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Donor natural killer cells trigger production of beta-2-microglobulin to enhance post bone marrow transplant immunity

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Abstract:
Allogeneic hematopoietic transplantation is a powerful treatment for hematologic malignancies. Post-transplant immune incompetence exposes patients to disease relapse and infections. We previously demostrated that donor alloreactive natural killer (NK) cells ablate recipient hematopoietic targets, including leukemia. Here, in murine models, we show that infusion of donor alloreactive NK-cells triggers recipient dendritic cells (DCs) to synthesize beta-2-microglobulin (B2M) that elicits the release of c-KIT-Ligand and interleukin-7 that greatly accelerate post-transplant immune reconstitution. An identical chain of events was reproduced by infusing supernatants of alloreactive NK/DC co-cultures. Similarly, human alloreactive NK-cells triggered human DCs to synthesize B2M that induced interleukin-7 production by thymic epithelial cells and thereby supported thymocyte cellularity in vitro. Chromatography fractionation of murine and human alloreactive NK/DC co-culture supernatants identified a protein with molecular weight and isoelectric point of B2M and mass spectrometry identified amino-acid sequences specific of B2M. Anti-B2M antibody depletion of NK/DC co-culture supernatants abrogated their immune rebuilding effect. B2M knock-out mice were unable to undergo accelerated immune reconstitution but infusion of (wild type) NK/DC coculture supernatants restored their ability to undergo accelerated immune reconstitution. Similarly, silencing the B2M gene in human DCs, before co-culture with alloreactive NK cells, prevented the increase in thymocyte cellularity in vitro. Finally, human recombinant B2M increased thymocyte cellularity in a TEC/thymocyte culture system.

Our studies uncover a novel therapeutic principle for treating post-transplant immune incompetence and suggest that, upon its translation to the clinic, patients may benefit from adoptive transfer of large numbers of cytokine-activated, ex-vivo expanded donor alloreactive NK-cells.

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Key points

1) Donor natural killer cells trigger recipient dendritic cells to synthesize beta-2-microglobulin which stimulates cKIT-L and IL7 production

2) Adoptive transfer of ex-vivo expanded donor alloreactive NK cells accelerates post bone marrow transplant immune reconstitution

Abstract

Allogeneic hematopoietic transplantation is a powerful treatment for hematologic malignancies. Post-transplant immune incompetence exposes patients to disease relapse and infections. We previously demostrated that donor alloreactive natural killer (NK) cells ablate recipient hematopoietic targets, including leukemia. Here, in murine models, we show that infusion of donor alloreactive NK-cells triggers recipient dendritic cells (DCs) to synthesize beta-2-microglobulin (B2M) that elicits the release of c-KIT-Ligand and interleukin-7 that greatly accelerate post-transplant immune reconstitution. An identical chain of events was reproduced by infusing supernatants of alloreactive NK/DC co-cultures. Similarly, human alloreactive NK-cells triggered human DCs to synthesize B2M that induced interleukin-7 production by thymic epithelial cells and thereby supported thymocyte cellularity in vitro. Chromatography fractionation of murine and human alloreactive NK/DC co-culture supernatants identified a protein with molecular weight and isoelectric point of B2M and mass spectrometry identified amino-acid sequences specific of B2M. Anti-B2M antibody depletion of NK/DC co-culture supernatants abrogated their immune rebuilding effect. B2M knock-out mice were unable to undergo accelerated immune reconstitution but infusion of (wild type) NK/DC coculture supernatants restored their ability to undergo accelerated immune reconstitution. Similarly, silencing the B2M gene in human DCs, before co-culture with alloreactive NK cells, prevented the increase in thymocyte cellularity in vitro. Finally, human recombinant B2M increased thymocyte cellularity in a TEC/thymocyte culture system.

Our studies uncover a novel therapeutic principle for treating post-transplant immune incompetence and suggest that, upon its translation to the clinic, patients may benefit from adoptive transfer of large numbers of cytokine-activated, ex-vivo expanded donor alloreactive NK-cells.

Introduction

Allogeneic hematopoietic cell transplantation is the most powerful therapy for high-risk leukemia (*1, 2*). Unresolved issues are leukemia relapse, graft-versus-host disease (GvHD) and prolonged immune incompetence. In pioneering major histocompatibility complex (MHC) haplotype mismatched ("haploidentical") transplantation (3), we discovered that donor-versus-recipient natural killer (NK) cell alloreactions play a beneficial role (*4, 5*). Human NK cell function is finely tuned by clonally distributed cell surface receptors including inhibitory receptors termed "Killer-cell Ig-like Receptors" (KIRs) that recognize human leukocyte antigen (HLA) class-I allele groups ("KIR ligands"), such as Bw4, C1 and C2 (*4-13*). In transplants that are KIR ligand-mismatched in the donor-versus-recipient direction donor NK cells, that express KIR(s) for HLA class-I allele group(s) present in the donor but absent in the recipient, sense the missing expression of the inhibitory self HLA class-I KIR ligand and are activated to kill recipient targets. NK cell alloreactions reduce leukemia relapse and improve survival (*4, 5, 13*). In murine F1 $H\text{-}2^{d/b}$→parent $H\text{-}2^{b}$ transplants or in $H\text{-}2^{d}$→$H\text{-}2^{b}$ transplants, donor NK cells that do not express the $H\text{-}2^{b}$-specific Ly49C/I inhibitory receptor (but instead bear $H\text{-}2^{d}$-specific Ly49A/G2 receptors) cannot be blocked by the mismatched recipient MHC-haplotype and are activated to kill recipient's targets (*4, 5*). In this model, the pre-transplant infusion of donor-versus-recipient alloreactive NK cells ablates leukemic cells, recipient T cells responsible for graft rejection, and recipient dendritic cells (DCs) which trigger GvHD (*4, 5*).

Here, in murine bone marrow transplant (BMT) models and in a human cell culture system, we show that donor-versus-recipient alloreactive NK cells triggered recipient DCs to synthesize a protein that played a key role in stimulating production of two master regulators of lymphocyte development, IL-7 and cKIT-ligand (L) that greatly accelerated post-BMT recovery of donor-derived B and T lineage cells and DCs. Proteomics analyses demonstrated the molecule produced by DCs to be beta-2-

microglobulin (B2M). Genetics analyses strengthened this finding and showed the *in vivo* role of B2M in immune recostitution.

Materials and Methods

Murine bone marrow transplants

Experiments were performed in accordance with the Italian Ethics Approval Document for Animal Experimentation. Initial experiments investigated the effects of alloreactive NK cell infusions on post-BMT immune reconstitution. Six to eight week old female C57BL/6 (*H-2*^{b}) mice (Charles River Laboratories, Calco, Italy) were conditioned with lethal TBI (8 Gy). One day later, mice received an intravenous infusion (through the tail vein) of alloreactive NK cells from 6 to 8 week old female Balb/c (*H-2*^{d}) mice (Charles River Laboratories). Control mice were given non-alloreactive (i.e., syngeneic) NK cells. NK cells were obtained from splenocytes by Ficoll-Hypaque gradient centrifugation and immunomagnetic selection using a mouse NK cell Isolation kit (Miltenyi, Bergisch Gladbach, Germany). Before infusion, NK cells were cultured for 4 days in the presence of 2,000 IU/ml human IL-2 (Miltenyi) at the concentration of $2x10^{6}$ cells/ml in RPMI culture medium supplemented with 10% fetal calf serum (Invitrogen, CA, USA). DX5 antibody staining of murine NK cell-specific α2-integrin showed ≥ 98% purity of ex-vivo expanded, IL2 activated NK cells used for infusions in bone marrow transplanted mice (DX5- cells were CD3+ T cells) (Figure S1). Before infusion, NK cells were washed. NK cell infusions contained 10^{6} alloreactive NK cells in a final volume of 500 µl phosphate buffered saline (PBS). NK cells were analyzed for expression of inhibitory receptors for MHC class-I by fluorescein-isothiocyanate (FITC)-conjugated anti-Ly49A, FITC-conjugated anti-Ly49G2, phycoerythrin (PE)-conjugated anti-Ly49C/I monoclonal antibodies (mAbs) (BD Biosciences, CA, USA). Multi-color immunofluorescence was analyzed by flow-cytometry using

a 2-laser FACScanto (BD). Such an analysis demonstrated the NK cells from *H-2*^{d} donor mice contained a population (~40%) that did not carry the *H-2*^{b}-specific Ly49C/I inhibitory receptor and, consequently, was potentially alloreactive against recipient *H-2*^{b} targets. Alloreactivity was tested using *H-2*^{b} mouse Concanavalin A (Sigma-Aldrich, Missouri, USA) T cell blasts as targets in a ^{51}Cr release cytotoxicity assay (4). One day after the NK cell infusion, mice received 10 x 10^{6} T cell-depleted BM cells collected by flushing the femur and tibia shafts of *H-2*^{d} mice. BM cells were T cell-depleted by negative immunomagnetic selection using anti-CD5 microbeads (Miltenyi) as previously described (4).

In order to investigate whether a specific recipient cell type triggered alloreactive NK cells to accelerate post-BMT immune reconstitution, four types of recipient chimeras were constructed in which hematopoietic and non-hematopoietic tissues differed in their MHC class I types so as to make tissues potentially susceptible (*H-2*^{b}) or resistant (*H-2*^{d} or *H-2*$^{d/b}$) to alloreactivity mediated by NK-cells from the *H-2*^{d} donor mouse. Chimera 1 displayed NK-susceptible non-hematopoietic tissues and NK-resistant hematopoietic cells. Chimera 2 displayed NK-resistant non-hematopoietic tissues and NK-susceptible hematopoietic cells**.** Chimera 3 displayed DCs that were potentially susceptible to donor alloreactive NK cells, while all other recipient hematopoietic and non-hematopoietic cells were resistant. Chimera 4 displayed NK-resistant DCs and NK-susceptible hematopoietic and non-hematopoietic cells. BM graft and NK cell numbers were the same as in the transplants described above. For a detailed description see "Construction of transplantation chimeras" in Supplemental Material.

In a further series of experiments, mice received an infusion of supernatants obtained from NK/DC co-cultures. Such experiments were designed to investigate whether soluble factor(s) contained in alloreactive NK/DC co-culture supernatants mediated biological effects. Therefore, these experiments were intentionally performed using either allogeneic or syngeneic donor-recipient transplant pairs, with

identical results. The details of such experiments are extensively outlined under the "Supplemental Data" section, in the paragraph entitled "In-vivo infusions of NK/DC coculture supernatants".

Statistical analysis

For statistical analyses see "Supplemental Methodology" section

Immune reconstitution

Immune reconstitution was evaluated by multicolor immune-fluorescence, ELISA and qPCR. For a detailed description of methodologies see the paragraph entitled "Immune reconstitution" in the "Supplemental Methodology" section.

Human cell cultures

For methodologies dealing with cloning of human alloreactive NK and T cells and cytotoxicity assays against allogeneic DCs (13) and generation of NK/DC coculture supernatants and the human thymocyte epithelial cells (TEC)/thymocyte culture system (14) see the paragraph entitled "Human cell cultures" in the "Supplemental Methodology" section.

Proteomics

The biochemical analyses were designed to identify a newly synthesized, biologically active protein ("the immune rebuilding factor") and to define its biochemical features. In order to biochemically define the "immune rebuilding factor", NK/DC coculture supernatants were subjected to HIC and RP chromatography, SDS-PAGE and autoradiography. SDS-PAGE bands displaying immune rebuilding activity were excised and subjected to protein digestion and peptide extraction before nano liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. For the detailed description

of such experiments see the paragraph entitled "Proteomics" in the "Supplemental Methodology" section.

Analysis of the role of the *B2M* gene

In order to document the role of B2M in accelerating post-transplant immune reconstitution, supernatants obtained from co-cultures of alloreactive NK-DCs or non-alloreactive NK/DC culture combinations were B2M immunodepleted using the polyclonal rabbit anti-B2M FL-119 Ab (Santa Cruz Biothecnology), followed by a secondary goat anti-rabbit Ab subsequently adsorbed on a sepharose-column. A non-B2M immune rabbit serum was used as a negative control. In order to obtain genetic evidence for B2M's role in accelerating immune reconstitution, the following experiments were performed: 1) *B2M*-KO *H-2*^{b} mice (Jackson Laboratories) were lethally-irradiated and were given an infusion of 10^6 alloreactive WT *H-2*^{d} NK cells before T cell-depleted BMT (10 x 10^6 cells) from WT *H-2*^{d} mice; 2) lethally-irradiated WT *H-2*^{b} mice were given an infusion of 500 µl supernatant from co-cultures of 5 x 10^6 alloreactive WT *H-2*^{d} NK cells and 5 x 10^6 DCs from *B2M*-KO *H-2*^{b} mice; 3) *B2M*-KO mice received an infusion of supernatants obtained from co-cultures of alloreactive WT *H-2*^{d} NK cells and WT *H-2*^{b} DCs. In order to obtain genetic evidence of the B2M's role in the human system, the *B2M* gene was silenced in human DCs. Human DCs were transfected using Amaxa P3 Primary cell 4D-NucleofectorTM X Unit (Lonza, Basel, Switzerland), in accordance with the manufacturer's instructions. Aliquots of 2 x 10^6 DCs were electroporated with 20 pmol of B2M-specific or scramble siRNA, re-suspended in cell culture medium and incubated overnight at 37^{o}C/5% CO_2. *B2M*-silenced DCs or DCs electroporated with scramble RNA or untreated DCs were co-cultured with alloreactive NK cell clones. NK/DC co-culture supernatants were added to the human TEC/thymocyte culture.

Gene Expression Profiling

In order to obtain information about the downstream pathways triggered by B2M on stromal cells, B2M KO mice were used in order to avoid any interference by endogenous B2M. They were lethally irradiated and seven days later they were infused with supernatants obtained from co-cultures of alloreactive WT *H-2*^{d} NK-cells and WT *H-2*^{b} DCs or non-alloreactive (syngeneic) NK/DC co-culture combinations. After 12 hours, RNA from murine BM was subjected to gene expression profiling. For the detailed description of such experiments see the paragraph entitled "Gene expression profiling" in the the "Supplemental Methodology" section.

Results

H-2^{b} recipient mice were conditioned with total-body irradiation (TBI) and received an infusion of interleukin (IL)-2 activated NK cells from *H-2*^{d} mice. Such NK cells contain a population that lacks the *H-2*^{b}-specific Ly49C/I inhibitory receptor and, consequently, is alloreactive against *H-2*^{b} lympho-hematopoietic cells (4). Subsequently, mice were transplanted with extensively T cell-depleted bone marrow (BM) from *H-2*^{d} mice (contaminating T cells in the BM graft were < 0,2 %) (see cartoon in Figure 1). The engrafted hematopoietic stem cells quickly gave rise to a greatly accelerated recovery of major players of the immune system, such as developing and mature T and B cells, as well as of DCs in the thymus, BM and spleen which quickly reached values comparable with those of donor mice (see Figure 1 and its legend for a detailed description of the various developing T, B and DC subsets). We next investigated whether interactions between donor NK cells and specific recipient cell types were responsible for accelerated post-BMT immune reconstitution. Because alloreactive NK cells are preferentially activated by hematopoietic cells (*4*), we constructed recipient chimeras in which hematopoietic lineage cells and non-hematopoietic tissues differed in their MHC class-I types so as to

make tissues potentially susceptible (*H-2*^{b}) or resistant (*H-2*^{d} or *H-2*$^{d/b}$) to alloreactivity mediated by donor *H-2*^{d} NK cells (see cartoons in Figure 2A). Recipient chimeras were lethally-irradiated, received an infusion of *H-2*^{d} NK cells and subsequently received a T cell-depleted BMT from the *H-2*^{d} donor mice. In chimeras with NK-susceptible non-hematopoietic tissues and NK-resistant hematopoietic cells, post-BMT immune reconstitution was not accelerated (Figure 2A: chimera 1). In contrast, chimeras with NK-resistant non-hematopoietic tissues and NK-susceptible hematopoietic cells displayed accelerated post-BMT immune reconstitution (Figure 2A: chimera 2). Because of the well-known key role of DCs in the activation of NK cell effector functions (15, 16), we constructed recipient chimeras in which DCs were the only recipient cell type that was either resistant or susceptible to donor NK cell alloreactivity (Figure 2A: chimera 3 and chimera 4). Accelerated post-BMT immune reconstitution did not occur in chimeras that had NK-resistant DCs (Figure 2A: chimera 3). In contrast, accelerated post-BMT immune reconstitution did occur in chimeras with NK-susceptible DCs (Figure 2A: chimera 4). Thus, recipient NK-susceptible DCs were necessary and sufficient for accelerated post-BMT immune recovery to occur. To investigate whether accelerated immune reconstitution was mediated by soluble factors released as a consequence of NK/DC interactions, we co-cultured alloreactive NK cells from *H-2*^{d} mice with NK-susceptible DCs from *H-2*^{b} mice and infused the co-culture supernatant into lethally-irradiated recipient mice prior to the infusion of T cell-depleted BMT (see cartoon in Figure 2B). As such experiments were designed to investigate the potential effects of alloreactive NK/DC co-culture supernatants, they were intentionally performed using either allogeneic or syngeneic donor-recipient transplant pairs, with identical results. Remarkably, unlike the non-alloreactive NK/DC co-culture supernatant (denoted "NK^{syn} + DC" in fig. 2B), the alloreactive NK/DC co-culture supernatant (denoted "NK^{allo} + DC" in fig. 2B) promoted accelerated reconstitution of donor thymocytes, B lineage cells and DCs (Figure 2B). Thus, the interaction between alloreactive NK cells and NK-susceptible DCs resulted in the production of a soluble factor that accelerated post-BMT

immune reconstitution. The factor appeared to be a newly synthesized protein as the supernatant's ability to accelerate donor immune reconstitution was abolished by trypsin treatment and because preformed intracellular proteins obtained by lysing NK cells or DCs did not promote accelerated immune reconstitution (supplemental Figure 2A). To further demonstrate the protein was newly synthesized and to identify the cellular source of the protein (NK cells versus DCs), DNA transcription was blocked with actinomycin D ("actD") either in NK cells or in DCs before their co-culture. Co-culture supernatants were infused into conditioned mice before BMT. Supernatants in which DNA transcription was blocked in DCs, but not in NK cells, failed to accelerate post-BMT immune reconstitution (Figure 2B). These results demonstrated the "immune rebuilding factor" was synthesized by DCs upon attack by alloreactive NK cells. Only the infusion of supernatants obtained by co-culturing DCs with alloreactive NK cells, and not, for example, with alloreactive cytotoxic T cells (CTLs), accelerated immune reconstitution (supplemental Figure 2B). Thus, it would appear that an NK cell-specific signaling was required in order to trigger DCs to activate DNA transcription and synthesis of the "immune rebuilding factor". Moreover, since the capacity to accelerate reconstitution of the immune system was lacking in supernatant of cultures in which *perforin*-knock-out (KO) NK cells were used as effectors, it appears that the NK cell-mediated killing of allogeneic DCs was indispensable for the release of the "immune rebuilding factor" in the supernatant (supplemental Figure 2B).

To probe the existence of a human homologue of "the immune rebuilding factor", NK cell clones were generated from individuals who possessed both HLA class-I C1 and C2 group alleles (C group heterozygous individuals) and, therefore, possessed NK cells that were potentially alloreactive against targets from HLA class-I C1 or C2 allele group homozygous individuals (4, 5, 6, 13). NK clones that were alloreactive against DCs from individuals homozygous for either the HLA C1 or C2 allele groups were co-cultured with target DCs. When added to cultures of human thymic epithelial

253 cells (TECs) and human thymocytes (*14*), supernatants from such NK/DC co-cultures increased the
254 cellularity of thymocytes (Figure 2C). As in mice, DNA transcription blockade in DCs, but not in NK
255 cells, prevented the increase in human thymocyte counts (Figure 2C). Also, trypsin treatment of human
256 alloreactive NK/DC co-culture supernatants abolished their ability to increase thymocyte cellularity
257 (supplemental Figure 2C). Likewise, NK cell or DC lysates were not effective at increasing thymocyte
258 cellularity (supplemental Figure 2C). Finally, only supernatants obtained by co-culturing DCs with
259 alloreactive NK cells, and not for example CTLs, were able to increase thymocyte counts
260 (supplemental Figure 2D).

261 To identify newly synthesized proteins with immune rebuilding activity, we performed two
262 parallel NK/DC co-culture experiments, one in the presence and the other in the absence of ^{35}S-
263 methionine. Culture supernatants were fractionated by hydrophobicity chromatography (HIC) and
264 reverse phase (RP) chromatography. One fraction (out of the 30 murine and 30 human fractions that
265 were obtained in the non-radioactive experiment) displayed the most robust biological effect. Such
266 fraction enhanced murine thymocyte (Figure 3A), T and B cell and DC counts *in vivo* (not shown) and
267 increased human thymocyte cellularity *in vitro* in the human TEC/thymocyte cultures (Figure 3B).
268 Then, HIC+RP chromatography fractions from the radioactive experiment were subjected to sodium
269 dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. An 11-12
270 kilodalton (kDa) molecular weight (MW) protein was detected in the murine and human fractions that
271 corresponded to the fractions that exerted maximal immune rebuilding activity in the non-radioactive
272 experiment (Figure 3C). Two-dimensional electrophoresis showed the 11-12 kDa MW protein had
273 isoelectric points (pI) of 7.5 and 8 in murine samples and 6-7 in human samples (Figure 3D).
274 Subsequently, proteins were extracted from the area of interest of the non-radioactive SDS-PAGE gel,
275 digested and analyzed by high-resolution mass spectrometry. To identify the molecular nature of the
276 "immune rebuilding factor" amongst the hundreds of proteins detected by mass spectrometry, the

following ranking criteria were applied: 1) ≥1.5-fold increase in protein content in supernatants from alloreactive NK/DC co-cultures in comparison with supernatants from non-alloreactive NK/DC co-cultures, 2) 11-12 kDa MW, 3) pI of 6-7 for the human and 7.5 and 8 for the murine protein, 4) detection of the protein displaying the above biochemical features in all murine (n= 3) and human (n= 2) experiments. Among the 30 top-ranking proteins (Table S1) out of the 853 that were detected by mass-spectrometry, we preliminarily tested FKBP1A because it is the target of tacrolimus and has a plethora of immune-modulatory functions (17), in spite of the fact that it did not fulfill all the ranking criteria because it had isoelectric points that differed from those detected in our experiments. We immune-depleted alloreactive NK/DC co-culture supernatant with anti-FKBP1A antibodies. The immune rebuilding effect of the supernatant was not affected (not shown).

Only one protein, i.e. B2M, fulfilled all the ranking criteria (18, 19) (supplemental table 1 and Figure 3E). We next sought functional evidence for B2M's role in accelerating immune reconstitution. In the mouse, anti-B2M antibody-depletion reduced the immune rebuilding ability of alloreactive NK/DC co-culture supernatants, thus indicating B2M played a role in post-transplant immune reconstitution (Figure 4A). Most importantly, genetic evidence was obtained in *B2M*-KO *H-2*^{b} mice (Figure 4B). *B2M*-KO *H-2*^{b} mice were lethally-irradiated and were given alloreactive NK cells and a T cell-depleted BMT from wild-type (WT) *H-2*^{d} mice. In this model, accelerated immune reconstitution was not observed. Accelerated post-BMT immune rebuilding was also not observed when lethally-irradiated WT *H-2*^{b} mice were given an infusion of supernatants from co-cultures of alloreactive WT *H-2*^{d} NK cells and *B2M*-KO *H-2*^{b} DCs. In contrast, infusion of supernatants obtained from co-cultures of alloreactive WT *H-2*^{d} NK cells and WT *H-2*^{b} DCs endowed *B2M*-KO mice with the capacity to undergo accelerated post-BMT immune reconstitution (Figure 4B).

Given that multiple cells of the hematopoietic lineage, such as B lineage cells, developing T cells and DCs were involved in the immune rebuilding effect, we hypothesized that the BM

environment played a primary role in mediating the accelerated immune recovery. Therefore, to identify the molecular mechanisms underlying the regenerative effects induced by B2M, we performed RNA-seq analyses (shown as a volcano plot in figure 4C) followed by Gene Set Enrichment Analysis (GSEA) on BM from mice that had been lethally-irradiated but not rescued by BMT and had received an infusion of alloreactive NK/DC co-culture supernatants versus infusion of non-alloreactive (syngeneic) NK/DC co-culture supernatants. The list of differentially regulated genes is shown in the BM gene expression profiling table (Supplementary Data 1). Amongst the 20 most upregulated genes, *IL-7* and *cKIT-L* stood out because they are well-known master regulators of lymphocyte development (20-22). Indeed, IL-7 and cKIT-L mRNA quantification by qPCR confirmed these cytokines were stably upregulated (for one week) in BM and thymus of lethally-irradiated, alloreactive NK cell-treated mice (Figure 4D). IL-7 and cKIT-L were indispensable for faster post-BMT immune reconstitution, as combined infusion of blocking anti-IL-7 and anti-cKIT-L antibodies hindered accelerated immune reconstitution of BMT-transplanted mice (Fig. 4E). Interestingly, GSEA revealed that several biological processes including cell cycle phase transition, response to cytokine, biosynthetic metabolic processes and leukocyte immunity were induced after infusion of alloreactive NK/DC co-culture supernatants (supplemental Figure 3 and BM gene expression profiling table, Supplementary data 2).

Comparable results were obtained using human alloreactive NK cells co-cultured with human DCs in which *B2M* gene expression was silenced by siRNA (Figure 5A). Supernatants from co-cultures of alloreactive NK cells and *B2M*-silenced DCs failed to increase thymocyte cellularity in the human TEC/thymocyte culture system (Figure 5B). Further evidence for the involvement of B2M in the accelerated immune rebuilding was obtained by the use of recombinant B2M. Unfortunately, the use of murine recombinant B2M did not provide informative results (data not shown), probably because commercially available murine recombinant B2M molecules are histidine-tagged, a feature which is known to result in diminished or altered biological activity (23, 24). In contrast, a commercially

available human recombinant B2M, which is not histidine-tagged and is expressed in Escherichia Coli as the 14.0 kDa B2M precursor (25), did exert a dose-dependent effect on the increase in thymocyte cellularity in the TEC/thymocyte culture system. However, the effect mediated by 14.0 kDa B2M precursor was smaller (one third) than that obtained by using alloreactive NK/DC co-culture supernatants (Figure 5C).

In the human TEC/thymocyte culture system, alloreactive NK/DC co-culture supernatants promoted IL-7 production by TECs (Figure 5D) and the addition of an anti-IL-7 antibody prevented the increase in thymocyte cellularity, thus indicating that thymocyte expansion promoted by alloreactive NK/DC co-culture supernatants was IL-7-dependent in this *in vitro* system (Figure 5E).

Discussion

The present study uncovers a novel, NK cell-based immunotherapeutic intervention to obviate post bone marrow transplant immune deficiency. In murine allogeneic bone marrow transplantation models, the infusion of ex-vivo expanded donor alloreactive NK cells triggered B2M production by recipient DCs that in turn elicited the release of *IL-7* and *cKIT-L* from thymic and BM stroma. *IL-7* and *cKIT-L* greatly accelerated reconstitution of donor-type, developing and mature T and B cells, as well as of DCs in the thymus, BM and spleen (see figure 6 for a graphic summary of data). Comparable results were obtained in a human *in vitro* thymocyte/TEC culture system, that is, a human alloreactive NK/DC co-culture supernatant promoted IL-7 production by TECs which in turn supported thymocyte cellularity.

Evidence for the unprecedented involvement of B2M in immune reconstitution was obtained by the observation that: 1) chromatography fractionation of alloreactive NK/DC co-culture supernatants identified a protein with molecular weight and isoelectric points of B2M. 2) A high-sensitive mass

spectrometry analysis identified a protein with the amino acid sequences specific of B2M. 3) Anti-B2M antibody depletion of NK/DC co-culture supernatants abrogated the immune rebuilding effect. 4) The B2M gene is indispensable for accelerated immune reconstitution. B2M KO mice were unable to undergo accelerated immune reconstitution and the infusion of (wild-type) NK/DC coculture supernatants reversed their inability to undergo accelerated immune reconstitution. Similarly, silencing the human B2M gene in human DCs before co-culture with alloreactive NK cells prevented the increase in thymocyte cellularity in the human in vitro system. 5) The human recombinant 14.0 kDa B2M precursor (25) increased thymocyte cellularity in an in vitro TEC/thymocyte culture system. The partial immune rebuilding effect exerted by the human recombinant B2M, compared with the B2M synthesized by DCs, might be due to the fact that the recombinant B2M had a molecular weight of 14.0 kDa, since it included the whole precursor sequence with the signal peptide for secretion. Moreover, in the E. Coli expression system, no post-translational modifications are usually inserted in the expressed proteins due to the diversity of the molecular machinery for post-translational modifications, including proteolytic cleavage. According to our analysis, the protein candidate responsible of the immune reconstitution and identified as B2M, had a molecular weight of about 12.0 kDa corresponding to the mature sequence of B2M as confirmed by SDS-PAGE and mass spectrometry (25). It is therefore possible that the actual effector is the mature protein and not the precursor. Furthermore, two-dimensional electrophoresis experiments showed that B2M is present as two spots with different isoelectric points, a possible indication of DC-specific post-translational modifications that may happen before secretion and may be involved in the immune reconstitution effect.

B2M, recently shown to play a role in inflammation (26, 27), is primarily known as key component of MHC class-I family molecules: it is necessary for their correct folding and cell surface expression enabling the formation of a stable peptide binding groove and, consequently, for recognition by CD8+ T cells and CD8+ T cell clonal expansion. Interestingly, B2M-deficiency in humans supports

373 the idea that B2M is involved in the maturation of various components of the immune system because
374 B2M-deficient patients exhibit broad immune incompetence involving not only CD8+ T cells but also
375 CD4+ and B cells (*28*). Thus, it is conceivable that the NK/DC "cross-talk" (16) and the consequent
376 NK cell activation and killing of autologous DCs (15) may lead to B2M production even under
377 physiological conditions (an effect that our control experiments using autologous NK/DC combinations
378 might not have been sensitive enough to detect).

379 To improve immune reconstitution after allogeneic hematopoietic cell transplantation a variety
380 of approaches have been proposed, such as adoptive T cell therapy with non-alloreactive and/or
381 pathogen-specific T cells, transfer of lymphoid progenitor cells, thymic grafts, enhancement of
382 thymopoiesis by sex-steroid blockade, administration of IL-7, keratinocyte growth factor, fms-like
383 tyrosine kinase-3 ligand, IL-22 or growth hormone (*29-38)*. Approaches thus far tested clinically have
384 significant limitations. Here, for the first time, we demonstrate that infusion of lage doses of
385 alloreactive NK cells promotes stricking acceleration of post bone marrow transplant immune
386 reconstitution. As it might have been expected, we did not find any difference in immune reconstitution
387 of patients transplanted from NK alloreactive donors and patients transplanted from non-NK
388 alloreactive donors. In fact, it should be noted that there are very remarkable differences between our
389 clinical transplantation protocol (3, 4) and the murine experiments. Human transplants were performed
390 using purified CD34+ hematopoietic progenitor cells which give rise to reconstitution of NK cells that
391 initially contain alloreactive cells in low frequencies. In contrast, the murine experiments were
392 performed with the infusion of very large numbers of ex-vivo expanded, IL2-activated donor NK cells
393 containing high frequencies (40-50%) of alloreactive cells.

394 In conclusion, our studies uncover a novel therapeutic principle for the treatment of the post
395 bone marrow transplant immune deficiency and suggest that future protocols that will include the

adoptive transfer of large numbers of cytokine-activated, ex-vivo expanded donor alloreactive NK cells may improve the survival rate of patients undergoing haploidentical hematopoietic transplantation.

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Author contributions: L.R. designed and performed experiments and wrote paper. E.U., S.C. and R. S. performed murine transplant experiments and human cell cultures. F.S. and P.L.O. performed biochemical analyses. E.B. performed murine immune reconstitution analyses. D.C. designed and performed proteomics analyses and wrote paper. S.R.P. performed proteomics analyses. S.B. performed human RNA silencing experiments. D.R. contributed to human thymocyte culture experiments. S.P., as heart surgeon at the Ospedale Santa Maria della Misericordia, Perugia, provided human thymic tissue samples obtained during corrective cardiovascular surgery. L.B. conceived, designed, supervised biochemical analyses and critically revised the manuscript. C.R.J. supervised proteomics analyses and critically revised the manuscript. G.A.H. designed experiments, provided constructive suggestions and wrote paper. E.V. and A.C performed GSEA and revised the manuscript, A.P. and F.L. revised the manuscript. A.V. conceived and supervised the project, designed experiments and wrote the paper.

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518

Figure legends

Figure 1. Infusion of donor alloreactive NK cells accelerates post-BMT immune reconstitution.

(A) *H-2*^{b} recipient mice were conditioned with TBI+IL2-activated alloreactive NK cells (NK^{allo}) from *H-2*^{d} mice or with TBI+IL2-activated syngeneic (non-alloreactive) H-*2*^{b} NK cells (NK^{syn}). Mice were then transplanted with T cell-depleted BM from *H-2*^{d} mice. "Cntrl" denotes cell counts in transplant donors. (B) Recovery of BM B220^{+}/Cytoplasmic μ (Cμ)$^{-}$ pro-B cells (black bars), B220^{+}/Cμ^{+}/Surface Immunoglobulins (SIg)$^{-}$ pre-B cells (white bars) and B220^{+}/SIg^{+} B cells (red bars). (C) Recovery of splenic B220^{+}/SIg^{+} B cells. (D) Recovery of CD4^{+}/CD8^{+} double-positive (red bars), CD4^{+} single-positive (white bars) and CD8^{+} single-positive (black bars) thymocytes. (E) Recovery of splenic CD3^{+} T cells, including gamma/delta T cells, regulatory T cells (Tregs), effector memory (EM) T cells, central memory (CM) T cells and naïve T cells. (F) Splenic CD3^{+} T cell proliferation in response to allogeneic *H-2*^{b} splenocytes and anti-CD3 antibody stimulation at 35 days after transplant. (G) Recovery of BM CD11c^{+} DCs. (H) Recovery of splenic CD11c^{int}/B220^{+}/GR1^{+} plasmacytoid DCs (pink bars) and CD11c^{high}/B220^{-}/GR1^{-} myeloid DCs (white bars). (I) Recovery of thymic CD11c^{high}/CD8^{+} DCs (blue bars) and CD11c^{high}/CD8^{-} DCs (white bars). Bars: mean ± SD of at least 3 independent experiments; statistics were performed with Student's test (GraphPad Prism 5). **$p < 0.01$.

Figure 2. Donor alloreactive NK cells trigger recipient DCs to synthesize proteins that accelerate post-BMT immune reconstitution.

(A) Recipient murine chimeras were constructed in which hematopoietic lineage cells and non-hematopoietic tissues differed in their MHC class-I thus rendering tissues either potentially susceptible (*H-2*^{b}) or resistant (*H-2*^{d} or *H-2*$^{d/b}$) to donor (*H-2*^{d}) NK-cell alloreactivity. Chimeras were conditioned and received NK cells and BMT from *H2*^{d} mice. Cartoons illustrate potential target cell susceptibility

(→) or resistance (→|) to NK cell alloreactivity for each individual chimera. Post-BMT cell counts showed that, unlike NK-susceptible non-hematopoietic tissue (Chimera 1), NK-susceptible hematopoietic cells were necessary for accelerated immune reconstitution (Chimera 2). In particular, NK-susceptible DCs were the only recipient hematopoietic lineage cell that was necessary for accelerated immune rebuilding: accelerated post-BMT immune reconstitution did not occur in chimeras that had NK-resistant DCs (Chimera 3), while accelerated post-BMT immune reconstitution did occur in chimeras with NK-susceptible DCs (Chimera 4). (B) Cartoon: *H-2*^{d} NK cells (NK^{allo}) were co-cultured with allogeneic *H-2*^{b} DCs to generate alloreactive NK/DC co-culture supernatants. In control experiments, in order to obtain non-alloreactive NK/DC co-culture supernatants, *H-2*^{d} NK cells (NK^{syn}) were co-cultured with syngeneic *H-2*^{d} DCs. The supernatants were infused into lethally irradiated mice before BMT. Infusion of alloreactive NK/DC co-culture supernatants accelerated post-BMT immune recovery. Infusion of supernatants from alloreactive NK/DC co-cultures in which DNA transcription was blocked in DCs by Actinomycin D (actD), but not in NK cells, failed to accelerate immune reconstitution (cell counts at day +20 are shown). (C) Human alloreactive NK/DC co-culture supernatants (NK^{allo}), obtained by co-culturing alloreactive NK cell clones (from HLA class-I C1/C2 group heterozygous individuals) and NK-susceptible DCs (from HLA class-I C group homozygous individuals) were added to thymocyte/TEC co-cultures. Unlike supernatants obtained from human non-alloreactive (autologous) NK/DC combinations (NK^{auto}), human alloreactive NK/DC co-culture supernatants increased human thymocyte counts *in vitro*. DNA transcription blockade in DCs, but not in NK cells, prevented the increase in human thymocyte counts (cell counts at day 10 of culture are shown).

Figure 3. Biohemical analyses demostrate the murine and human "immune rebuilding factor" is B2M.

(A, B) Murine and human NK/DC co-culture supernatants were fractionated by HIC+RP. One out of the 30 fractions (fraction 22) displayed the most robust biological effect with enhanced murine thymocyte, T and B cell and DC counts *in vivo* and increased human thymocyte cellularity *in vitro* in the human TEC/thymocyte cultures (the *in vivo* increase in murine T and B cells and DCs is not shown). (c, d) Two parallel NK/DC co-culture experiments were performed, one with and one without ^{35}S-methionine, and supernatants were fractionated by HIC+RP chromatography. (C) Fractions from the radioactive experiment were subjected to SDS-PAGE and autoradiography. An 11-12 kDa MW protein was detected in the fractions that corresponded to those that exerted maximal immune rebuilding activity in the non-radioactive experiment. (D) Two-dimensional electrophoresis showed the 11-12 kDa MW protein had pI of 7.5 and 8 for murine and 6-7 for human samples. (E) Panel e illustrates the 14 top ranking proteins identified by mass-spectrometry that displayed ≥1.5-fold increase in protein content in alloreactive NK/DC co-culture supernatants as compared to non-alloreactive NK/DC co-culture supernatants. Proteins were ranked according to presence of: 1) 11-12 kDa MW, 2) pI of 7.5 and 8 for murine and 6-7 for human samples (on top of panel e the combination of the above biochemical features is denoted as "MW-pI"), 3) detection of the protein displaying the above biochemical features in all murine and human experiments. The only protein that possessed all these features was B2M.

Figure 4. **B2M triggers accelerated post-BMT immune reconstitution.**

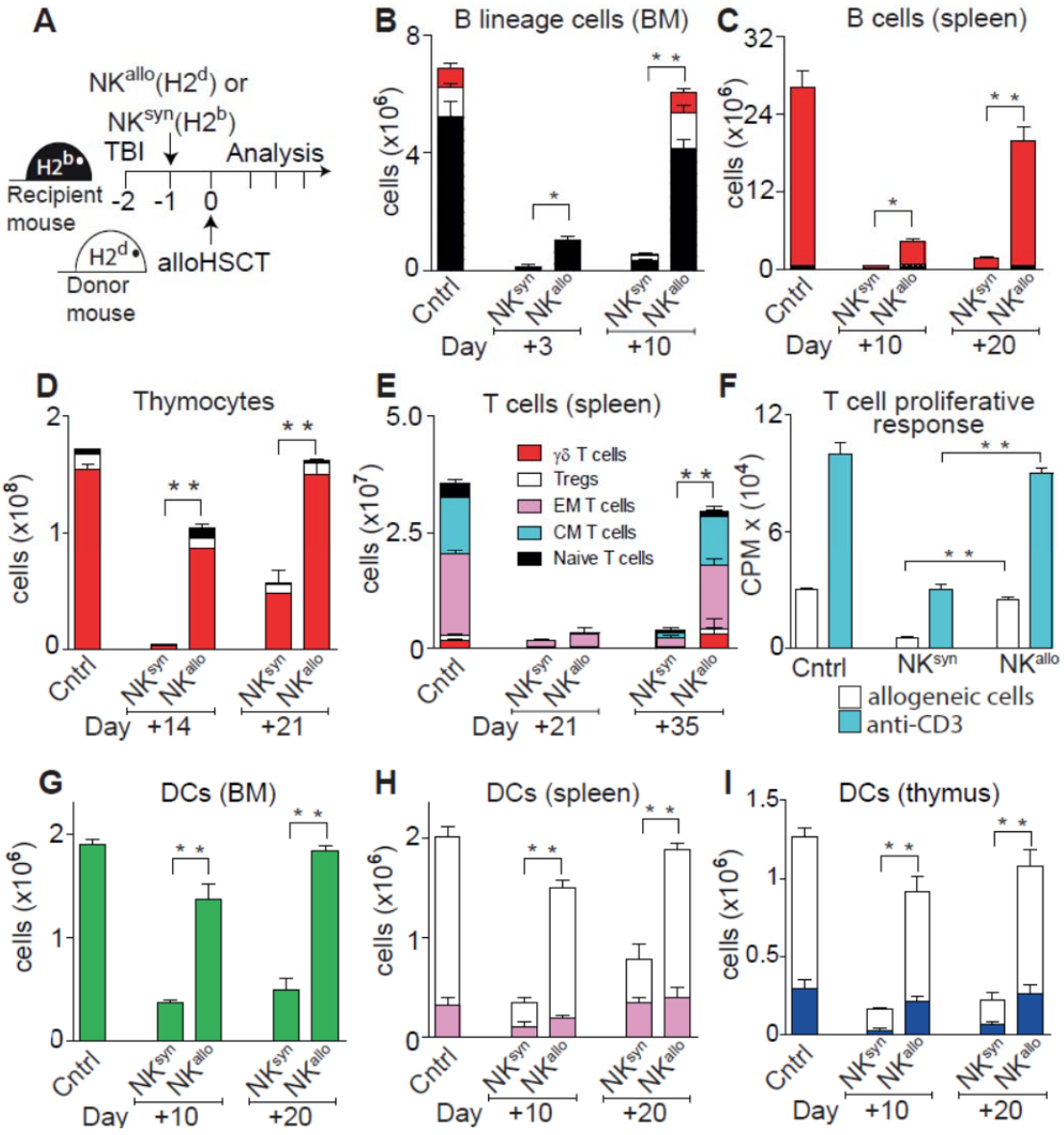
(A) Anti-B2M antibody-depletion reduced the immune rebuilding ability of alloreactive NK/DC co-culture supernatants. (B) When *B2M*-KO *H-2*^{b} mice were lethally-irradiated and were given alloreactive NK cells and a T cell-depleted BMT from WT *H-2*^{d} mice, no accelerated post-BMT

590 immune reconstitution occurred. Also, no accelerated post-BMT immune reconstitution occurred
591 when supernatants from co-cultures of alloreactive WT *H-2*^{d} NK cells and *B2M*-KO DCs were infused
592 in WT recipient mice. In contrast, infusion of supernatants from co-cultures of alloreactive WT *H-2*^{d}
593 NK cells and WT *H-2*^{b} DCs restored the *B2M*-KO mouse ability to undergo accelerated post-BMT
594 immune reconstitution. (C) RNA-seq volcano plot analysis displayed genes that were differentially
595 expressed in the BM of mice seven days after that had received TBI and infusion of alloreactive
596 NK/DC co-culture supernatants (without BMT rescue) versus infusion of non-alloreactive NK/DC co-
597 culture supernatants. RNA-seq analysis revealed significant up-regulation of two master regulators of
598 lymphocyte development namely *IL-7* and *cKIT-L.* (D) qPCR showed *IL-7* and *cKIT-L* were stably
599 upregulated (for one week) in BM and thymus of lethally-irradiated, alloreactive NK cell-treated mice.
600 (E) Infusion of anti-IL-7 plus anti-cKIT-L antibodies into BMT-transplanted mice prevented
601 accelerated immune reconstitution.
602
603 **Figure 5. Genetic and functional evidence that human B2M triggers IL-7 production by human**
604 **TECs and augments thymocyte counts.**
605 (A) Western-blot of B2M developed by Enhanced ChemiLuminescence shows absence of the B2M
606 protein in DCs in which the human *B2M* gene was silenced with siRNA versus its presence in control
607 DCs (untreated or electroporated or treated with scramble siRNA). (B) Supernatants from co-cultures
608 of human alloreactive NK cells and *B2M*-silenced DCs failed to increase human thymocyte cellularity
609 (see lane labelled "B2M-siRNA"). (C) Increase in human thymocyte cellularity upon addition of
610 human recombinant B2M in the TEC/thymocyte culture system. (D) Human alloreactive or non-
611 alloreactive NK cell clones were co-cultured with human DCs. NK/DC co-culture supernatants were
612 added to human TECs. Unlike supernatants from non-alloreactive (auto) NK/DC co-cultures, the
613 supernatants from alloreactive NK/DC (allo) co-cultures promoted IL-7 production by TECs. (E)

When thymocytes were cultured with TECs, the addition of an anti-IL-7 Ab prevented the increase in thymocyte counts mediated by an alloreactive NK/DC co-culture supernatant. Bars: mean ± SD of at least 3 independent experiments; statistics were performed with Student's test (GraphPad Prism 5). *p < 0.05; **p < 0.01.

Figure 6. Graphic summary of data.

During the interaction between NK cells and MHC-mismatched DCs, the mismatched MHC may not be recognised by NK cell inhibitory receptors and the lytic action elicited by activating receptors is allowed to proceed (*4, 5*). The NK cell alloreaction triggers DCs to transcribe the *B2M* gene and to synthesize and release B2M. B2M signals thymic and BM stroma to produce cKIT-L and IL-7 that mediate accelerated post-BMT immune reconstitution.


A
NK^allo(H2^d) or
NK^syn(H2^b)
TBI
Analysis
H2^b
Recipient mouse
-2 -1 0
H2^d
alloHSCT
Donor mouse
B
B lineage cells (BM)
cells (x10^6)
Cntrl
NK^syn
NK^allo
Day +3 +10
C
B cells (spleen)
cells (x10^6)
Day +10 +20
D
Thymocytes
cells (x10^8)
Day +14 +21
E
T cells (spleen)
cells (x10^7)
γδ T cells
Tregs
EM T cells
CM T cells
Naive T cells
Day +21 +35
F
T cell proliferative response
CPM x (10^4)
allogeneic cells
anti-CD3
G
DCs (BM)
cells (x10^6)
Day +10 +20
H
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cells (x10^6)
Day +10 +20
I
DCs (thymus)
cells (x10^6)
Day +10 +20

Figure 1

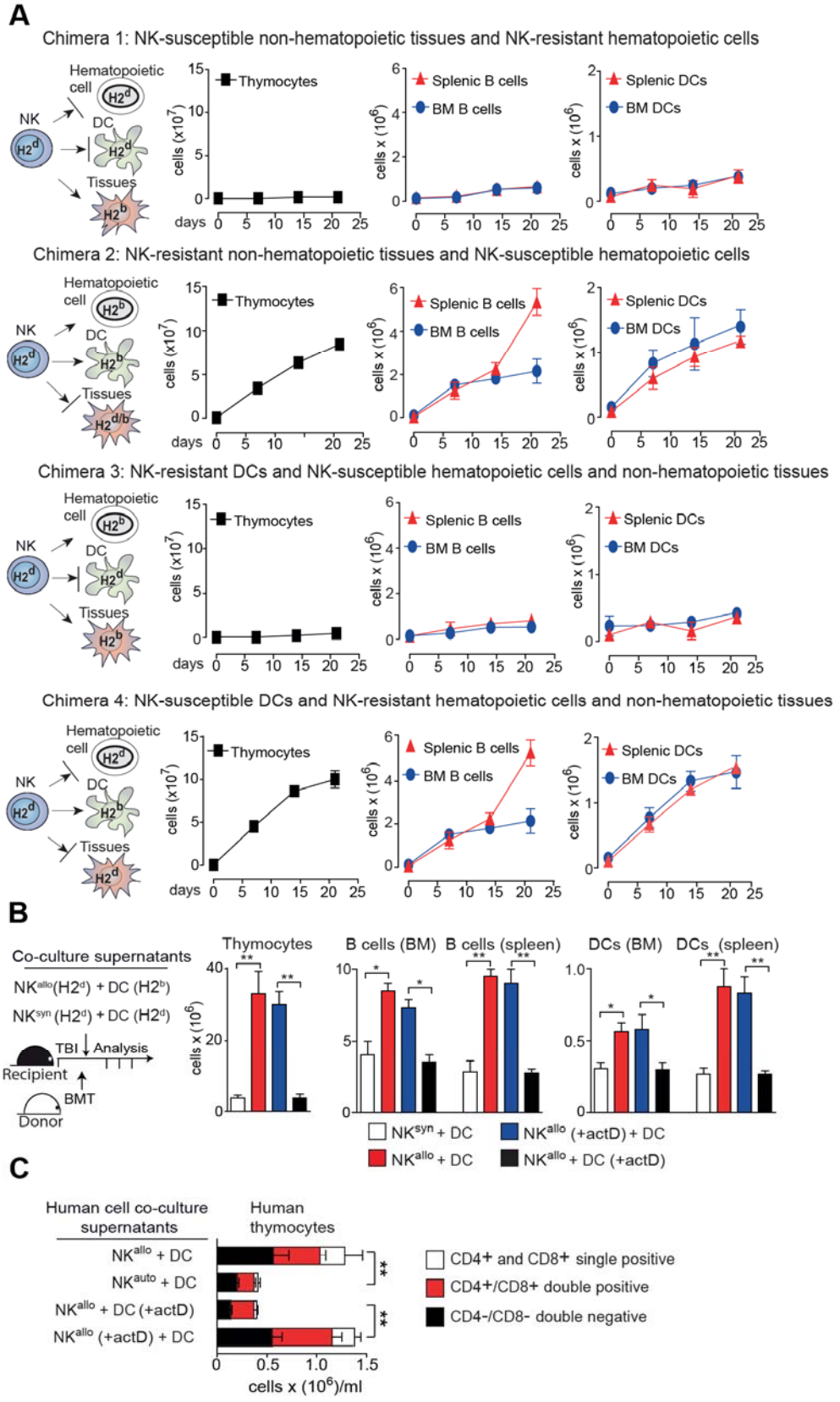
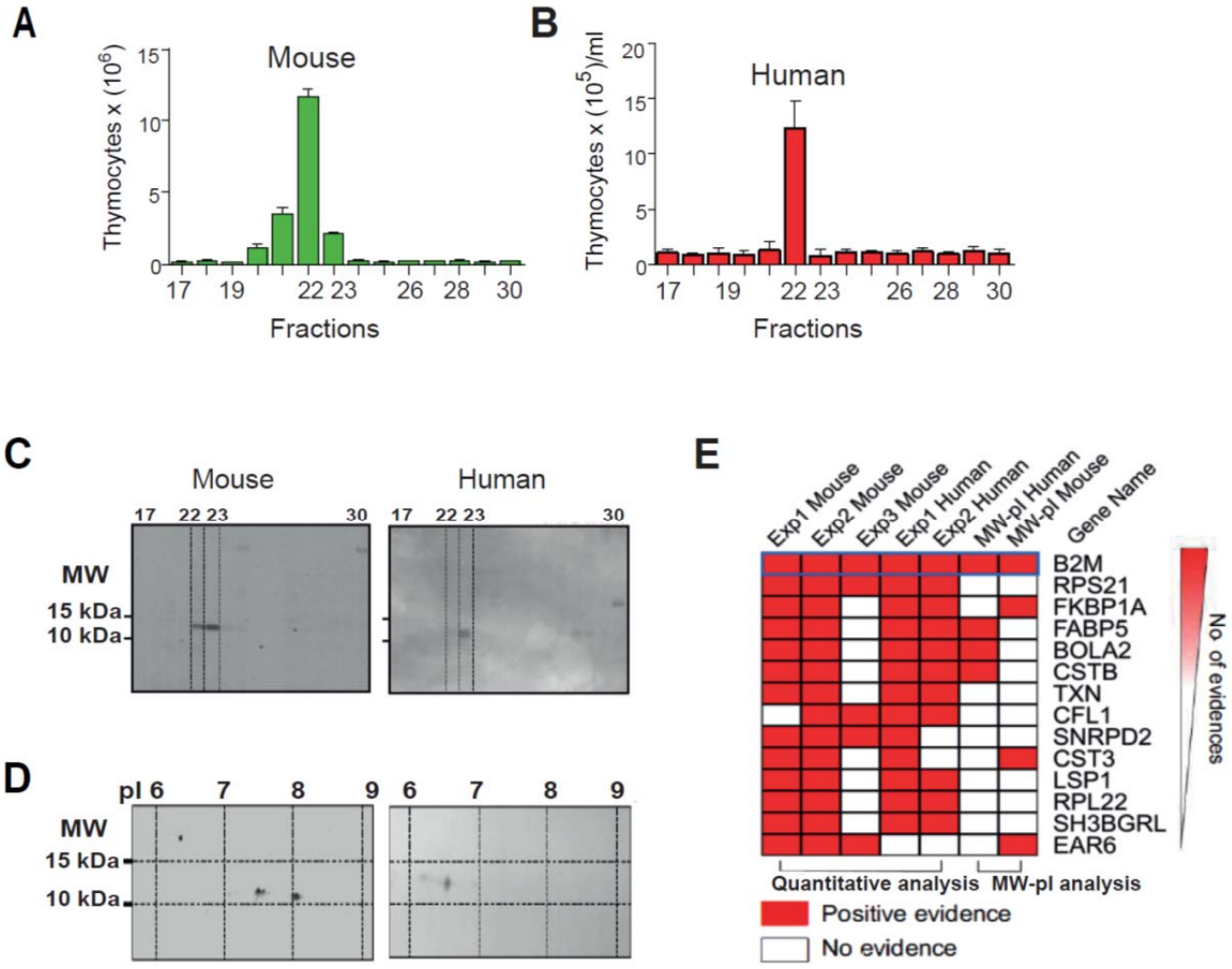
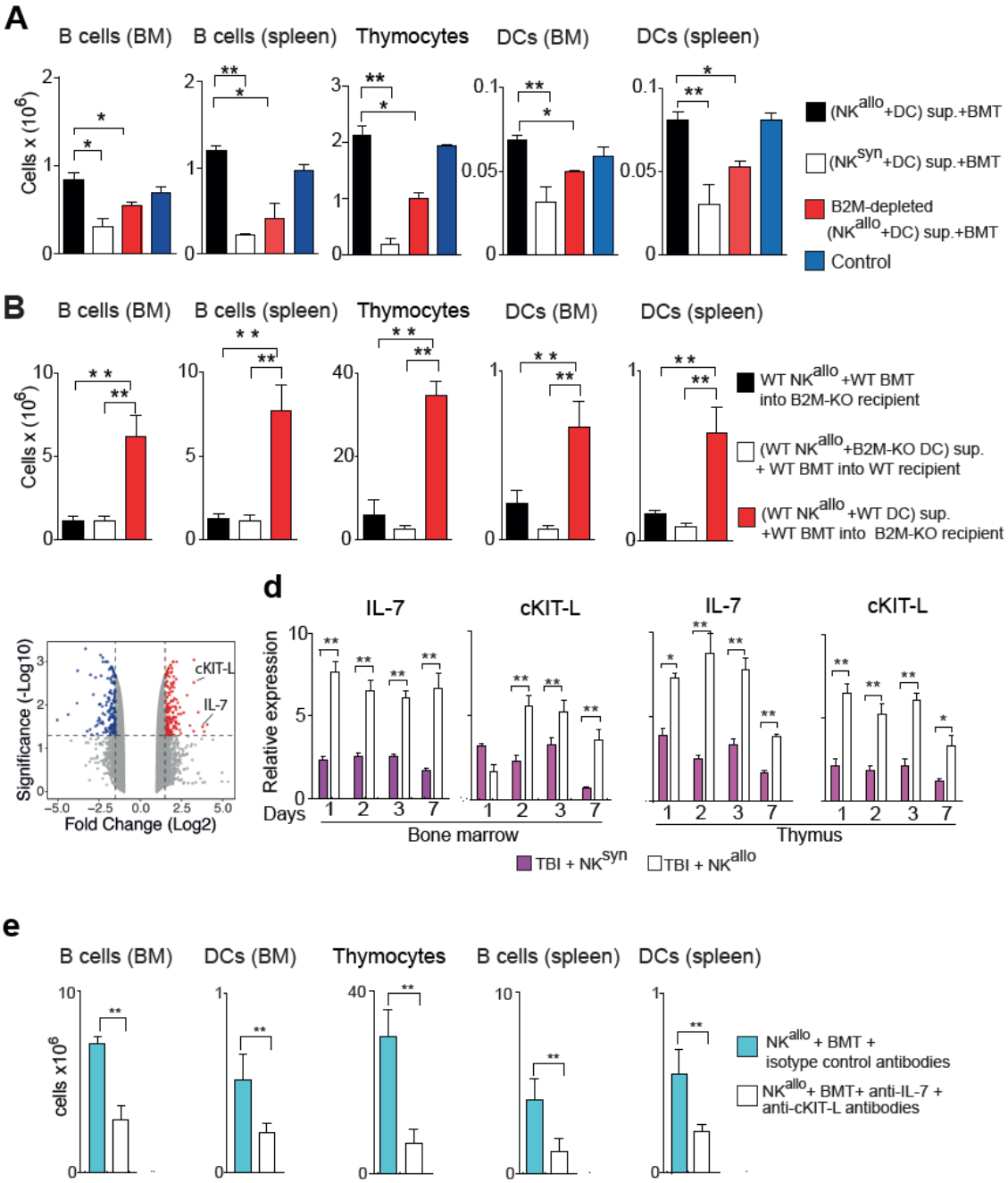

A
Chimera 1: NK-susceptible non-hematopoietic tissues and NK-resistant hematopoietic cells
Hematopoietic cell
NK
DC
Tissues
Thymocytes
Splenic B cells
BM B cells
Splenic DCs
BM DCs
cells (x10^7)
cells x (10^6)
days
Chimera 2: NK-resistant non-hematopoietic tissues and NK-susceptible hematopoietic cells
Chimera 3: NK-resistant DCs and NK-susceptible hematopoietic cells and non-hematopoietic tissues
Chimera 4: NK-susceptible DCs and NK-resistant hematopoietic cells and non-hematopoietic tissues
B
Co-culture supernatants
NK^allo(H2^d) + DC (H2^b)
NK^syn (H2^d) + DC (H2^d)
TBI
Analysis
Recipient
BMT
Donor
Thymocytes
B cells (BM)
B cells (spleen)
DCs (BM)
DCs (spleen)
cells x (10^6)
NK^syn + DC
NK^allo + DC
NK^allo (+actD) + DC
NK^allo + DC (+actD)
C
Human cell co-culture supernatants
Human thymocytes
NK^allo + DC
NK^auto + DC
NK^allo + DC (+actD)
NK^allo (+actD) + DC
CD4+ and CD8+ single positive
CD4+/CD8+ double positive
CD4-/CD8- double negative
cells x (10^6)/ml

Figure 2



630

631 **Figure 3**



632

633

634 **Figure 4**

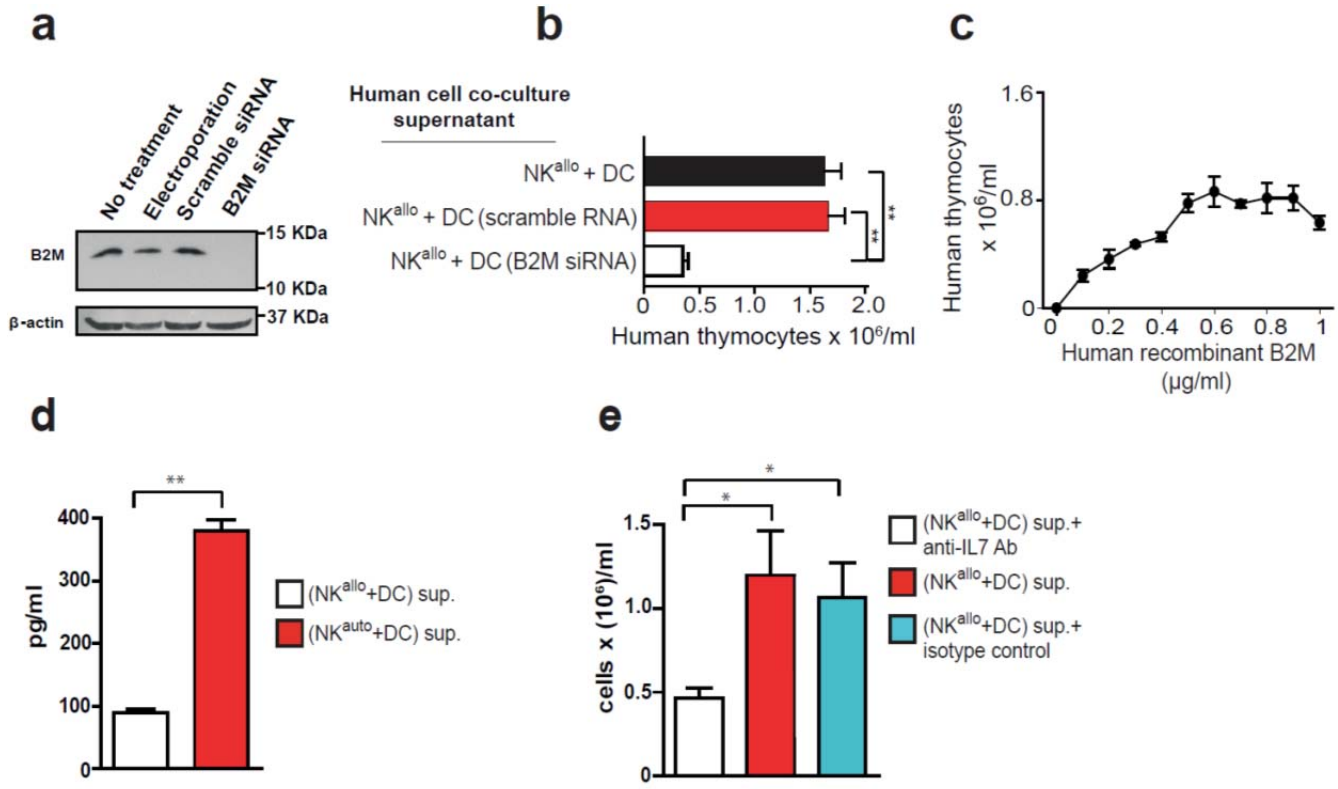
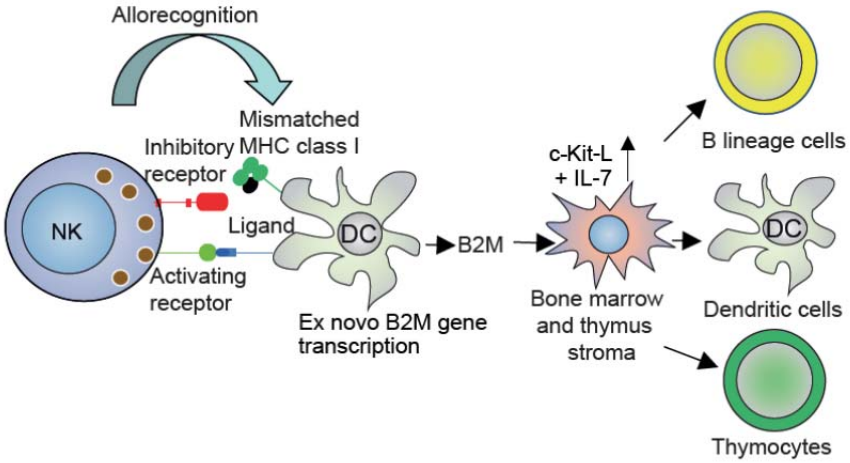

a
No treatment
Electroporation
Scramble siRNA
B2M siRNA
B2M
15 KDa
10 KDa
β-actin
37 KDa
b
Human cell co-culture supernatant
NKallo + DC
NKallo + DC (scramble RNA)
NKallo + DC (B2M siRNA)
**
**
0 0.5 1.0 1.5 2.0
Human thymocytes x 10⁶/ml
c
Human thymocytes x 10⁶/ml
1.6
0.8
0
0 0.2 0.4 0.6 0.8 1
Human recombinant B2M (µg/ml)
d
**
400
300
200
100
0
pg/ml
(NKallo+DC) sup.
(NKauto+DC) sup.
e
*
*
1.5
1.0
0.5
0
cells x (10⁶)/ml
(NKallo+DC) sup.+ anti-IL7 Ab
(NKallo+DC) sup.
(NKallo+DC) sup.+ isotype control

Figure 5



643

644 **Figure 6**