Increased numbers of circulating hematopoietic stem/progenitor cells (HSPC) are chronically maintained in patients treated with the CD49d blocking antibody Natalizumab

Running title: Circulating HSPC in anti-CD49d-treated MS patients

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Abstract:
Blockade of CD49d-mediated lymphocyte trafficking has been used therapeutically for certain autoimmune diseases, such as Multiple Sclerosis (MS). In addition to negative effects on the trafficking of mature lymphocytes to sites of inflammation, CD49d blockade in mice and monkeys rapidly mobilizes HSPC capable of short- and long-term engraftment. Here we aimed to ascertain the effects of treatment with anti-functional anti-CD49d antibody in humans (MS patients receiving infusions of the CD49d-blocking antibody Natalizumab) on levels of circulating HSPC after a single dose of antibody or after long-term treatment. On average, 6-fold elevated levels of circulating CD34+ cells and colony-forming unit-culture (CFU-C) were achieved within one day of the first dose of Natalizumab and similar levels were continuously maintained under monthly Natalizumab infusions. The blood of Natalizumab-treated subjects also contained SCID-repopulating cells. The fate of these circulating HSPC and their clinical relevance for MS patients remains to be determined.
Introduction:
In non-human primates, CD49d-blockade with humanized anti-functional antibodies results in rapid (peak <1 day) and prolonged (>10 days) increase in circulating HSPC.\textsuperscript{1,2} Elevated circulating HSPC were likewise observed in anti-CD49d-antibody treated mice.\textsuperscript{2} Furthermore, mice conditionally ablated for CD49d sustain >8-fold elevated levels of circulating HSPC without evidence of progressive accumulation or of BM depletion for their life span.\textsuperscript{3} Even though the homing efficiency of anti-CD49d mobilized HSPC was reduced, if sufficient numbers of cells were used these cells provided short- and long-term engraftment. Studies in humans treated with anti-CD49d antibody, particularly after protracted blockade of CD49d, have not been reported, nor have any other studies of prolonged administration of mobilizing agents been performed. A clinical-grade humanized mouse-anti-human function-blocking CD49d-antibody (Natalizumab, Tysabri\textsuperscript{®}) is available under a special restricted distribution program for treatment of relapsing-remitting MS who failed to respond to or did not tolerate first-line therapeutics.\textsuperscript{4} As trials of Natalizumab in healthy normal volunteers are not justifiable, because of prolonged immune-modulating effects of the antibody, including the possibility of rare, but potentially fatal progressive multifocal leukencephalopathy,\textsuperscript{5} we made observations in a cohort of MS patients receiving/scheduled to receive disease-modifying monotherapy with Natalizumab.

Methods:
Human Subjects and Protocol:
Adults with MS receiving/scheduled to receive disease-modifying therapy (DMT) with Natalizumab (300 mg i.v. once every month) at the University of Washington (UW) Departments of Neurology/Rehabilitation Medicine were eligible for participation. Exclusion criteria were other DMT, steroids or lithium. After written informed consent, immediately preceding the next scheduled Natalizumab infusion blood was drawn from “untreated” patients (before the first infusion) and “chronic” patients (≥5 prior doses). In some patients, a second blood draw was done after infusion, generally on the subsequent day. A cohort of normal controls was also recruited. Blood draws were anonymous; except for classification as “untreated/first-dose” or “chronic” recipient no subject information was collected. The study was approved by the UW Internal Review Board.

HSPC assays:
CFU-C assays were performed as described.\textsuperscript{6} Side-scatter low/CD34bright (CD34+) cells were quantified by flow cytometry, as described.\textsuperscript{7} The presence of competitive repopulating units (CRU) was tested in xenotransplants 9-10 weeks post-transplant, as described. Transwell migration of CFU-C towards SDF-1 (100 ng/mL) was enumerated as described.\textsuperscript{6} Cell cycle status on flow-sorted CD34+ cells was analyzed by Acridine Orange staining.\textsuperscript{8}

Results and Discussion:
Prior to the first Natalizumab dose, circulating CD34+ cells and CFU-C in “untreated” MS patients (Fig. 1A,D) were within the range reported for normal
subjects and our normal controls studied concurrently, i.e. MS per se is not associated with elevated circulating HSPC. Subjects on “chronic” Natalizumab treatment had 5- to 7-fold elevated circulating CD34+ cells and CFU-C (Fig. 1A,D) one month after infusion. In a subgroup of “chronic” subjects, circulating HSPC were analyzed before and one day after the monthly dose of Natalizumab. Renewed infusion did not result in significant further augmentation of circulating CD34+ cells or CFU-C (analyzed one day after the infusion, Fig. 1C,F), indicating continuous functional satiation of CD49d on BM-HSPC with standard Natalizumab dosing.

Comparison in Natalizumab-recipients before and one day after the first infusion (“1st dose”) revealed 5- to 6-fold increased CD34+ cells and CFU-C after the first infusion (Fig. 1B,E). Mean post-infusion values after the first dose were no different from those in “chronic” Natalizumab-recipients before or after repeated Natalizumab infusions, documenting achievement of maximal levels of circulating HSPC within 24 hours of a single Natalizumab infusion. Since circulating CD34+ cells and CFU-C in untreated MS patients were normal, the increased frequency of circulating HSPC in Natalizumab-treated MS patients is apparently due to drug effects.

CD34+ counts in Natalizumab-treated MS patients were thus approximately 1/6 of those in MS patients mobilized with G-CSF. However, the relative frequency of clonogenic cells appeared to be higher among Natalizumab-mobilized than among G-CSF-mobilized CD34+ cells (1 in 3 CD34+ cells for Natalizumab vs. 1 in 10 for G-CSF mobilized CD34+ cells), likely because the G-CSF induced proliferation/differentiation dilutes clonogenic cells with more mature CD34+ subsets. Thus the difference in HSPC mobilization potency may be less pronounced than CD34+ numbers suggest. CD34+ counts in Natalizumab-patients were one third of those achieved with the CXCR4-antagonist AMD3100 in normal volunteers.

The sustained HSPC levels in Natalizumab recipients contrast with mobilization kinetics after G-CSF, in which after the peak on day 5, circulating HSPC numbers are regressive despite continued administration. It indicates that without CD49d-mediated BM retention a new equilibrium is established between HSPC in BM and those in circulation. In Natalizumab-recipients a total of ca. 50x10E6 CD34+ cells (8 CD34+ cells/µL * 6L of blood) are in circulation at any given time. Assuming that the transit time of normal circulating HSPC is short and not very different from normal in Natalizumab-treated patients (supported by unpublished data for CD49d-/- mice), several million CD34+ cells are trafficking through the blood of the average Natalizumab recipient every day. A similar pattern of sustained elevation of circulating HSPC was observed in CD49d deficient mice. Most CD34+ cells in blood from Natalizumab-patients are quiescent (Fig. 1H), a phenotype associated with favorable homing and/or engraftment. Similar data were reported for unmobilized or G-CSF-mobilized CD34+ cells, i.e. may represent a general feature of circulating HSPC. Peripheral blood CFU-C from Natalizumab-treated patients did not migrate in in-vitro transwell assays, whether spontaneously or SDF-1-directed, in contrast to normal BM HSPC (Fig. 1G). We attribute the lack of migration to continuous saturation by Natalizumab, as
several previous observations show absence of migration with anti-CD49d antibody treated cells.
To establish whether peripheral blood of Natalizumab-treated patients contained any CRU, xenotransplants of mononuclear cells from 7-8 ml of blood (containing 50-60,000 CD34+ cells) were performed. Human engraftment of >1% was observed in 3/3 recipients (not shown), although no quantitative conclusions about CRU can be drawn from these studies. The engraftment data are of interest in view of the cells’ inability to migrate in vitro. Similar to our data, cells mobilized with the chemokine Groβ did not migrate in vitro but engrafted well, reinforcing the view that in vitro behavior of HSPC can not predict their in vivo performance.
The role, or clinical consequence, of elevated circulating HSPC for Natalizumab-treated MS patients is unclear. Potential effects of HSPC on remodeling/repair of non-hematopoietic organs have been reported. It is thus tempting to hypothesize that circulating HSPC may contribute to the anecdotal cases of neurological improvement under therapy, which may not be explained by the main mechanism of Natalizumab in MS, i.e. attenuated lymphocyte recruitment. Although in Natalizumab-treated MS patients significant numbers of HSPC continuously circulate outside the protective BM environment, their likelihood to acquire deleterious mutations is probably small, given their G0 cycling status, but this possibility needs to be considered. Thus far an increased propensity of Natalizumab-patients for hematological malignancies has not been reported; however, further long-term observations are needed.
The usefulness of Natalizumab as a priming agent for HSPC peripheralization is likely restricted only to patients receiving Natalizumab for their underlying illness, given the risk of immunization against Natalizumab and prolonged immune-modulating effects. However, CD49d-blockade with short-acting small-molecule CD49d-inhibitors, if efficacious, could be theoretically considered as treatment strategy for patient groups intolerant or unresponsive to G-CSF, especially in combination with other mobilizing agents, such as the CXCR4 antagonist AMD3100.

Support: Studies were supported by a Core Center of Excellence in Hematology Grant from the FHCRC (NIH DK56465) (HB) and by NIH grant HL58734 (TP).

Acknowledgements: The support of the nursing staff of the 8SE infusion station at UWMC is gratefully acknowledged.

Author contribution: HB conceived of the studies, wrote the IRB protocol, obtained informed consent, performed experiments, collected and analyzed data, and wrote the paper. AW wrote the IRB protocol and recruited subjects. KHC performed experiments. SL recruited subjects. TP analyzed data and wrote the paper.

Conflict of interest disclosure: SL serves on the Speaker’s Bureau for Biogen/Idec, manufacturers of Natalizumab. The other authors have no declarations to make.
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Figure Legend:

Fig. 1: Elevated numbers of circulating HSPC in the blood of Natalizumab-treated MS patients

A, D: Circulating HSPC in normal controls, not Natalizumab-treated MS patients and long-term Natalizumab-treated MS patients: Circulating CD34+ cells (1.8±0.4/µL, p=0.36 vs. control) and CFU-C (638±128/mL, p=0.4 vs. control) were normal in MS patients prior to the first Natalizumab infusion (“untreated”), and significantly elevated in patients who had received at least 5 prior doses of Natalizumab, measured immediately before application of the next dose (“chronic”; 9.0±1.2/µL CD34+ cells, 3243±332/mL CFU-C, p<0.005 vs. control). (Normal controls (“nl.ctrl.”): 1.3±0.1/µL CD34+ cells, 608±129/mL CFU-C, on the left).

B, E: First-dose Natalizumab-patients: After the first Natalizumab infusion (“after”), peripheral blood CD34+ cells and CFU-C were significantly increased over pre-treatment values (“before”; 1.6±0.2/µL vs. 8.0±2.1/µL CD34+ cells, 414±161/mL vs. 2560±726/mL CFU-C, p<0.005).

C, F: Chronic Natalizumab-patients: Renewed Natalizumab infusion (“after”) in “chronic” Natalizumab-recipients did not result in additional mobilization, compared to CD34+ cell and CFU-C values just prior to that infusion (7.9±1.7/µL vs. 7.9±0.9/µL CD34+ cells, p=0.99; 3133±335/mL vs. 3525±305/mL CFU-C, p=0.27). CD34+ cells/µL (A-C) or CFU-C/mL (D-F) are plotted on the Y-axis. Each diamond represents values from one patient (for CFU-C: mean values from replicates from one patient); bars and whiskers indicate mean values ± SEM.

G: CFU-C migration: In contrast to normal BM HSPC, peripheral blood CFU-C from Natalizumab-recipients did not migrate towards SDF-1 in in-vitro transwell assays (p<0.001).

H: Cell cycle status of Natalizumab-mobilized HSPC: Natalizumab-mobilized CD34+ cells were almost exclusively quiescent, and overwhelmingly in G0 phase of cell cycle (flow cytometry histogram; RNA displayed on the X-axis, DNA on the Y-axis).
Figure 1
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