To the editor:

Targeting host B-cell immune responses by persistent donor NK-cell alloreactivity following nonmyeloablative allogeneic stem cell transplantation

Allogeneic bone marrow transplantation (BMT) is an established treatment for a variety of hematological and immunological disorders. It is postulated that the beneficial effects after myeloablation and haploidentical BMT are mediated by alloreactive donor natural killer (NK) cells, and host-reactive NK-cell clones have been isolated from human transplant recipients up to 3 months after transplantation.[1,2] However, little is known about the ability of graft-derived NK cells to target host immune cells after nonmyeloablation and haploidentical BMT. Likewise, the persistence of donor NK-cell alloreactivity in chimeric hosts is poorly defined in this setting.[3] Here, we report the ability of alloreactive donor NK cells to eradicate host immune cells after haploidentical BMT in mice, without inducing graft-versus-host disease (GVHD).

The Experimental Animal Commission of Leiden University Medical Center (Leiden, The Netherlands) approved the experiments described in this letter.

Recently, we showed the effective treatment of B-cell–mediated autoimmune disease (AID) in mice by allogeneic BMT.[4] We postulated the rapid eradication of host B cells produced pathogenic autoantibodies by donor NK cells. To further analyze donor NK-cell alloreactivity toward antibody-secreting B cells, we used a model of haploidentical (F1→P) BMT involving host B cells recognizing a model antigen, ovalbumin (OVA). In this model, donor T cells are tolerant for host cells,[5,6] but donor NK cells are activated as they lack inhibitory signals provided by self–major histocompatibility complex (MHC) class I.[7,8] After immunization, high levels of anti-OVA antibodies were detected. Subsequently, the mice were treated with nonmyeloablative conditioning (ie, low-dose total-body irradiation (6 Gy) and anti-CD40L antibodies (0.5 mg), and BMT from syngeneic, haploidentical or fully MHC-mismatched donor mice. Both fully MHC-mismatched and haploidentical BMT resulted in the early elimination of OVA-specific B cells (Figure 1A) and anti-OVA antibodies (Figure 1B), in contrast to syngeneic BMT. Recipient mice had no signs of acute GVHD (ie, no weight loss and/or detectable histologic abnormalities of liver, gut, and skin), supporting the notion that donor NK cells can mediate beneficial anti–host B-cell alloresponses, without causing GVHD.

Next, we examined whether host-reactive NK-cell alloresponses after haploidentical BMT are still present 3 months after BMT using an in vivo cytotoxicity assay.[9,10] In this experiment, differentially carboxyfluoroscein succinimidylester (CFSE)-labeled splenocytes from syngeneic (host) and haploidentical (control) mice were injected into chimeric hosts (4% to 5% host Gr-1$^+$ cells). Prior to the challenge with CFSE-labeled splenocytes, NK cells in recipient mice were (or were not) depleted by anti-NK1.1 antibodies. At day +7, peripheral-blood cells were analyzed for the presence of CFSE$^+$ cells, and the extent of host-cell elimination was determined. Syngeneic cells were eliminated in control mice, while this elimination was inhibited after depletion of NK1.1$^+$ cells (Figure 1C). Although the effect of depletion of NK1.1$^+$ cells is probably underestimated, as not all F1-derived NK-cells express NK1.1, these data indicate that host-reactive NK-cells contribute to the eradication of host immune cells, and that this alloreactivity persists up to 3 months after BMT.

In conclusion, our data suggest that donor NK-cell alloreactivity toward host immune cells may be exploited in the setting of allogeneic BMT to treat AID in which B cells play a pathogenic role.

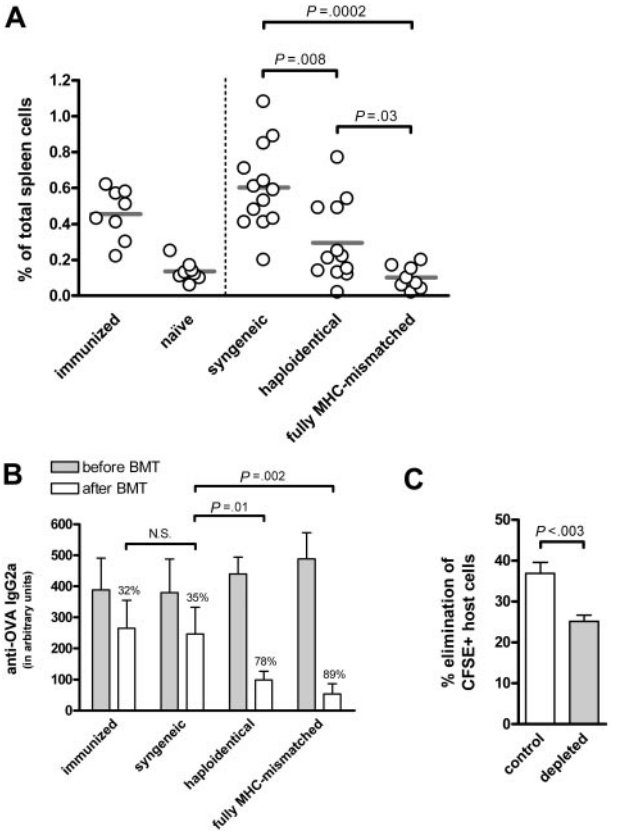

Figure 1. Persistent donor NK-cell alloreactivity toward host B cells after haploidentical BMT. (A,B) Haploidentical BMT results in a significant decrease in OVA-specific B cells and anti-OVA antibodies. DBA/1 mice were immunized with OVA, and were either left untreated or were subjected to nonmyeloablative conditioning consisting of low-dose total body irradiation (6 Gy) and a single injection of 0.5 mg of anti-CD40L antibodies (MR1), followed by transplantation with 10^7 syngeneic, haploidentical (F1→P) or fully MHC-mismatched BM cells from DBA/1, (DBA/1 x C57BL/6) F1 or C57BL/6 donor mice, respectively. The percentage of OVA-specific B cells in spleen was analyzed 2 weeks after BMT. Compared to syngeneic controls, both haploidentical (F1→P) and fully MHC-mismatched BMT recipients showed a significant decrease in the percentage of OVA-specific B cells ($P = .008$ and $P = .0002$, respectively). Sera were taken before and after BMT, and tested by ELISA for the presence of anti-OVA IgG2a antibodies. At the start of treatment, no differences in anti-OVA antibody levels were observed between the groups of mice. After treatment, all groups displayed a decrease in anti-OVA antibodies; syngeneic BMT-treated mice showed a decrease in OVA-specific antibody levels of 35%, similar to that of controls (32%), while both haploidentical (F1→P) and fully MHC-mismatched BMT resulted in a strong decrease in antibodies against OVA of 78% ($P = .01$) and 89% ($P = .002$), respectively. As negative control, naive mice were analyzed, while immunized mice served as positive control. (N.S. = statistically not significant.) (C) Donor NK cells persistently eradicate host immune cells after haploidentical BMT. At 3 months after BMT, chimeric C57BL/6 mice treated with haploidentical (F1→P) BMT from (BALB/c x C57BL/6) F1 were injected with 10^7 CFSE-labeled splenocytes (containing 50% to 60% B cells) from syngeneic (CFSE^{dim}) and haploidentical (CFSE^{high}) mice mixed at a 1:1 ratio. Before adoptive transfer, NK cells in recipient mice were depleted with 0.25 mg anti-NK1.1 antibodies (PK136), while PBS was used as control. At day +7 after adoptive transfer, the presence of CFSE$^+$ cells in peripheral blood was analyzed by FACS. Error bars indicate mean ± SEM.

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To the editor:

Effect of the new HJV-L165X mutation on penetrance of HFE

We present a new homozygous truncating mutation, L165X, of the *hemojuvelin* gene (*HJV*), observed in one male patient with severe juvenile hemochromatosis (JH). Because the C282Y-variant in the *hemochromatosis* gene (*HFE*) was also common in this family, we investigated whether the inactivating mutation HJV-L165X influenced penetrance of HFE-C282Y homozygosity.

The proband, born in 1956 and diagnosed in 1972 by family screening (B III-54),[1] presented with increased serum iron values and heavy iron accumulation in the hepatocytes.[1,2] There was no consanguinity between his parents or grandparents,[1] and linkage to *HLA* was excluded in his familybranch, providing the first clue of genetic heterogeneity in hemochromatosis.[3] In late 2005, he provided contact information on relatives. The institutional review board approved this study and informed consent was obtained from all participants (n = 20). Non-fasting blood and urine samples from the proband and his relatives were collected between 7 and 9 PM on the same day. Information on the number of phlebotomies and the time between the last phlebotomy and sample collection was provided. Urinary hepcidin was measured by mass spectometry.[4] For the proband, we sequenced the *hepcidin* (*HAMP*) and *HJV* genes. Mutations in the *HAMP* gene were absent. The *HJV* gene was sequenced using previously reported primers.[5] For exon 3 we designed new primers: Ex3a1935F 5′-GCAAACTACACTCCGATAGAG-3′ and Ex3a2253R 5′-GCTGGATCATCAGGTCTTCG-3′, resulting in a 319 bp product, and Ex3b2202F 5′-GACCTCGCCTTCCATTCG-3′ and Ex3b2603R 5′-GAATCTCATGAGGTGGATCGG-3′, leading to a 402 bp product (GenBank NT_004 434/gi:88 943 080). We observed a novel homozygous mutation in exon 3 of the *HJV* gene. The 494T→A transversion leads to a premature stop codon at position 165 of the HJV protein: L165X. This probably leads to nonsense-mediated decay of the corresponding messenger RNA. If the aberrant message is translated, however, it would code for a protein that lacks the GPI anchor signal, such that it remains in the endoplasmic reticulum.[6] In both cases, it can be anticipated that upstream regulation of hepcidin is impaired.[7]

Relatives of the proband were investigated for the HFE-C282Y and the HJV-L165X mutation by a restriction fragment length polymorphism analysis using the Ex3b2202F and Ex3b2603R primers and the restriction enzyme HpyCH4V (New England Biolabs, Ipswich, MA).

HJV-L165X homozygosity was only present in the proband, while heterozygosity was common among relatives (allele frequency: 14/40 = 35.0%). Furthermore, the HFE-C282Y mutation was observed frequently (allele frequency: 27/40 = 67.5%) (Table 1). Phlebotomies were only reported in individuals later found to be homozygous for either HJV-L165X (n = 1; proband) or HFE-C282Y (n = 8). Iron indices (current and from the early seventies[1,2]; J. P. G., unpublished data, early 1970s) are copied into Table 1.

Current serum iron parameters are not appropriate as a measure of iron burden, as most relatives have been adequately phlebotomized. We found an alternative in the following parameters: quantity of iron removed by phlebotomies (iron removed/age),[8] a rough estimate hampered by the probability that intestinal iron uptake may increase upon phlebotomy; transferrin saturation (TS) values and desferrioxamine (DFO) test results from the early seventies, before treatment; and urinary hepcidin levels, measured with our improved MS assay.[4] The iron removed/age, urinary hepcidin levels, and iron indices from the early seventies were similar for HJV-heterozygotes (L165X) and the HJV-wildtypes, also when stratified by *HFE* genotype, indicating the absence of a clinically relevant modifying effect. Others reported HJV a modifier,[9,10] although not consistently.[11] Furthermore, our data agree with recent findings that HFE and HJV participate, at least partially, in distinct regulatory pathways.[12] Finally, against a background of multiple small variations, we cannot exclude a minor effect of the heterozygous HJV-L165X mutation on iron homeostasis.

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