

causing local vasodilatation and by attracting and activating neutrophils, eosinophils, mast cells, monocytes, and macrophages.<sup>5</sup> DCs are known to express NK1R regardless of their maturation status, and SP has been reported to sustain the survival of DCs in culture and in living animals.<sup>6</sup> On the other hand, immunologic impacts of NK1R agonists on DCs remain relatively unknown. Using natural SP, homokinin-1, and a synthetic NK1R-specific SP homolog, Janelsins et al<sup>1</sup> sought to fill in this gap. These investigators found that NK1R agonists increase surface expression of major histocompatibility complex (MHC) class II molecule, costimulatory molecules (CD40, CD80, and CD86), adhesion molecules (CD11b, CD18, and CD54), and C-C chemokine receptor type 7 (CCR7) in murine bone marrow–derived DCs. Although NK1R agonists failed to alter the secretion of IL-1, IL-6, TNF $\alpha$ , and IL-12 by DCs, they potently inhibited IL-10 production. When injected subcutaneously after antigen loading, those DCs stimulated with NK1R agonists migrated efficiently to skin-draining LNs and elicited robust activation of CD4 and CD8 T cells producing interferon- $\gamma$  and antigen-specific cytotoxic T cells. *How can those DCs that secrete only negligible amounts of IL-12 initiate type 1 immunity?* The DCs pretreated with NK1R agonists were found to induce marked recruitment of Ly6C<sup>+</sup> inflammatory DCs to the skin-draining LNs, where they physically interacted with inflammatory DCs and promoted their production of IL-12. Janelsins et al<sup>1</sup> elegantly determined the source of IL-12 by transferring SP-pretreated DCs from IL-12 p35-deficient mice into wild-type recipient mice—these recipient mice still exhibited robust Th1-polarized immunity. Their findings demonstrate that NK1R agonists counteract with anti-inflammatory neuropeptides by directing DC functionality into contrary directions (see figure).

At the same time, the above findings raise several important questions. What are the underlying mechanisms by which anti-inflammatory neuropeptides and proinflammatory neuropeptides produce opposing outcomes in DCs? VIP,  $\alpha$ -MSH, and CGRP are known to downregulate the transcription of inflammatory cytokine genes by activating the cyclic AMP (cAMP)–protein kinase A pathway, thereby inhibiting the nuclear factor- $\kappa$ B (NF $\kappa$ B) pathway.<sup>2</sup>

Interestingly, Janelsins et al<sup>1</sup> showed that NK1R agonists triggered NF $\kappa$ B-dependent gene transactivation in DCs. This may corroborate a previous report showing that VIP and CGRP, but not SP, elevated intracellular cAMP levels in DC preparations isolated from skin.<sup>7</sup>

*Do NK1R agonists polarize T-cell differentiation only into the type 1 phenotype?* Recently, SP and hemokinin-1 have been reported to switch noncommitted human memory CD4 T cells into Th17 cells producing large amounts of IL-17A and interferon- $\gamma$ .<sup>8</sup> Thus, it will be interesting to determine whether SP-treated DCs can also promote Th17-polarized immune responses. *What is the clinical significance?* Increased levels of SP and elevated NK1R expression have been detected in lesions of inflammatory bowel disease and rheumatoid arthritis, suggesting pathogenic roles for SP in autoimmune inflammatory disorders.<sup>5</sup> It is, therefore, tempting to speculate that tissue-resident DCs may undergo Th1-biased maturation in the SP-rich microenvironment at the inflammatory lesions. If so, currently available peptide and nonpeptide NK1R antagonists<sup>9</sup> may be used to redirect DC maturation in those autoimmune diseases. Alternatively, NK1R agonists may serve as unique DC-targeted adjuvants specifically designed to harness Th1-polarized immunity against cancer cells and intracellular pathogens. In fact, this concept has been proven by local administration of a synthetic NK1R-specific SP homolog into the vaccination sites.<sup>10</sup> Obviously, many questions remain to be addressed. Nevertheless, the study by Janelsins et al

provides key information for our understanding of immunologic impacts of NK1R antagonists.

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## REFERENCES

1. Janelsins BM, Sumpter TL, Tkacheva OA, et al. Neurokinin-1 receptor agonists bias therapeutic dendritic cells to induce type 1 immunity by licensing host dendritic cells to produce IL-12. *Blood*. 2013;121(15):2923–2933.
2. Gonzalez-Rey E, Chorny A, Delgado M. Regulation of immune tolerance by anti-inflammatory neuropeptides. *Nat Rev Immunol*. 2007;7(1):52–63.
3. Reinke E, Fabry Z. Breaking or making immunological privilege in the central nervous system: the regulation of immunity by neuropeptides. *Immunol Lett*. 2006;104(1-2):102–109.
4. Seiffert K, Granstein RD. Neuroendocrine regulation of skin dendritic cells. *Ann N Y Acad Sci*. 2006;1088:195–206.
5. O'Connor TM, O'Connell J, O'Brien DI, Goode T, Bredin CP, Shanahan F. The role of substance P in inflammatory disease. *J Cell Physiol*. 2004;201(2):167–180.
6. Janelsins BM, Mathers AR, Tkacheva OA, Erdos G, Shufesky WJ, Morelli AE, Larregina AT. Proinflammatory tachykinins that signal through the neurokinin 1 receptor promote survival of dendritic cells and potent cellular immunity. *Blood*. 2009;113(13):3017–3026.
7. Asahina A, Moro O, Hosoi J, Lerner EA, Xu S, Takashima A, Granstein RD. Specific induction of cAMP in Langerhans cells by calcitonin gene-related peptide: relevance to functional effects. *Proc Natl Acad Sci USA*. 1995;92(18):8323–8327.
8. Cunin P, Caillon A, Corvaisier M, et al. The tachykinins substance P and hemokinin-1 favor the generation of human memory Th17 cells by inducing IL-1 $\beta$ , IL-23, and TNF-like 1A expression by monocytes. *J Immunol*. 2011;186(7):4175–4182.
9. Douglas SD, Leeman SE. Neurokinin-1 receptor: functional significance in the immune system in reference to selected infections and inflammation. *Ann N Y Acad Sci*. 2011;1217:83–95.
10. Mathers AR, Tkacheva OA, Janelsins BM, Shufesky WJ, Morelli AE, Larregina AT. In vivo signaling through the neurokinin 1 receptor favors transgene expression by Langerhans cells and promotes the generation of Th1- and Tc1-biased immune responses. *J Immunol*. 2007;178(11):7006–7017.

## ● ● ● HEMATOPOIESIS & STEM CELLS

Comment on Corselli et al, page 2891

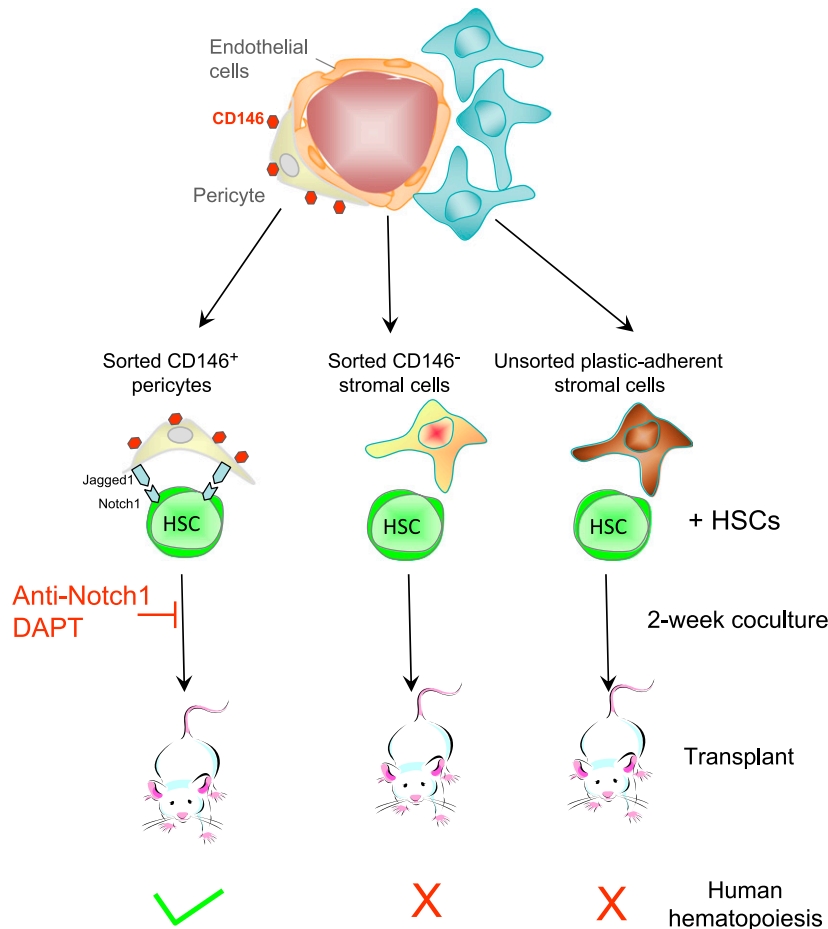
# A niche in a dish: pericytes support HSC

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In this issue of *Blood*, Corselli et al<sup>1</sup> purify CD146<sup>+</sup> pericytes from human adipose or fetal bone marrow and demonstrate that these cells are capable of supporting the self-renewal and proliferation of transplantable human cord blood hematopoietic stem cells (HSCs).

**C**ord blood is an easily accessible source of HSCs for transplantations. However, the absolute number of HSCs per cord blood

unit is generally too low to ensure rapid engraftment and immune reconstitution in adults after transplantation. The ability to



Human adipose or fetal bone marrow. CD146<sup>+</sup> pericytes from human adipose and fetal bone marrow support human HSCs in vitro. CD146<sup>+</sup> pericytes that ensheath and nurture blood vessels in adipose and fetal bone marrow are sorted as well as CD146<sup>-</sup> stromal cells. Sorted cells, as well as unfractionated plastic-adherent mesenchymal stromal cells, from these tissues are cocultured with human cord blood CD34<sup>+</sup> hematopoietic stem and progenitor cells for 2 weeks, and then transplanted into immunodeficient mice. Only cocultures with CD146<sup>+</sup> pericytes generate human HSCs capable of reconstituting human hematopoiesis in mice with long-term self-renewal in serial transplantations. The HSC-supportive effect of pericytes is mediated in part by Jagged expressed on pericytes and Notch1 expressed on HSCs as anti-Notch1 antibodies and the  $\gamma$ -secretase inhibitor DAPT block the supportive effect.

expand cord blood ex vivo in a bioreactor would overcome this limitation; however, to date, this has proven difficult to achieve. The main reason for the difficulty? Once isolated from supportive niches in the bone marrow, HSC proliferation is accompanied by rapid differentiation into lineage-committed progenitors that have lost their long-term self-renewal potential and ability to regenerate the whole hematopoietic system after transplantation. Therefore, ex vivo expansion of genuine long-term reconstituting HSCs requires the prior identification of the missing factors from the niche, which is much harder than it sounds because (1) the bone marrow is encased in a hard, mineralized bone and is, consequently, not easy to access and process for cellular and molecular studies and (2) there is a lack of

molecular markers for HSC-supportive niche cells.

The first marker identified was STRO-1, an antibody that binds to pericytes that wrap around the vasculature in human bone marrow and dental pulp.<sup>2,3</sup> STRO-1<sup>+</sup> pericytes can differentiate into osteoblasts and adipocytes and, most importantly, can support the production of human colony forming unit-mix for a few weeks in cocultures.<sup>2,3</sup> However, STRO-1 has major shortcomings that prevented its use in clinical applications and further functional characterization of STRO-1<sup>+</sup> pericytes. Indeed, STRO-1 (1) identifies a glycosylation motif and (2) is an immunoglobulin M that loses its binding properties upon purification or biochemical modifications (such as covalent binding to a fluorophore, a magnetic

bead or biotin). The next breakthrough came from the realization that CD146, also called melanoma cell adhesion molecule or MUC18, is expressed at the surface of pericytes from many different human tissues such as bone marrow, adipose, pancreas, and placenta.<sup>3-5</sup> CD146<sup>+</sup> human pericytes have myogenic and osteogenic potential and can form ectopic bones containing an ectopic bone marrow when transplanted subcutaneously in immunodeficient mice.<sup>4</sup> Although these findings are suggestive that CD146<sup>+</sup> pericytes could support hematopoiesis, this has never been experimentally tested.

In this article,<sup>1</sup> Corselli et al first demonstrate that CD146<sup>+</sup> pericytes from human adult adipose and fetal bone marrow express molecules that are well-known markers of HSC-supportive pericytes in the mouse bone marrow. In cocultures with cord blood lineage-negative CD34<sup>+</sup> HSCs, sorted CD146<sup>+</sup> pericytes from these 2 tissues are much better at maintaining production of human leukocytes and CD34<sup>+</sup> hematopoietic stem and progenitor cells than CD146<sup>-</sup> stromal cells or unfractionated plastic-adherent mesenchymal stromal cells (see figure). Most importantly, sorted CD146<sup>+</sup> pericytes could maintain human HSCs that engraft immunodeficient mice and serially transplant, the hallmarks of long-term reconstituting human HSCs. In sharp contrast, CD146<sup>-</sup> and unfractionated bone marrow mesenchymal stromal cells could not.

Another important point of the Corselli article is that human HSCs did not require the addition of exogenous recombinant cytokines to be maintained by CD146<sup>+</sup> pericytes for 2 weeks. Therefore, these pericytes provide the necessary factors for HSCs to survive, proliferate, and generate differentiated leukocytes while maintaining a pool of undifferentiated reconstituting HSCs. Finally, the supportive effect of CD146<sup>+</sup> pericytes required cell-cell contact and was mediated in part by the transmembrane Notch ligand Jagged-1, abundantly expressed at the surface of these pericytes, interacting with its receptor Notch1 expressed on HSCs. Indeed, anti-Notch1 antibodies and DAPT (*N*-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester), an inhibitor of  $\gamma$ -secretase that is necessary to Notch signaling, both inhibited HSC support by sorted pericytes. These results provide

compelling evidence that CD146<sup>+</sup> from adult and fetal tissues support and maintain human HSCs *in vitro*. These conclusions are congruent with observations in the mouse also showing that pericytes are necessary to maintain HSCs in the mouse bone marrow *in vivo*. Indeed, conditional deletion of the *Kitl* gene, which encodes the ligand of the tyrosine kinase c-kit, in pericytes,<sup>6</sup> or ablation of nestin-positive pericytes<sup>7</sup> compromise the maintenance of HSCs in the mouse bone marrow.

However, a number of questions remain to be further investigated. The authors did not determine whether CD146<sup>+</sup> pericytes could support the actual *ex vivo* expansion of human reconstituting HSCs by quantifying content in reconstituting HSCs before and after coculture. It would also be interesting to evaluate the supportive effect of pericytes combined with bone marrow endothelial cells. Indeed, studies in the mouse clearly indicate that pericytes are not the sole HSC-supportive stromal cells in the bone marrow as endothelial cells are also essential to maintain HSC in their niche via expression of Kit ligand<sup>6</sup> or regulate their proliferation via E-selectin.<sup>8</sup> Similar to pericytes, human umbilical vein endothelial cells transformed with an Akt kinase activating adenoviral gene are able to expand transplantable human HSCs.<sup>9</sup> Therefore by analogy with mouse bone marrow niches, in which both endothelial cells and pericytes act in concert to maintain HSCs, cultures with a combination of human pericytes and endothelial cells may support human HSC *ex vivo* expansion longer or more efficiently. Finally, there is some irony in the findings of this article as HSC-supportive pericytes were isolated from adipose collected from liposuctions. While adipocytes seem to be detrimental to HSCs,<sup>10</sup> the pericytes that maintain the vasculature feeding adipocytes are beneficial to HSCs once separated from the fat. One of the most abundant and wasteful human tissues in the developed world could finally be put to good use to recipients of HSC transplantations.

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## REFERENCES

1. Corselli M, Chin CJ, Parekh C, et al. Perivascular support of human hematopoietic cells. *Blood*. 2013;121(15):2891-2901.

2. Simmons PJ, Torok-Storb B. Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. *Blood*. 1991;78(1):55-62.

3. Shi S, Gronthos S. Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *J Bone Miner Res*. 2003;18(4):696-704.

4. Sacchetti B, Funari A, Michienzi S, et al. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell*. 2007;131(2):324-336.

5. Crisan M, Yap S, Casteilla L, et al. A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell*. 2008;3(3):301-313.

6. Ding L, Saunders TL, Enikolopov G, Morrison SJ. Endothelial and perivascular cells maintain hematopoietic stem cells. *Nature*. 2012;481(7382):457-462.

7. Méndez-Ferrer S, Michurina TV, Ferraro F, et al. Mesenchymal and hematopoietic stem cells form a unique bone marrow niche. *Nature*. 2010;466(7308):829-834.

8. Winkler IG, Barbier V, Nowlan B, et al. Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. *Nat Med*. 2012;18(11):1651-1657.

9. Butler JM, Gars EJ, James DJ, Nolan DJ, Scandura JM, Rafii S. Development of a vascular niche platform for expansion of repopulating human cord blood stem and progenitor cells. *Blood*. 2012;120(6):1344-1347.

10. Naveiras O, Nardi V, Wenzel PL, Hauschka PV, Fahey F, Daley GQ. Bone-marrow adipocytes as negative regulators of the hematopoietic microenvironment. *Nature*. 2009;460(7252):259-263.

## ● ● ● THROMBOSIS & HEMOSTASIS

Comment on Bakchoul et al, page 2821, and on Lee et al, page 2828

# Protamine-induced thrombocytopenia?

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In this issue of *Blood*, Bakchoul et al and Lee et al describe and characterize a common but only recently recognized immune response to protamine after cardiopulmonary bypass (CPB) surgery with potential important clinical implications.<sup>1,2</sup>

**P**rotamines are small, positively charged, DNA-binding proteins found in the sperm of invertebrate and vertebrate animals. Protamine sulfate, derived from salmon sperm, is used to reverse the anticoagulant activity of unfractionated heparin during CPB and as a stabilizer in neutral protamine Hagedorn (NPH), a long-acting formulation of insulin.

As reported by Bakchoul et al and Lee et al, the immune response to protamine in the CPB population bears several similarities to the immune response to complexes of platelet factor 4 (PF4) and heparin, the antigenic target in heparin-induced thrombocytopenia (HIT). Only 1% to 3% of patients are seropositive at the time of surgery for anti-protamine<sup>1,2</sup> or anti-PF4/heparin IgG.<sup>3</sup> Formation of these antibodies is common after CPB, occurring in 25% to 29%<sup>1,2,4</sup> and 39%<sup>3</sup> of individuals, respectively, by 4 to 6 weeks. Like anti-PF4/heparin antibodies, a minority of anti-protamine antibodies induce platelet activation *in vitro* in a FcγRIIA-dependent manner.<sup>1,2,4</sup>

However, important differences between the anti-PF4 and anti-protamine immune response also exist. Chief among these is the nature of the antigen. PF4 is an endogenous

chemokine stored in the α granules of platelets. PF4 monomers polymerize to form non-covalently-linked tetramers with a molecular weight of ~32 kDa. Salmon protamine is a ~4 kDa xenogeneic protein that bears scarce similarity in its linear amino acid sequence to human protamine.

The onset and persistence of antibody formation to these proteins after CPB may differ as well. Anti-PF4/heparin IgG seroconversion occurs at a median of 4 to 6 days after CPB.<sup>5</sup> This early response suggests preimmunization to antigenic epitopes on PF4, which may reflect prior binding to the vasculature<sup>6</sup> or certain bacteria.<sup>7</sup> The immune response to protamine is more delayed. In the study by Lee et al, of 143 patients in whom anti-protamine IgG developed, 96% (137) seroconverted between hospital discharge and day 30 after CPB,<sup>2</sup> a time course more consistent with a typical naive immune response. Anti-PF4/heparin antibodies become undetectable at a median of 85 days after heparin withdrawal, although titers persist beyond 100 days in 40% of patients with HIT.<sup>8</sup> In the study by Bakchoul et al, anti-protamine IgG was present in only 5% of patients at day >120.<sup>1</sup> Continued exposure to an endogenous antigen (PF4) and finite