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IMMUNE REGULATORY CELLS IN UMBILICAL CORD BLOOD: T REGULATORY CELLS AND MESENCHYMAL STROMAL CELLS

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A major goal in hematopoietic cell transplantation (HCT) is to retain the lymphohematopoietic potential of the cell transfer without its side effects. In addition to the physical injury caused by conditioning regimen, donor T cells can react to alloantigens of the recipient and cause graft-versus-host disease (GVHD), which accounts for the largest share of morbidity and mortality after HCT. Immune modulator cells such as regulatory T cells (Tregs) and mesenchymal stromal cells (MSCs) have shown promise in their ability to control GVHD and yet, in preclinical models, preserve the graft-versus-malignancy effect. Initially, MSCs and Tregs have been isolated from adult sources such as bone marrow or peripheral blood, respectively. More recent studies have indicated that umbilical cord blood (UCB) is a rich source of both cell types. We will review the current data on UCB-derived Tregs and MSCs and their therapeutic implications.

T REGULATORY CELLS

To guard against foreign pathogens and tumor cell growth as well as to prevent aberrant responses to self-antigens, the immune system has evolved to encompass several non-redundant regulatory mechanisms. The focus of this section will be on the biology and clinical applications of UCB $CD4^{+}25^{+}$ T regulatory cells (Tregs). Sakaguchi and colleagues first reported that $CD25^{+}$-depleted CD4 T cells transferred into nude mice resulted in autoimmune disease(Sakaguchi, *et al* 1995) which could be reversed by adding Tregs. For more than 10 years, the critical immune modulatory properties of Tregs have been well-described in mice(Asano, *et al* 1996, Suri-Payer, *et al* 1998, Takahashi, *et al* 1998, Thornton and Shevach 1998). In solid organ transplant settings, tolerance induced by the combined administration of donor-specific transfusions and costimulatory pathway blockade in vivo was dependent upon donor Tregs(Thornton and Shevach 1998), while Tregs present in the recipient at the time of skin or cardiac allografting were critical to achieving and maintaining allogeneic cell tolerance (reviewed in(Wood and Sakaguchi 2003)). Extending studies by Sakaguchi et al, investigators have shown that the adoptive transfer of Tregs suppressed pancreatic islet allograft rejection(Davies, *et al* 1999, Hara, *et al* 2001, Sanchez-Fueyo, *et al* 2002).

On the flip side, removal of Tregs from the donor allograft accelerated graft-versus-host disease (GVHD) and solid organ graft rejection(Anderson, *et al* 2004, Cohen, *et al* 2002,

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Hoffmann, *et al* 2002, Taylor, *et al* 2002), while freshly isolated or ex vivo expanded donor Treg infusion potently inhibited acute or chronic GVHD(Taylor, *et al* 2002, Zhao, *et al* 2008). In addition, donor or host Tregs were able to ameliorate ongoing chronic GVHD(Anderson, *et al* 2004, Zhao, *et al* 2008). In sublethally irradiated recipients of T cell-depleted allogeneic bone marrow, host anti-donor alloreactive T cells were able to mediate donor bone marrow graft rejection, which was inhibitable by donor Treg infusion(Hanash and Levy 2005, Joffre, *et al* 2004, Taylor, *et al* 2004). Donor Tregs also prevented GVHD-induced thymic atropy and in the process thereof accelerated the time course of T cell immune recovery(Trenado, *et al* 2003). In contrast to other approaches for regulating adverse T cell alloresponses in hematopoietic stem and progenitor cell transplant recipients, Tregs have been uniformly effective in various rodent models in independent laboratories and thus represent one of the most important advances in immune modulation in the past several decades. Given the striking results in rodent allogeneic transplantation models and high degree of morbidity and mortality associated with allogeneic cell transplantation, hematopoietic stem and progenitor cell transplant proved to be a reasonable first venue for donor Treg infusion.

Despite compelling preclinical data, clinical applications of donor Treg infusions have not come easy. Treg infusional trials in humans have lagged behind rodents due to the fact that $CD4^{+}25^{++}$ cells are present in a relatively low frequency population (~1–2%) in adult peripheral blood (PB). In rodents, a clearly distinguishable population of $CD4^{+}$ cells with a high antigen density of CD25 ($CD4^{+}25^{++}$) enables readily achievable separation from $CD4^{+}CD25^{-}$ population. In contrast, human PB contains an additional population of $CD4^{+}$ cells that express an intermediate density of CD25 ($CD4^{+}25^{+}$), do not express Foxp3, and function as previously activated effector cells (Fig 1A). Clinical testing adult peripheral blood Tregs also has been hampered of by the availability of GMP reagents for rigorous Treg purification. Foxp3, a helix-loop-helix transcription factor, is expressed in Tregs with potent suppressor cell activity, as well as some activated conventional T cells in humans. Moreover, isolation of $FoxP3^{+}$ cells requires permeabilization of T cells and hence is incompatible with their viability. Other markers such as CD27 and CD127 (IL7Ra chain) may denote fractions with the $CD4^{+}25^{++}$ population enriched for suppressor cell function, though GMP reagents are not yet available for widespread testing.

In contrast to peripheral blood, UCB T cells are largely naïve and as such, there is a distinct $CD4^{+}CD25^{bright}$ subset that exists in relatively high frequency compared to PB. Therefore, the likelihood for co-purification of activated or memory $CD4^{+}25^{+}$ T cells in UCB units is reduced when compared to PB perhaps as a result of the relatively low exposure of the fetus to environmental pathogens and vaccines as compared to the adult. The skewing of $CD4^{+}25^{+}$ T cells toward a Treg suppressor cell phenotype versus conventional T cell may explain, at least in part, the relatively low incidence of acute GVHD using UCB compared to similar HLA-matched bone marrow grafts. As such, $CD4^{+}25^{++}$ Tregs can be isolated with a less cumbersome isolation procedure that relies upon the high density of CD25 antigen predominately on Tregs. The final UCB Treg isolation products contain ~50% CD4/CD25/FoxP3 flow cytometry, consistent with a Treg phenotype.

As with conventional T cells, Tregs require T cell receptor (TCR) ligation and costimulation. These signals can be provided by anti-CD3 and anti-CD28 antibodies, attached to microbeads produced under good manufacturing production (GMP) conditions that can cross-link the TCR and CD28 molecules (Fig 1B). Exogenous IL-2 is critical as Tregs do not produce a sufficient amount of IL-2 for their own expansion. Using these antibody-coated beads, UCB Tregs can be expanded under GMP conditions by ~200-1000 fold in < 3 weeks(Godfrey, *et al* 2005). In contrast, PB Treg expand only 50–100 fold because Rapamycin must be added to suppress the outgrowth of contaminating activated or

memory T cells (Fig 1B). Such polyclonally expanded UCB and PB Treg are routinely ≥50% $CD4^{+}25^{++}Foxp3^{+}$ and can potently suppress host and third-party alloresponses in vitro, currently at ratios of 1:16–1:64 of Tregs to conventional T cells when manufactured under GMP conditions. In vivo, UCB Tregs can suppress unrelated donor peripheral blood mononuclear cell responses at ratios of 1:1–1:3 in a model of xenogenic GVHD. In clinical trials, non-myeloablated or myeloablated recipients of two unrelated UCB units have been given standard GVHD prophylaxis (cyclosporine A; mycophenolate mofetil) also received third-party, HLA partially-matched Tregs.

Although $CD25^{bright}$ Tregs are more readily purified from UCB units than PB(Godfrey, *et al* 2004, Godfrey, *et al* 2005), only ~5–7.5 × 10^{6} Tregs can be isolated from a single frozen UCB unit. Moreover, the number of Tregs available for infusion are limited by those that can be isolated from that unit alone and therefore, multiple infusions or use of Tregs for both GVHD prevention and subsequent therapy, if needed, would be problematic. Therefore, alternative expansion procedures have been explored. Toward that end, we have explored the use of a cell-based universal artificial antigen-presenting cell (aAPC) system to provide costimulatory signals for Treg expansion and survival. K562 erythromyeloid leukemia cells were engineered to stably express CD32 (low-affinity FcgRII). UCB Tregs expanded equally well on these aAPCs loaded with anti-CD3/28 mAbs as with anti-CD3/28 mAb-loaded microbeads(Hippen, *et al* 2008). However, in vivo, xenogeneic GVHD suppression was favored by cell-based aAPCs, likely due to the provision of ligands present on the former and not the latter. When the human TNF/TNFR family costimulatory ligands OX40L or 4-1BBL were co-expressed, such anti-CD3/28 mAb-loaded cell-based aAPCs were more effective than microspheric beads in favoring the expansion of Tregs. These genetically modified aAPCs permitted > 1250-fold expansion of UCB Tregs in <3 weeks without loss of in vitro suppressor cell potency. In vivo adoptive transfer of expanded UCB Tregs indicated that cell-based aAPC expansion cultures were comparable to beads in suppressing xenogeneic GVHD despite very high Treg cell yields. Thus, these studies suggest a novel and more effective strategy for UCB Treg expansion than the currently widely used bead-based expansion approach. Indeed, aAPC cell-based expansion techniques for Tregs are likely to move into the clinical trial expansion practices in the near future.

In preclinical xenogeneic GVHD studies, UCB Tregs expanded using anti-CD3/28 microbeads or cell-based aAPCs persisted in the circulation for about 7–11 days. Dependent upon the conditions used for Treg expansion, there was a correlation between the duration of Treg persistence in the blood and potency of GVHD inhibition. Therefore, it may be important to ensure that adequate numbers of infused Tregs are present early post-BMT. Improved expansion rates and avoidance of GVHD prophylactic such as lympholytic (steroids, anti-thymocyte globulin, or CAMPATH-IH) or anti-proliferative agents (e.g. calcineurin inhibitors) that inhibit IL-2 production needed to drive Treg expansion will increase the likelihood for successful Treg suppression. Attention as to whether expanded Tregs have retained the capacity to home to secondary lymphoid organs where GVHD is initiated should be taken into account when considering the final product for infusion.

In summary, UCB Tregs are a readily accessible source of highly suppressive Tregs. Improvements in isolation approaches using new discriminatory cell surface antigens that are continually being identified to enrich for Tregs with more potent suppressor cell capacity expansion procedures by magnetic beads or high speed cell sorting and expansion procedures including those described above using aAPCs and multiple restimulations with these cells will facilitate clinical applications in hematopoietic stem and progenitor cell transplant. Only with randomized trials, particularly those conducted with minimal or no use of calcineurin inhibitors such as cyclosporine A or preferably with the complete absence of immune suppression, will the exact worth of UCB Treg be uncovered

MESENCHYMAL STROMAL CELLS

Initially, MSCs were reported as absent from UCB(Wexler, *et al* 2003), but later investigations showed that UCB MSCs can be isolated and expanded in vitro(Bieback and Kluter 2007, Erices, *et al* 2000, Flynn, *et al* 2007, Tondreau, *et al* 2005). In a fashion analogous to bone marrow-derived MSCs, spindle-shaped UCB cells attach to tissue culture plastic and differentiate to cells capable of expressing markers of bone, cartilage, and fat. MSCs can be derived from multiple hematopoietic (e.g., BM, UCB, peripheral blood) and non-hematopoietic tissues (e.g., fat, liver, muscle)(Phinney and Prockop 2007, Zuk, *et al* 2002). These rare cells (<0.01% of total cellular content) are plastic-adherent and have a remarkable capacity to expand rapidly in vitro and still, within several early cell culture passages, maintain the ability to differentiate into a number of mesenchymal cellular phenotypes at a clonal level(Pittenger, *et al* 1999). This MSC ability to form single-cell-derived clones led to their initial definition as colony-forming units-fibroblastic (CFU-F) (Friedenstein, *et al* 1970). MSCs typically express surface proteins integrin beta 1 (CD29), hyaluronate receptor (CD44), SH-3/SH-4 (CD73), Thy-1 (CD90), endoglin (CD105), and vascular cell adhesion molecule-1 (CD106), while they lack expression of hematopoietic markers such as monocyte surface protein CD14, hematopoietic stem cell antigen sialomucin CD34, and leukocyte common antigen CD45(Deans and Moseley 2000, Pittenger, *et al* 1999).

Identity and function on MSCs in vivo remains an enigma, even though multiple possibilities have been proposed, such as MSCs as resident cells in the vascular wall (termed pericytes)(Caplan 2009, or MSCs as parenchymal cells responsible for replenishing and physiological turning-over of adult mesenchymal tissues(Cetrulo 2006), Weiss and Troyer 2006).

In a study comparing human MSCs derived from BM, fat, or UCB, UCB MSCs had the lowest frequency of CFU-F but their proliferation rate was the highest of the three(Kern, *et al* 2006). Comparison of gene-expression signatures of BM MSCs and UCB MSCs revealed dominant osteogenic phenotype in BM MSC while UCB MSC expression was characterized by activation of IL-1 and TNF alpha angiogenic pathways(Flynn, *et al* 2007, Panepucci, *et al* 2004). When compared to bone marrow derived MSCs, UCB MSCs differentiate equally well to osteocytes and adipocytes, but demonstrate less adipogenic potential(Bieback, *et al* 2004, Kern, *et al* 2006). Importantly, the expression profiles of proteins in general, and cytokines in particular, in bone marrow and UCB MSCs are very similar(Feldmann, *et al* 2005, Liu and Hwang 2005). Taken together with the functional data, it appears that bone marrow and UCB MSCs are more similar than different, and thus their major properties and functional domains will be considered together.

Totality of evidence suggests that MSC cultures are heterogeneous cell populations of uncertain composition, as is evidenced by the multiple terms describing these adherent cell cultures: MSCs, mesenchymal stem cells, marrow stromal cells, and multipotent mesenchymal stromal cells(Phinney and Prockop 2007). Synthesis of available evidence also suggests that MSC progeny does not differentiate across germinal boundaries (i.e., into ectodermal and endodermal tissues) as do more immature embryonal stem cells and induced pluripotent stem cells. In addition, there are no definitive human marker panels to date that would allow prospective isolation of MSCs, and even MSCs derived from the same tissues are not functionally equivalent. Multiple isolation and expansion protocols exist and even slight differences among them (or even within them, as MSCs derived using the same isolation and expansion technique may differ) result in gene expression and phenotypic changes that make direct comparison of data difficult.

Equally challenging has been extrapolation of the MSCs in vitro multidifferentiation potential to the in vivo behavior of MSCs and definition of MSC function in live tissues(da Silva Meirelles, *et al* 2008). Significant clinical expectations have been associated with three functional aspects of MSCs.

1. Tissue repair: In cell transfer preclinical models of bone, skin, myocardium, kidney, pancreas, and lung, MSCs function as reparative cells with a remarkable ability to home to sites of tissue injury and to aid in tissue regeneration(Kunter, *et al* 2006). Contrary to initial expectations that MSCs will function as a reservoir to replace damaged cells, however, donor MSCs do not replace the damaged cells of the recipient(Phinney and Prockop 2007). Rather, they appear to exert their healing effects by secreting large quantities of tissue mediators in response to injury, by limiting apoptosis, and by recruiting the cells of the recipient to productive repair(Prockop 2007. This mechanistically not-yet-understood paracrine effect, whereby regenerative microenvironment is established, is frequently associated with enhanced angiogenesis(Caplan 2009), Prockop and Olson 2007). Clinical trials are underway for treatment of the damaged myocardium following an acute myocardial infarction and for the treament of chronic obstructive pulmonary disease.

2. Hematopoietic engraftment support. MSCs are closely physically and functionally associated with blood-forming cells in the bone marrow hematopoietic stem cell niche(Calvi, *et al* 2003). MSCs are rich source of growth factors, adhesion molecules, and homing cytokines. The MSCs' trophic effects (e.g., via stromal derived factor-1 production), their capacity to provide angiogenic support (e.g., via secretion of vascular endothelial growth factor, platelet-derived growth factor, and basic fibroblast growth factor) and perhaps even neurogenic support to the highly vascularized and innervated bone marrow,—together with experimental evidence that MSCs and other stromal cells are capable of mediating a modest expansion of hematopoietic cells in co-culture in vitro(Reese, *et al* 1999, Robinson, *et al* 2006, Wang, *et al* 2004)—led to the intriguing possibility that co-infusion of MSCs and hematopoietic cells can shorten time to engraftment and reduce graft failure after hematopoietic cell transplantation(Caplan 2009. While murine experimentation and small clinical series seemed to confirm this possibility, larger well-controlled clinical trials showed beneficial effects of MSCs in the engraftment of some hematopoietic grafts (e.g., haploidentical transplants)(Ball, *et al* 2007, Le Blanc, *et al* 2007) with less clear evidence at this time as to whether MSCs are equally supportive of other grafts (e.g., UCB transplants). It is likely, though, that different trial designs, cell doses, or MSC sources need to be used to optimize or uncover the full potential of MSCs in hematopoietic cell transplantation.

3. Immune modulation. MSCs are immune modulatory cells that do not elicit alloreactive lymphocyte proliferative response(Aggarwal and Pittenger 2005). In fact, MSCs inhibit proliferation of T cells and B cells, and suppress dendritic cells(Beyth, *et al* 2005, Rasmusson, *et al* 2005). This function of MSCs has been elegantly demonstrated in clinical trials using MSCs to treat GVHD that is resistant to standard therapy with steroids, calcineurin inhibitors, and anti-thymocyte globulin. Standard-therapy-resistant GVHD is an almost always lethal complication of HCT. Thus, MSCs have been quickly applied for this purpose in GVHD clinical trials and an initial phase III trial has completed enrollment(Le Blanc, *et al* 2004, Ringden, *et al* 2006). Trials of MSC infusion also are underway for autoimmunity indications such as Crohn's disease and type I diabetes. Almost all the clinical information available, however, is based on the use of bone marrow MSCs, and whether UCB MSCs have similar beneficial effects is unknown.

It is increasingly accepted that the functional complexity of MSCs may be a reflection of the fact that multiple effects of MSCs are specific to cellular subpopulations concealed in the bulk MSC cultures. Dissection of these specialized MSC populations could illuminate ways to make the MSC therapy more targeted to specific injured organs or disease processes. In addition, this could lead to expansion of the MSC uses beyond current indications. For example, systemic MSC infusion may enable cross-correction of soluble protein (e.g., enzyme iduronidase in mucopolysaccharidossis type I, Hurler syndrome)(Koc, *et al* 2000) or structural protein (e.g., extra cellular matrix collagen 7 in epidermolysis bullosa)(Tolar, *et al* 2009) deficiency, cross-correction that is at present possible only by using myeloablative hematopoietic cell transplantation. In addition, MSCs can be gene modified to enhance their tissue repair and cross-correction abilities(Bartholomew, *et al* 2001, Egermann, *et al* 2006, Gnecchi, *et al* 2005). Furthermore, MSCs can be useful in targeting tissues in body sites resistant to correction by hematopoietic cell transfer (such as brain, bone, and heart valves in hematopoietic cell transplantation recipients with Hurler syndrome). Lastly, pluripotency of MSC can be extended beyond mesenchyme either by reprogramming into embryonic-stem cell-like induced pluripotent stem cells(Park, *et al* 2008) or by isolation of subpopulations of UCB MSC with superior “stemness”: such as unrestricted somatic stem cells(Kogler, *et al* 2004, Kogler, *et al* 2006), embryonic-like stem cells(McGuckin, *et al* 2005), and very small embryonic-like cells(Kucia, *et al* 2007, Kucia, *et al* 2006).

There is a tremendous enthusiasm for the application of MSCs to clinical cell therapy and tissue engineering, especially when envisioned as a simple-to-isolate, third-party, versatile, off-the-shelf therapy for diverse congenital, immune, and ischemic medical conditions(Burt, *et al* 2008, Phinney and Prockop 2007). Conducting parallel clinical trials with well-defined end points and controls, and gaining insights from mechanistic laboratory research are necessary to make this optimistic vision a reality. Furthermore, MSC therapy can in theory lead to significant adverse outcomes, such as immunosuppression and higher risk of infections, and tumorigenesis either in the form of teratomas or sarcomas derived from the infused MSCs or in the form of donor MSC-mediated stimulation of tumor cells in the recipient or both(Djouad, *et al* 2003, Ning, *et al* 2008, Rubio, *et al* 2005, Tolar, *et al* 2007). Nevertheless, therapeutic advances in MSC therapy taking advantage of their trophic and immunoregulatory functions can fulfill major unmet needs in tissue regeneration.

Thus, we know with great certainty now that both Tregs and MSCs are active in key HCT pathophysiological processes, such as GVHD and tissue injury, resolution of which is likely to favorably impact the overall outcome of HCT. Number of prophylactic and therapeutic approaches is currently under investigation that has a potential to enhance their healing properties separately or, perhaps, in combination. Ultimately, advances in T reg and MSC biology offer great promise in safer and more effective treatment of a variety of malignant and non-malignant diseases treatable by HCT.

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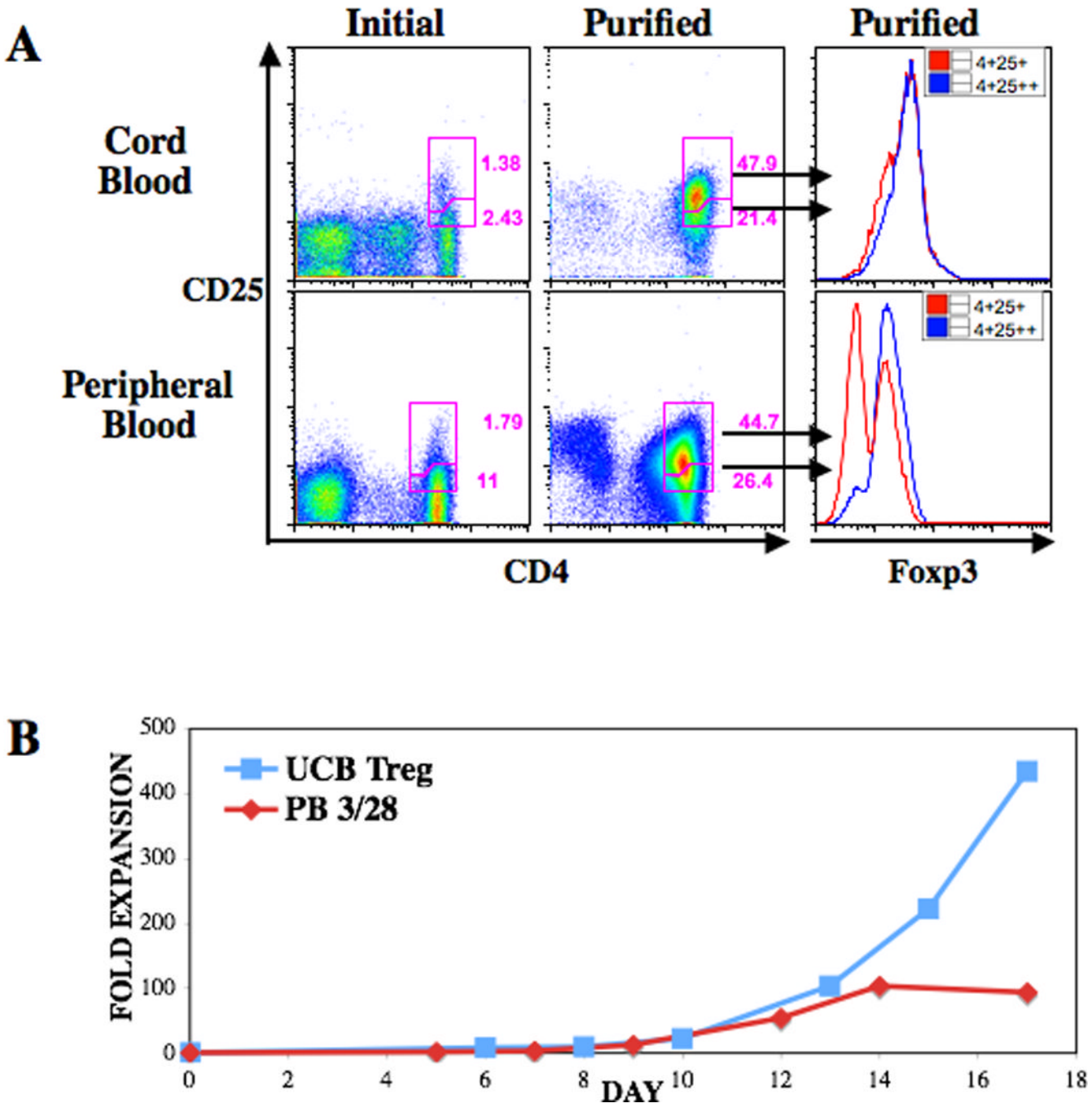

Fig. 1.

Purification and Expansion of Treg from UCB and PB. (A) Regulatory T cells were purified from UCB or PB using GMP grade to isolate $CD4^{+}25^{++}$ Treg cells. Representative example of flow cytometric phenotyping before and after purification, focusing on the increased abundance of $CD4^{+}25^{+}$ cells present in the initial and purified PB samples. (B) Regulatory T cells purified from UCB or PB were expanded in vitro with anti-CD3/28 beads in the presence of high dose IL-2 (300U/ml) for 17 days (+ Rapamycin for PB Treg cultures).