stem Cell, Inc., provided FT-VI and FT-VII used in the studies reported in this manuscript.

**Discussion**—While we found that FT-VI and FT-VII were similarly able to fucosylate and enhance the engraftment of CB CD34<sup>+</sup> cells, differences in their ability to fucosylate lymphocytes cells may modulate graft-*versus*-tumor and/or graft-*versus*-host effects and may allow further optimization of CB transplantation.

## Introduction

For effective homing and engraftment of hematopoietic stem and progenitor cells (HSPC) to the bone marrow (BM), it is thought that specific cell surface ligands expressed by the HSPC interact with receptors expressed by the endothelial cells lining the blood vessels of the hematopoietic system. While the expression of certain cell surface glycoproteins by HSPC might be sufficient for homing to hematopoietic tissues (1–9), there is evidence that the activity of specific ligands is improved when they are fucosylated (10–17). Fucosylation is the addition of fucose moieties by fucosyltransferase (FT)-directed, site-specific processes. Previous studies have revealed that CB HSPC have consistently lower levels of endogenous fucosylation than BM or mobilized peripheral blood (mPB) progenitor cells and that *ex vivo* fucosylation using recombinant human FT-VI enhances the adhesion, homing and engraftment of CB HSPC in xenografts (18,19). From these data it appears that *ex vivo* fucosylation using FT-VI may be used to mitigate the delayed engraftment that is currently associated with CB transplantation. Thus, a clinical trial is underway testing the effect of *ex vivo* fucosylation of CB using recombinant human FT-VI prior to transplantation (NCT01471067).

However, FT-VI is not normally expressed on hematopoietic cells, but rather in endothelial, epithelial, gastrointestinal and some malignant cells. In contrast, FT-VII is widely expressed on hematopoietic cells including BM CD34<sup>+</sup> cells (20). Indeed FT-VII appears to be the dominant fucosyltransferase responsible for producing leukocyte selectin ligand activity (21), and a spontaneous FT-VII mutation impairs selectin binding (22). Importantly FT-VII expression is unexpectedly low in CB HSPC (23), suggesting that fucosylation with FT-VII may provide a more physiologic approach to restoring fucosylated proteins to CB HSPC. The aim of this study was to compare the activities of FT-VI and FT-VII to identify any qualitative differences in the rate, magnitude, multi-lineage and multi-tissue engraftment of human CB HSPC *in vivo*.

## Materials and Methods

Two  $\alpha$ -(1,3)-fucosyltransferase enzymes, FT-VI and FT-VII (provided by American Stem Cell Inc, Floresville, TX), were compared for their ability to fucosylate CB HSPC in an *ex vivo* setting. Fucosylation was revealed by flow cytometry through the binding of HECA-452 (BD Biosciences), a directly conjugated (FITC), rat IgM antibody that reacts against fucosylated (sialyl Lewis X (sLe<sup>X</sup>)-modified) cell surface glycoproteins, including P-selectin glycoprotein ligand (PSGL)-1 (CD162) (24). HECA-452 was originally described as detecting a “cutaneous lymphocyte antigen” (CLA).

## Hematopoietic Cells

Fresh CB units were obtained and all animal work conducted under M. D. Anderson Cancer Center Institutional Review Board and Institutional Animal Care and Use Committee approved protocols, respectively. CB mononuclear cells (MNC) were isolated from fresh CB units by Ficoll-Histopaque density separation and CB CD34<sup>+</sup> cells enriched by magnetic-activated cell sorting (MACS) according to manufacturer’s instructions (Miltenyi Biotec, Auburn, CA.). MACS-selected CD34<sup>+</sup> cells were then pooled and divided into (i) untreated, (ii) FT-VI-treated or (iii) FT-VII-treated fractions. *Ex vivo* fucosylation was performed as previously described (18). Briefly, CB CD34<sup>+</sup> cells were treated at 10<sup>6</sup> cells/ml for 30

minutes at room temperature with 1 mM GDP  $\beta$ -fucose (EMD Biosciences, San Diego, CA.) in Phosphate Buffered Saline (PBS) containing 1% human serum albumin (HSA, Baxter Healthcare Corp., Westlake Village, CA.) and in the previously optimized concentrations of 100 mU/ml FT-VI, or 75  $\mu$ g/ml FT-VII. Untreated cells were incubated as above except no enzyme was added. After incubation, cells were washed in PBS containing 1% HSA, cellularity determined by hemocytometer and cells diluted in saline prior to intravenous injection into sublethally-irradiated NOD-SCID IL-2R $\gamma^{\text{null}}$  (NSG) mice (Jackson Laboratories, Bar Harbor, ME). A  $^{137}\text{Cs}$  source delivered a total sublethal radiation dose of 300 cGy over one minute (J. L. Shepherd and Associates Mark I-25 Irradiator, San Fernando, CA). In each group, mice received  $10^5$  CB CD34 $^+$  cells.

### **Assessment of human engraftment in peripheral blood (PB), BM and spleen (SP) of NSG recipients**

Human engraftment was determined once or twice *per* week by withdrawal of 40 $\mu$ l of PB from the retro-orbital sinus of anesthetized mice and red blood corpuscles were lysed (Pharm Lyse, BD Pharmingen). At >100 days after transplant, BM and spleen (SP) were also harvested. Samples were assessed for the presence of human and murine CD45 positive cells by flow cytometry (BD FACSCalibur) using PE-conjugated rat anti-mouse CD45 and APC-conjugated mouse anti-human CD45 (both BD Biosciences). Mouse PB, human CB and antibody isotypes provided appropriate controls. Data were acquired and analysis performed using CellQuest Pro software (BD Biosciences). The percentage of human engraftment was calculated as: [Percent human CD45  $\div$  (Percent human CD45 + Percent murine CD45)]  $\times$  100

### **Secondary transplantation of human CD34 $^+$ cells from the bone marrow of primary CB CD34 $^+$ recipients**

When recipients of FT-VI-treated or FT-VII-treated CB CD34 $^+$  cells were euthanized at >100 days after transplant (as previously described), femoral BM from individuals in each group ('primary' recipients) was pooled and transplanted into 3 groups of sublethally-irradiated NSG mice (n=5 mice/gp, 'secondary' recipients). Equivalent numbers of nucleated BM cells (approximately  $3 \times 10^7$ /mouse) were transplanted intravenously and the number of human CD34 $^+$  cells (approximately  $2 \times 10^6$ /mouse) retrospectively assessed by flow cytometry of the pooled BM samples (as previously described). Mice were euthanized >10 weeks after transplantation and human engraftment assessed in PB, BM and SP (as previously described). This would reveal any impact of the initial *ex vivo* fucosyltransferase treatment on long-term engraftment potential.

### **Additional flow cytometric analyses of CB MNC to compare the activities of FT-VI and FT-VII**

While this study was focused primarily on the impact of FT-VI and FT-VII on CB CD34 $^+$  cells, limited analysis of the HECA-reactivity of CB MNC following treatment with FT-VI or FT-VII was performed allowing a comparison of fucosylation of non-CD34 $^+$  cell types. The HECA-reactivity of total CB MNC (CD45 $^+$ ), CB T-lymphocytes (CD3 $^+$ ), CB B-lymphocytes (CD19/20 $^+$ ) and CB myeloid cells (CD14 $^+$ ) was assessed (all antibodies from BD). Data were acquired using a FACSCalibur (Becton Dickinson) and analysis performed using CellQuest Pro software (BD Biosciences).

### **Statistical analysis**

Data are presented as mean  $\pm$  SEM and compared using the Student's T-test with significance assumed at *P* 0.05.

## Results

### Fucosylation of CB CD34<sup>+</sup> cells with FT-VI or FT-VII

The HECA-452 anti sLe<sup>x</sup> antibody revealed that <25% of CB CD34<sup>+</sup> HSPC were ‘fucosylated’ endogenously (Figure 1A). Treatment with either FT-VI or FT-VII, significantly increased (>90%) the proportion of HECA-reactive (‘fucosylated’) CB CD34<sup>+</sup> cells (Figures 1B and 1C, respectively). The fucosylation profiles of FT-VI and FT-VII had similar maximal values, although the distribution of fucosylation (HECA-452) intensities was broader for FT-VI, providing a greater range (low to high) of fucosylation on a *per cell* basis than was observed for FT-VII.

### Human engraftment measured in PB of sublethally-irradiated NSG recipients

Cohorts of sublethally-irradiated NSG were intravenously injected with 10<sup>5</sup> untreated, FT-VI-treated or FT-VII-treated CB CD34<sup>+</sup> cells (Figure 2). As reported previously, FT-VI treatment significantly enhanced the rate and magnitude of engraftment of CB CD34<sup>+</sup> cells in this xenograft assay (18). For the first time, we report here, that FT-VII treatment of CD34<sup>+</sup> CB HSPC led to a similarly robust magnitude of engraftment when compared to FT-VI treated CB HSPC. Mice receiving FT-VII-treated CB CD34<sup>+</sup> cells demonstrated a significantly higher level of engraftment by day 32 post-transplant compared to recipients of untreated CB CD34<sup>+</sup> cells (12±3% vs. 5±1%, *P*=0.04). Similarly, recipients of FT-VI-treated CB showed a trend toward increased engraftment by day 32 (8±1% vs. 5±1%, *P*=0.05). Mice receiving either FT-VI or FT-VII-treated CD34<sup>+</sup> cells both sustained a significantly improved (approximately 3-fold) engraftment over mice receiving untreated CB CD34<sup>+</sup> cells. Although recipients of FT-VII-treated CB CD34<sup>+</sup> cells demonstrated consistently greater engraftment than mice receiving FT-VI-treated CB CD34<sup>+</sup> cells, this difference was only statistically different on day 74 (“#” in Figure 2). By the peak engraftment at the day 86 time point, recipients of untreated CB CD34<sup>+</sup> cells had achieved 15±4% human engraftment, while engraftment was 2.7-fold greater (40±6%, *P*=0.01) in the recipients of FT-VI-treated CB CD34<sup>+</sup> cells and 3.5-fold greater (52±5%, *P*=0.001) in the recipients of FT-VII-treated CB CD34<sup>+</sup> cells. *Ex vivo* treatment of human CB CD34<sup>+</sup> cells with either FT-VI or FT-VII significantly enhanced the rate and magnitude of engraftment in this NSG engraftment model. Magnitude of engraftment trended in favor of FT-VII treatment at most timepoints including early and peak engraftment timepoints.

### Human engraftment in BM and spleen

When mice were euthanized, human engraftment in the BM and spleens of the engrafted mice was assessed (Table 1). Flow cytometric assessment of BM of mice receiving untreated CB CD34<sup>+</sup> cells revealed 68±4% human engraftment. Human engraftment was significantly (*P*=0.006), 1.3-fold increased to 88±3% in the recipients of FT-VI-treated CB CD34<sup>+</sup> cells and significantly (*P*=0.0001) 1.4-fold increased to 96±1% in the recipients of FT-VII-treated CB CD34<sup>+</sup> cells when compared to recipients of untreated CB CD34<sup>+</sup> cells. A significant difference in the human engraftment between recipients of FT-VI or FT-VII-treated CB CD34<sup>+</sup> cells was observed (88±3% vs. 96±1%, respectively, *P*=0.04). Flow cytometric assessment of human engraftment in the spleens of mice receiving untreated CB CD34<sup>+</sup> cells revealed 77±3% human engraftment. Human engraftment was not significantly different from this value in the recipients of FT-VI-treated CB CD34<sup>+</sup> cells (84±4%, *P*=0.219), but was significantly (*P*=0.004) 1.2-fold increased at 91±1%, in the recipients of FT-VII-treated CB CD34<sup>+</sup> cells.

Multi-lineage analysis revealed that in recipients of CB CD34<sup>+</sup> treated with FT-VI or FT-VII, human B-lymphocyte engraftment was similarly, significantly increased (14–15% of BM and 30–33% of the spleen) as compared to approximately 10% BM and 15% of spleen,

in recipients of untreated CB CD34<sup>+</sup> cells (Table 1). Outside of human B-lymphocyte engraftment, there were no further differences in the patterns of multi-tissue, multi-lineage human engraftment between recipients of untreated and FT-VI treated CB CD34<sup>+</sup> cells. However, recipients of FT-VII treated CB CD34<sup>+</sup> cells showed greater (approximately 2-fold) levels of human myeloid cells in BM (approximately 17% vs. 9%) and human T-lymphocytes in the spleen (approximately 4% vs. 2%) than those found in recipients of untreated or FT-VI treated CB CD34<sup>+</sup> cells. Therefore, although human engraftment was similarly enhanced when comparing the BM and spleens of recipients of FT-VI and FT-VII treated CB CD34<sup>+</sup> cells, FT-VII treatment was associated with elevated human total and myeloid engraftment in the BM and elevated human T cell engraftment in the spleen.

### Secondary transplantation of human CD34<sup>+</sup> cells from the bone marrow of primary CB CD34<sup>+</sup> recipients

Flow cytometric assessment >10 weeks after secondary transplantation revealed that there was no significant difference between the levels of human engraftment measured in the peripheral blood (0.56±0.22% vs. 0.69±0.13,  $P=0.657$ ), BM (2.86±1.46% vs. 1.08±0.25%,  $P=0.273$ ) and spleen (2.94±1.20% vs. 1.59±0.34%,  $P=0.708$ ).

### Comparison of FT-VI and FT-VII activities on non-CD34<sup>+</sup> CB MNC

The *in vivo* experiments reported used CD34-selected CB cells to isolate the effects of fucosylation on HSPC homing and engraftment, however, in the majority of clinical CB transplantation protocols transplant the entire CB unit. Thus we compared the effects of FT-VI and FT-VII treatment on total CB MNC which included CD3<sup>+</sup> T-cells, CD19<sup>+</sup>/CD20<sup>+</sup> B-cells, and CD14<sup>+</sup> myeloid cells in addition to CD34<sup>+</sup> cells. Flow cytometric analysis of HECA-reactivity for untreated CB MNC revealed that the majority of CD45<sup>+</sup> cells were not endogenously fucosylated (Figure 3A). Consistent with these data, multi-lineage analysis revealed that CD3<sup>+</sup> T-cells, CD19<sup>+</sup>/CD20<sup>+</sup> B-cells, and CD14<sup>+</sup> myeloid cells (Figures 3D, 3G and 3J, respectively) were not endogenously fucosylated. When CB MNC were treated with FT-VI, two distinct CD45<sup>+</sup> peaks were revealed by flow cytometry (Figure 3B) indicating that while a proportion of the CB MNC were fucosylated by FT-VI, a proportion were not. Multi-lineage analysis revealed that while the majority of CD14<sup>+</sup> myeloid cells were fucosylated following treatment with FT-VI (Figure 3K), CD3<sup>+</sup> T-lymphocytes and CD19<sup>+</sup>/20<sup>+</sup> B-lymphocytes were not (Figures 3E and 3H, respectively). In contrast, the *ex vivo* treatment of CB MNC with FT-VII yielded a 100% HECA-reactive CD45<sup>+</sup> CB MNC product (Figure 3C). Consistent with this observation, multi-lineage analysis revealed that CD14<sup>+</sup> myeloid cells, CD3<sup>+</sup> T-lymphocytes and CD19<sup>+</sup>/20<sup>+</sup> B-lymphocytes were all fucosylated (Figures 3L, 3F and 3I, respectively).

## Discussion

In this study, we performed a direct comparison of FT-VI and FT-VII treatment of CB HSPC to assess whether any functional and/or qualitative differences would be seen in terms of the rate, magnitude, multi-lineage and multi-tissue engraftment *in vivo*. We demonstrate that the two enzymes similarly fucosylate CB CD34<sup>+</sup> cells *ex vivo*, and similarly improve the rate and magnitude of engraftment *in vivo*, with a trend toward higher engraftment following FT-VII treatment. As delayed engraftment and graft loss are significant obstacles for CB transplant patients, this may provide significant benefit. Furthermore, when a multi-lineage, multi-tissue assessment was made of the pattern of human engraftment in recipient mice, there was evidence that the transplantation of FT-VII (rather than FT-VI) treated CB CD34<sup>+</sup> cells was associated with improved human myeloid and T-lymphocyte engraftment. These data suggest that the treatment of CB CD34<sup>+</sup> cells with FT-VII (rather than FT-VI) may enhance the *de novo* generation of myeloid and lymphoid cells in the recipient,

potentially improving both neutrophil and T-cell recovery post-transplant. Given these results, FT-VII treatment appears to be a promising potential therapeutic approach for further enhancing cord blood transplantation. However, there are limitations to using these *in vivo* xenograft models to predict engraftment and post-transplant recovery. In particular, the murine hematopoietic microenvironment lacks several critical myeloid growth factors which do not cross species including interleukin (IL)-3, stem cell factor (SCF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). In addition the role of fucosylation on graft-*versus*-tumor and/or graft-*versus*-host are not addressed with this CD34-selected/immunodeficient mouse model.

In conclusion, these results further support the potential role of *ex vivo* fucosylation of CB HSPC to improve engraftment, and reveal differences in the fucosyltransferase activities of FT-VI *versus* FT-VII on the magnitude of multi-tissue engraftment, including enhanced myeloid and T-cell recovery. Further studies into improving CB transplant through enhanced homing/engraftment are needed.

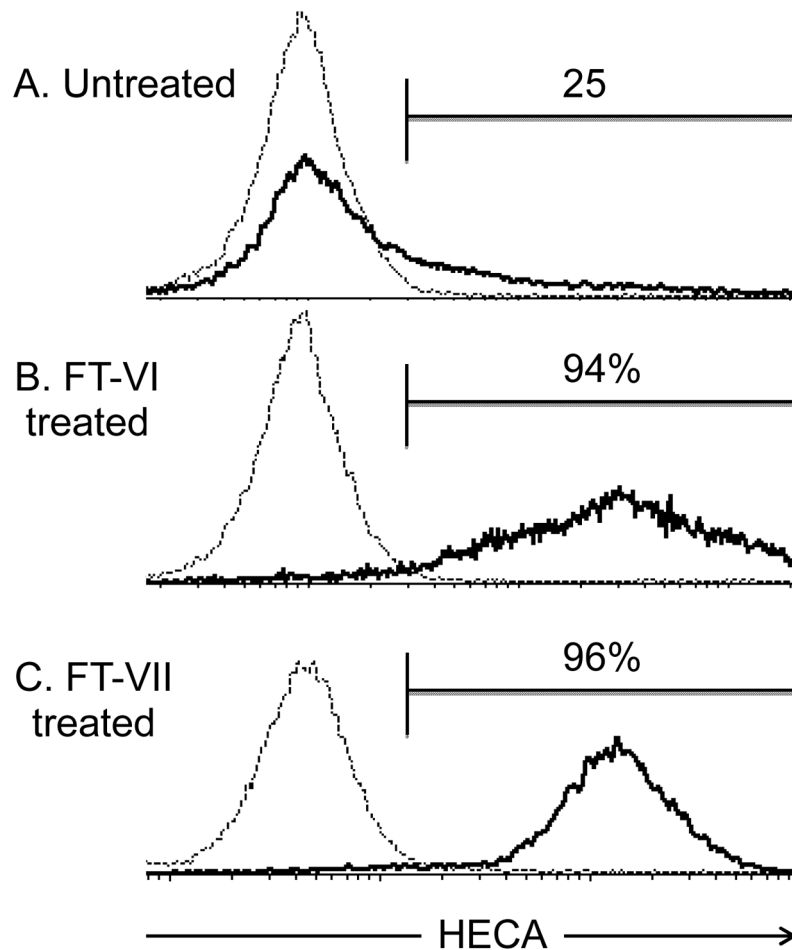
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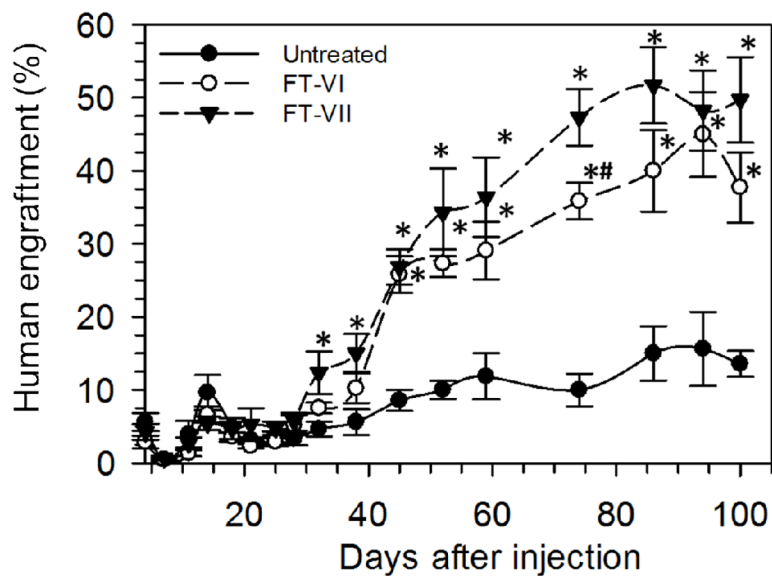
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**Figure 1. *Ex vivo* treatment of CB CD34<sup>+</sup> cells with FT-VI and FT-VII**

The HECA-452 antibody was used to reveal fucosylation. In a pool of untreated CB CD34<sup>+</sup> cells isolated from fresh CB units, approximately 25% were HECA-reactive (Figure 1A). This was increased to 94% (Figure 1B) and 96% (Figure 1C), respectively following *ex vivo* treatment with FT-VI or FT-VII.

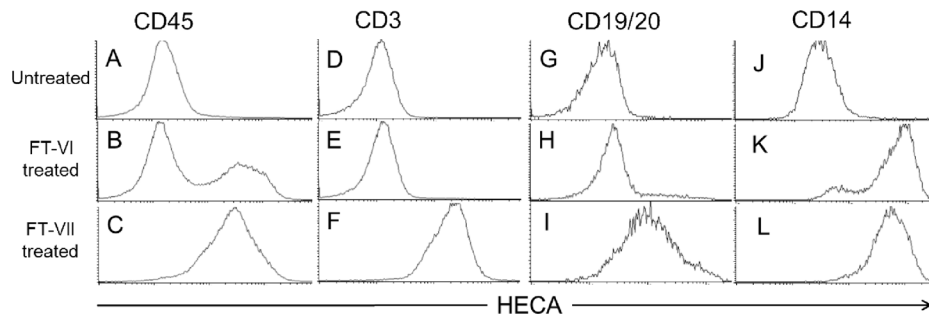




\*Significant difference from Untreated  
 #Significant difference between FT-VI & FT-VII

**Figure 2. Human engraftment measured in the peripheral blood (PB), bone marrow (BM) and spleens (SP) of sublethally-irradiated NSG mice following the transplant of untreated, FT-VI-treated or FT-VII-treated CB CD34<sup>+</sup> cells**

Significantly improved levels of human engraftment were observed in PB of sublethally-irradiated mice receiving  $10^5$  CB CD34<sup>+</sup> cells treated with FT-VI or FT-VII as compared to mice receiving  $10^5$  untreated CB CD34<sup>+</sup> cells.



**Figure 3. *In vitro* comparison of FT-VI and FT-VII activities against non-CB CD34<sup>+</sup> cells present in CB MNC**

*Ex vivo* treatment of CB MNC with FT-VI or FT-VII revealed a marked difference between the activities of the two  $\alpha$ -(1,3)-fucosyltransferases. Endogenous levels of fucosylation of CD45<sup>+</sup>, CD3<sup>+</sup>, CD19/20<sup>+</sup> and CD14<sup>+</sup> cells contained in CB MNC are shown (**3A**, **3D**, **3G** and **3J**). FT-VI-treatment fucosylated only part of the CD45<sup>+</sup> cell population (**4B**), which included CD14<sup>+</sup> myeloid cells (**3K**). It did not fucosylate CD3<sup>+</sup> (**3E**) or CD19/20<sup>+</sup> (**3H**) lymphocytes. In contrast, FT-VII fucosylated all CD45<sup>+</sup> cells (**3C**, **3F**, **3I** and **3L**).

Table 1

Multi-lineage, multi-tissue human engraftment.

CB CD34 <sup>+</sup> treatment	Human Engraftment (%)							
	Total (CD45)		B-cell (CD19/20)		Myeloid (CD14)		T-cell (CD3)	
	BM	SP	BM	SP	BM	SP	BM	SP
Untreated	68±4	77±3	10±1	15±2	10±2		1.8±0.2	
FT-VI	<b>88±3*</b>	84±4	<b>14±1*</b>	<b>30±0.4*</b>	9±2		2.1±0.5	
FT-VII	<b>96±1**</b>	<b>91±2*</b>	<b>15±1*</b>	<b>33±2*</b>	<b>17±2**</b>		<b>4.3±1.2</b>	

\* Significantly different from untreated

# Significantly different from FT-VI