

ORIGINAL ARTICLE

SCID repopulating cells derived from unmobilised adult human peripheral blood

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SUMMARY

Severe combined immunodeficient (SCID)-repopulating cells (termed SRC) with lymphohaematopoietic differentiation potential reside at an extremely low frequency in unmobilised adult human peripheral blood. Recently, an *ex vivo* method of increasing the relative numbers of at least four distinct human stem cell classes, that include CD34⁺ haematopoietic progenitor cells, in mononuclear cells (MNC) obtained from unmobilised adult human peripheral blood has been described. This process is triggered by a monoclonal antibody (mAb) against the human monomorphic region of the beta chain of HLA-DP, DQ and DR (clone CR3/43). Herein, we assess the ability of human male donor-derived MNC, following *ex vivo* culturing for 3 hr in haematopoietic-conductive conditions (HCC) (3-hr MNC/HCC), to form SRC in female non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. All 3-hr MNC/HCC-recipient animals exhibited significant levels (>0.5%) of human cell engraftment in the

bone marrow, thymus and spleen when compared to animals receiving MNC cultured in the absence of CR3/43. Phenotypic characterisation of the bone marrow cell populations of engrafted mice demonstrated significant levels of human lymphohaematopoietic cell lineages, comprised of T lymphocytes, monocytes, erythrocytes and megakaryocytes, including platelets. In addition, significant levels of clonogenic human CD34⁺ cells were also detected by *in vitro* surrogate assay. The thymus of engrafted animals contained maturing human thymocytes, while the spleen consisted mainly of T lymphocytes. Fluorescence *in situ* hybridisation (FISH) further identified the presence of human male X and Y chromosomes at engrafted sites, whilst the human origin of the cells was confirmed by a specific PCR assay for the human Cart-1 gene. In conclusion, the conversion of MNC to SRC in response to treatment with CR3/43 for 3 hr could have far-reaching clinical implications especially where time and donor-histocompatibility are limiting factors.

Introduction

The backcrossing of the *scid* mouse mutation onto the non-obese diabetic (NOD/Lt) mouse background has provided an improved *in vivo* surrogate animal model host to assay for repopulation of human haematopoietic stem cells. The resulting NOD/LtSz-*scid/scid* (NOD/SCID) mice have shown long-term engraftment and differentiation over the SCID model when transplanted with human multipotent haematopoietic stem cells (termed SCID-repopulating cells; SRC) derived from human bone marrow, cord blood and mobilised peripheral blood¹⁻⁴. In these tissues, haematopoietic stem cell activity had originally been confined to cells expressing the CD34 antigen⁵⁻⁸.

Further characterisation of the CD34 compartment in order to reveal the true identity of SRC using this animal model has provided evidence that such cells are developmentally heterogeneous as reflected by their immunophenotype and pluripotency. For example, CD34⁺ cells co-expressing the CD38 marker (CD34⁺CD38⁺) have rapid, but short-term, repopulation potential when compared to CD34⁺ cells not expressing CD38 (CD34⁺CD38⁻)⁹⁻¹³. This latter population of cells, although initially slow in developing, have much wider, long-term and secondary repopulation potentials. In addition, a much higher frequency of late T-cell precursor development is found among SRC in the CD34⁺CD38⁻ subpopulation¹².

In contrast, further subsets of haematopoietic stem cells devoid of expressing the classical stem cell marker, CD34, or haematopoietic lineage-associated antigens, including CD38, have been shown to possess SRC potential in the NOD/SCID model¹⁴⁻¹⁵. This finding challenges the previously accepted dogma that SRC are exclusively confined to the CD34 compartment commonly found within conventional sources of haematopoietic stem cells.

An *ex vivo* method has recently been reported that increases the relative numbers of at least four different progenitor stem cell classes: haematopoietic, pluripotent, neuronal and cardiomyogenic derived from a source mononuclear cell (MNC) population obtained from unmobilised adult human peripheral blood¹⁶. The method includes *ex vivo* culturing MNC in haematopoietic-conductive conditions (HCC) consisting of Dexter's long-term culture (LTC) medium¹⁷ supplemented with purified CR3/43 monoclonal antibody (mAb) (MNC/HCC). Under such conditions, there is an increase in the relative number of CD34⁺ haematopoietic stem cells within the heterogeneous MNC starter population to previously unreported levels (up to 66.4%). This unfractionated heterogeneous population produces high proliferative potential

colony forming cells (HPP-CFCs) with a clonogenic efficiency of approximately 1 in 30 that, upon purification for CD34⁺ progenitors, markedly increases to 1 in 2.5. Immunophenotypic analysis of purified and unpurified cell extracts showed the presence of CD34⁺CD45^{lo}CD38^{+/+}CD133^{+/+} populations. The detection of high levels of CD34⁺CD38⁻ precursors in both unfractionated and fractionated cell populations reflects the presence of primitive stem cells that warrant their assay in the well-established NOD/SCID model. The further presence of CD133 in such cell fractions could be of particular significance since this novel marker has recently been identified in a rare CD34⁺CD38⁻Lin⁻ subpopulation that contained primitive precursors of CD34⁺ cells. Moreover, this rare subpopulation has been reported to acquire SRC properties *in vivo* following 3-day *ex vivo* culturing under defined serum-free conditions¹⁵.

In light of the aforementioned findings, we chose unfractionated heterogeneous MNC/HCC. Source MNC were obtained from human male donors and cultured *ex vivo* for 3 hr (3-hr MNC/HCC) prior to their functional assessment of engraftment and lymphohaematopoietic differentiation in female NOD/SCID animals. We report the detection of HLA-ABC⁺CD45⁺ human cells of the lymphohaematopoietic system that included CD3⁺ T lymphocyte (including CD8⁺ and CD4⁺ cells), B lymphocyte (CD19⁺), erythrocyte (glycophorin A⁺) and myeloid (CD33⁺CD61⁺, CD33⁺CD61⁻) subsets in either the bone marrow, spleen or thymus of 3-hr MNC/HCC-transplanted recipients, thus suggesting the presence of primitive human repopulating cells with a wide developmental potential in the infusate. In this manner, the engrafted human cells were further shown to contain (a) male X and Y chromosomes; (b) CD34⁺ and HLA-ABC⁺CD45⁻ cell populations, the latter indicating a stromal-supporting element important for stem cell proliferation and differentiation and (c) human-specific Cart-1. In contrast, animals receiving MNC cultured in LTC alone (MNC/LTC) did not engraft with human cells.

We conclude that unfractionated 3-hr MNC/HCC does indeed possess SRC activity when compared to its counterpart MNC/LTC as measured by the NOD/SCID long-term repopulation assay. Moreover, significantly, we report thymopoietic reconstitution in this model under such conditions in the absence of co-administered growth factors. In conclusion, the generation of cells having SRC potentials within a short time frame from unmobilised adult human peripheral blood could have profound clinical implications and warrants further characterisation, as well as extension, into other pre-clinical models of human disease.

Materials and methods

Animals

Upon arrival, 6–8-week-old female immunodeficient NOD/SCID mice (Jackson Laboratories) were maintained in quarantine in positively pressurised isolator cages with barrier filtering (Techniplast) at the Animal Research Facility of the George Washington University Medical Center. Mice were irradiated with 250 cGy of a ¹³⁷Cs-gamma source (Shepherd Mark I Model 25) prior to infusion of cells.

Production of Haematopoietic Progenitor Stem Cells

The haematopoietic progenitor stem cells were generated under the haematopoietic-conductive conditions (HCC) described previously¹⁶. Briefly, MNC were obtained by density gradient centrifugation from either solicited human male blood donors under an existing George Washington University Medical Center IRB-approved protocol or separately from two buffy coat samples of healthy male donors (kindly provided by the American Red Cross, Rockville, MD, USA). The MNC were resuspended in Dexter's LTC medium¹⁷ containing 3.5 µg per ml of purified mouse monoclonal anti-human HLA-DR, DP, DQ (clone CR3/43) generated in the absence of azide and antibiotics by DakoCytomation (MNC/HCC) and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

Infusion of Haematopoietic Progenitor Stem Cells

All animal experiments were approved by the Animal Care Committee of the George Washington University Medical Center. Three hours post-incubation, 3–5 × 10⁶ of separately prepared MNC/HCC haematopoietic progenitor stem cell population (3-hr MNC/HCC) were resuspended in 0.2–0.3 ml PBS and infused into the lateral tail vein of the NOD/SCID animals. In contrast, MNC cultured for 3 hr in LTC in the absence of purified CR3/43 treatment (MNC/LTC) were infused as a relative time control. All donor cells infused were distributed equally between control and test animal groups.

Tissue Collection

Animals were humanely sacrificed by carbon dioxide asphyxiation at weeks 6–7 post-infusion. Tissues were collected as follows: bone marrow was collected under sterile conditions using a 21-gauge needle by flushing femora and tibiae with Rosewell Park Memorial Institute

(RPMI) RPMI-1640 medium (Sigma) containing 2% fetal bovine serum (FBS) (Sigma). The cell suspension was centrifuged at 400 g for 10 min and the supernatant discarded. The cells were resuspended in PBS containing 10% FBS and an aliquot taken for immunophenotypic analysis (see below). A further aliquot was taken for X and Y chromosome analysis by fluorescence *in situ* hybridisation (FISH) (see below) and the remainder of the cell suspension was cryopreserved in 95% FBS : 5% dimethylsulphoxide (DMSO) (Sigma). Thymocyte and splenocyte suspensions were prepared under sterile conditions by mincing thymus and spleen, respectively, using the blunt end of a sterile syringe in RPMI-1640 containing 2% FBS. Aliquots were removed for immunophenotypic analysis and FISH and the residual cell suspensions were cryopreserved. Further tissues and organs were excised and cryopreserved in freezing medium (Triangle Biomedical Sciences) by snap-freezing in liquid nitrogen. These included: heart, lungs, liver, kidney and pancreas.

Flow Cytometry

Human-specific monoclonal antibodies against HLA-ABC and CD45 (common leukocyte antigen), conjugated with RPE and FITC (both DakoCytomation), respectively, were used to identify cells of human origin in mouse bone marrow, thymocyte and splenocyte cell suspensions. Further characterisation analysis was performed using the following conjugated human-specific monoclonal antibodies: CD3–FITC, CD4–FITC, CD8–RPE, CD14–RPE, CD19–RPE, CD33–RPE, CD34–FITC, CD38–FITC, CD61–FITC and glycophorin A–RPE (all DakoCytomation). Dual-labelled RPE/FITC IgG₁ and IgG_{2a}-PE isotype (both DakoCytomation) were used as a negative control stain and autofluorescence levels were determined. Twenty to one hundred thousand events with time were acquired and saved as a list mode file for analysis. Cell analysis was performed with a FACSCalibur system (BD Biosciences) using Cell Quest software version 3.3.

Analysis of Human Cell Engraftment by FISH

Analysis to detect human-specific X and Y chromosomes in cells of the bone marrow, thymus and spleen of the female NOD/SCID mice was performed using the CEP X SpectrumOrange/Y SpectrumGreen DNA Probe Kit (Vysis Inc.) according to the manufacturer's instructions.

Analysis of Human Cell Engraftment by PCR

Total DNA was isolated from cells at recorded time intervals using the Qiagen Maxi Kit (Qiagen) as per the

manufacturer's instructions. The DNA served as a template for analysis of human Cart-1 by PCR in a Perkin-Elmer 2400 (Perkin-Elmer) machine using the thermostable Taq polymerase (Promega) according to published literature¹⁰. The PCR reaction amplifies a 156-bp product from the untranslated region of the human Cart-1 gene and fails to amplify a product from mouse genomic DNA.

Purification of CD34⁺ Cells by Positive Selection

Bone marrow cells taken from both 3-hr MNC/HCC and 3-hr MNC/LTC-infused animals were purified using the CD34 MultiSort Kit (Miltenyi Biotec) according to manufacturer's instructions. Briefly, prior to isolation, cells were subjected to Fc receptor blocking to prevent non-specific binding followed by direct labelling of cells with anti-human class II CD34-coated microbeads. The labelled cells were passed twice through a MS+/RS+ Midimacs separation column in the MACS separator and bound cells were gently flushed and collected for assessment of clonogenic potential.

Clonal Assays

Purified cells prepared as described above were seeded in semisolid MethoCult GFH4434 according to manufacturer's instructions (StemCell Technologies Inc.) containing recombinant human growth factors. Differentiation into haematopoietic cell colonies was assessed and colonies were counted and scored with time using phase contrast microscopy (Olympus CK-40).

Immunohistochemical Analysis of Human CD45 in Haematopoietic Colonies generated by Engrafted CD34⁺ Cells

Individual myeloid-containing colonies were plucked and analysed for human CD45 expression using anti-human CD45-FITC conjugate (DakoCytomation) and confocal microscopy. Briefly, plucked colonies were washed once with HBSS (Ca⁺⁺ and Mg⁺⁺ free) containing 2% FCS (both Sigma). Prior to confocal analysis, single cell suspensions were resuspended in Iscoves Modified Dulbecco's Medium (IMDM) culture medium (Invitrogen) and deposited in organ culture dishes specially designed to have a coverslip at their base. Fifteen microlitres of the monoclonal antibody conjugate was added to the organ culture dish and cells stained for human CD45 antigen were analysed using confocal microscopy.

Results

Determination of 3-hr MNC/HCC Optimal Time Point

Our decision to elect for the 3-hr MNC/HCC was based upon a preliminary study aimed at determining the optimum time at which MNC are rendered SRC in response to treatment with CR3/43 cultured in LTC. In this pilot study, a total of 24 animals (3–4 weeks old) organised in groups of six were infused with either MNC/LTC, 3-hr MNC/HCC, 24-hr MNC/HCC or 4 days MNC/HCC post-irradiation at an age-related lethal dose of greater than 500 cGy where the MNC had been obtained from blood of the same human donor. At this elevated radiation dose, a significant mortality rate was observed by weeks 1–2 (22 out of 24), most likely due to radiation-induced toxicity in agreement with published literature^{10,18}. Nevertheless, all animals infused with MNC/HCC showed significant levels of multi-lineage human haematopoietic cell engraftment and differentiation in the bone marrow (18 out of 18), which was more pronounced in animals infused with 3-hr MNC/HCC. The predominant lineage repopulation was observed for B lymphocyte and myeloid cell subsets. In contrast, animals receiving MNC/LTC did not engraft. Interestingly, two animals out of the 24 had survived the high radiation dose administered and these were from the group of animals receiving the 24-hr MNC/HCC. Survival of these two remaining animals could have been due to an anomalous or radioprotection-induced effect.

Immunophenotypic Analysis of Human Cell Engraftment as Detected by HLA-ABC and Human CD45 Expression following Infusion of 3-hr MNC/HCC into Animals Receiving a 250-cGy Radiation Dose

Eighteen out of 24 female NOD/SCID mice aged 6–8 weeks were infused with 3-hr MNC/HCC separately obtained from two human male donors (Figure 1), following their irradiation at the reduced dose of 250 cGy. For the duration of the experiment, all animals survived, gained weight and showed normal social behaviour. Six to 7 weeks post-infusion, animals were humanely sacrificed and bone marrow, spleen and thymus were analysed for human cell engraftment and differentiation. Immunophenotypic analysis at these anatomical sites was initially determined using antihuman antibodies against the panhuman leukocyte marker, CD45, and human HLA-ABC. Eighteen out of 18 animals infused with 3-hr MNC/HCC showed significant (> 0.5%) levels of human

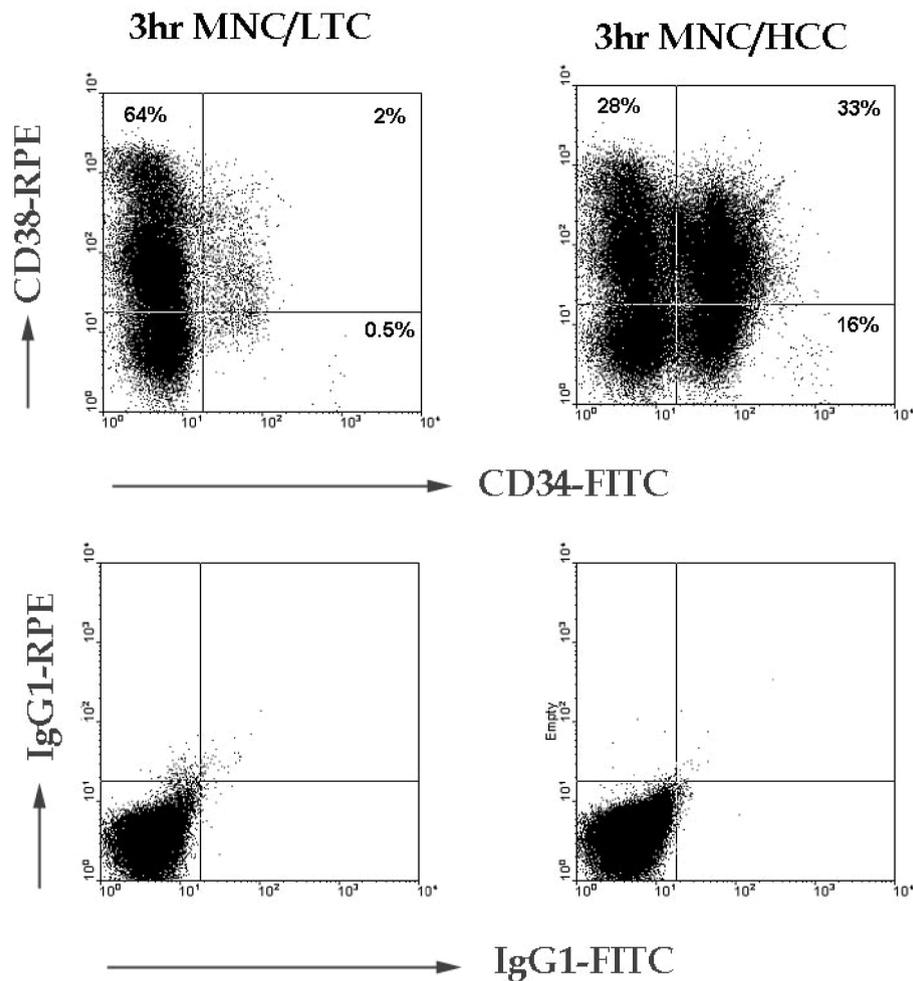


Figure 1. Immunophenotypic profiles as depicted by CD34 and CD38 expression of 3-hr MNC/LTC and 3-hr MNC/HCC cells, infused in NOD/SCID mice, in relation to their respective isotype-negative controls

haematopoietic as well as non-haematopoietic cell engraftment in the bone marrow as measured by HLA-ABC⁺CD45⁺ (Figure 2) and HLA-ABC⁺CD45⁻ populations, respectively (Figure 4A). However, human cell populations could not be detected in the six out of six (0%) animals receiving 3-hr MNC/LTC.

Further analyses of the spleen and thymi of nine of the engrafted animals revealed the extended presence of HLA-ABC⁺CD45⁻ and HLA-ABC⁺CD45⁺ subset populations at these sites (Figure 2). The level of HLA-ABC⁺CD45⁻ detection was 0.5–20% and 0–25.4% for spleen and thymus, respectively (data not shown). This latter cell population suggests the presence of a stromal/non-lymphoid element. In contrast, 3-hr MNC/LTC-infused animals did not contain sufficient numbers of cells to allow analysis by flow cytometry. This was also observed for animals that were not infused with human cells (data not shown) in agreement with published data¹. It should be noted that engraftment at the various anatomical sites analysed did not correlate with a donor-specific effect.

Immunophenotypic Analysis of Lymphohaematopoietic Subsets in Engrafted NOD/SCID Animals

Further analysis revealed the presence of significant levels of a variety of mature lymphohaematopoietic cell lineages including CD34⁺ cells in the bone marrow of all engrafted animals, some of which coexpressed the erythrocyte marker, glycophorin A (Figure 3A and Figure 4A). In addition, monocytes, as measured by the CD14 marker, were detected in the bone marrow of engrafted animals (Figure 3A and Figure 4A). Megakaryocytes (CD33⁺CD61⁺), including platelets (CD33⁻CD61⁺) and other myeloid lineages (CD33⁺CD61⁻), were also found in the bone marrow of engrafted animals (Figure 3A and Figure 4B). B-lymphocyte reconstitution in the bone marrow and spleen (Figures 3A,B, 4C), as assessed by expression of the CD19 surface marker, was detected, but at reduced levels when compared to engrafted animals receiving a

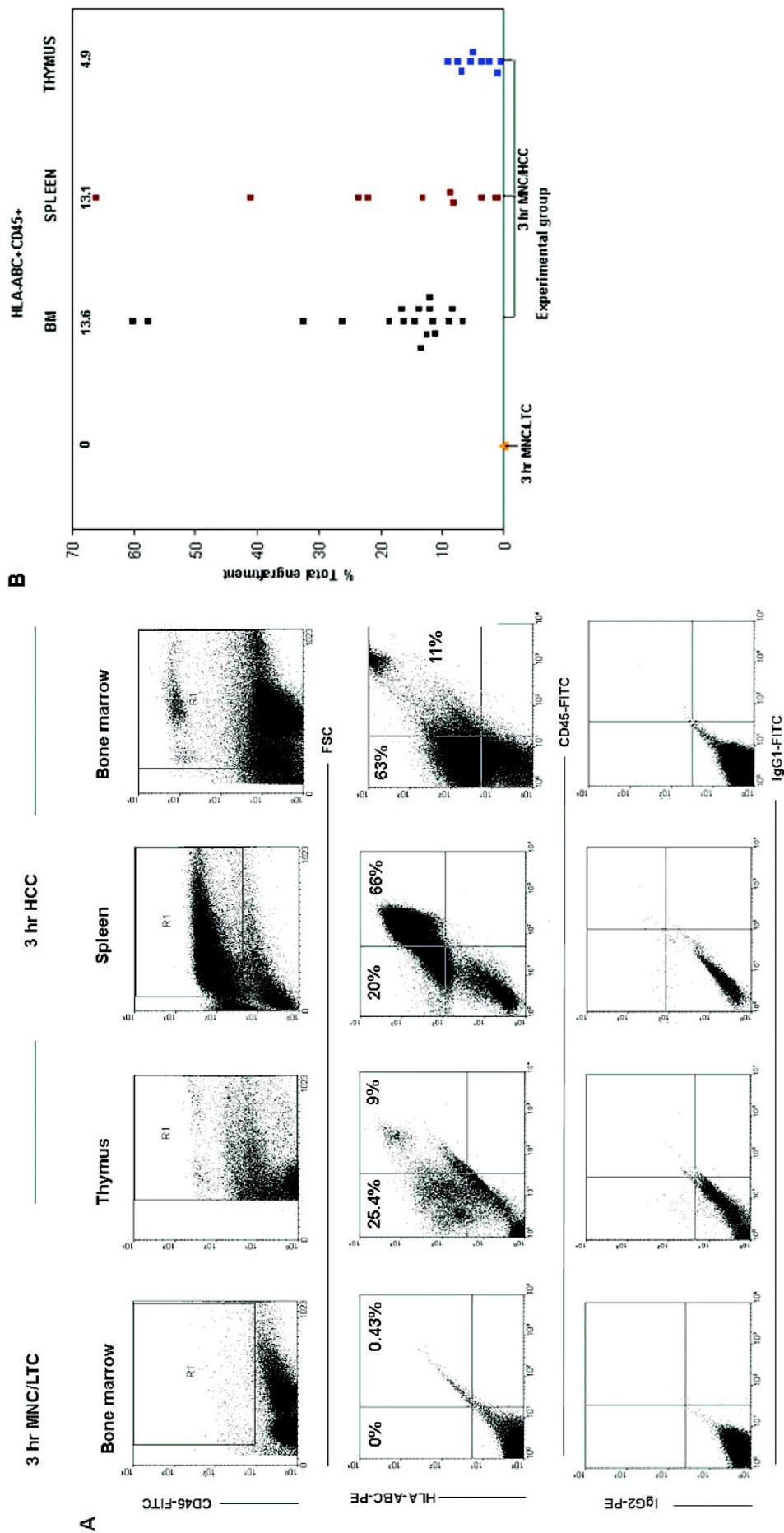


Figure 2. (A) Engraftment of 3-hr MNC/HCC (labelled 3-hr HCC) in the thymus, spleen and bone marrow of NOD/SCID mice as measured by CD45 and forward scatter (FSC) or HLA-ABC and CD45 expression. The lower panel is represented by the relevant isotype-negative control. (B) Percentage of human cell engraftment in the bone marrow (n = 18), spleen (n = 9) and thymus (n = 9) as measured by HLA-ABC and CD45 coexpression in animals receiving 3-hr MNC/HCC and six receiving non-treated 3-hr MNC/LTC. Median values are shown in bold typeface above each plot

A

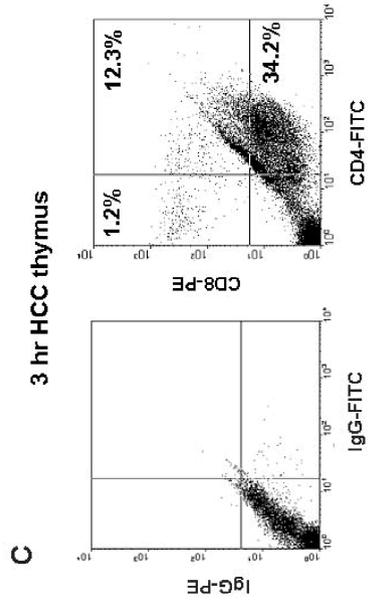
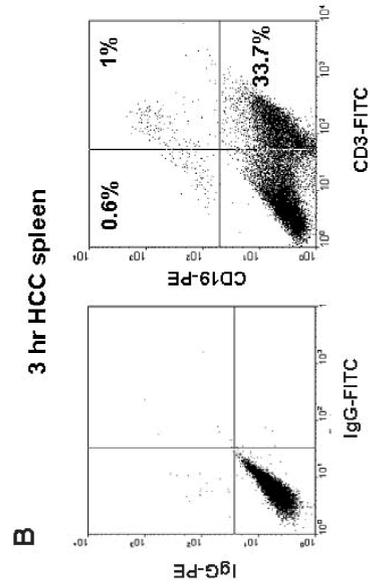
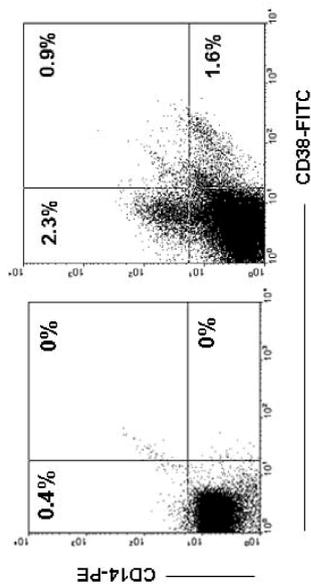
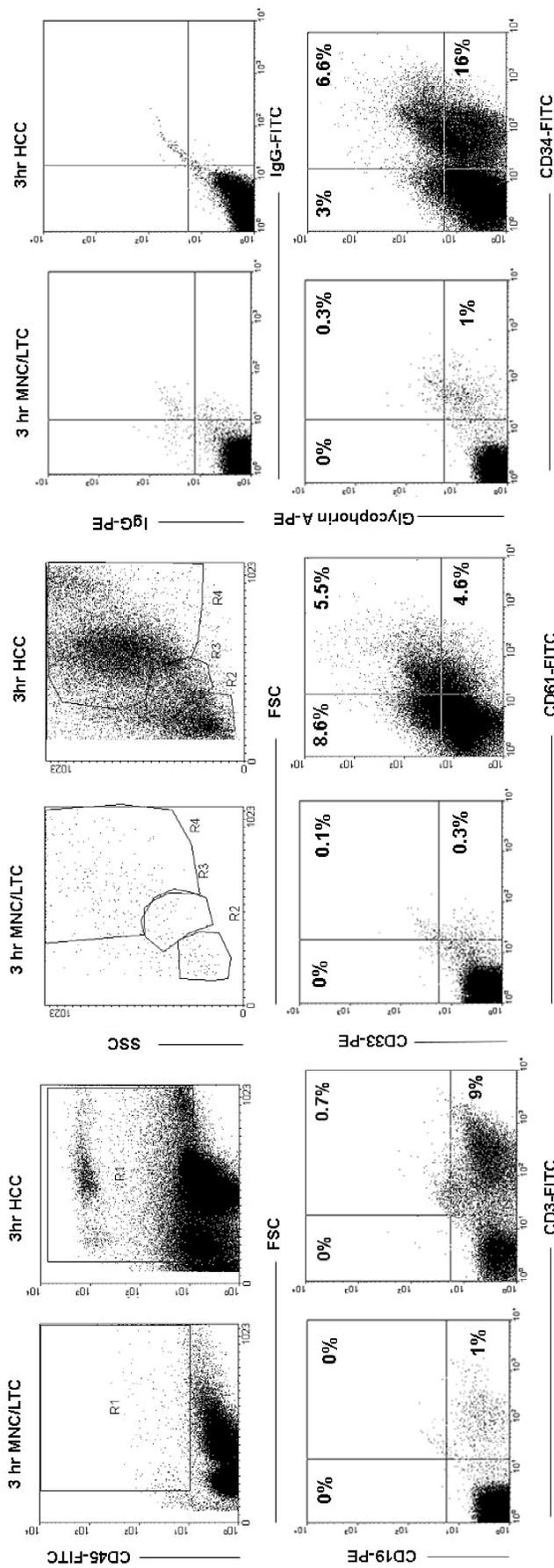


Figure 3. (A) Human lymphohaematopoietic reconstitution of the bone marrow of an animal infused with 3-hr MNC/HCC (column labelled 3-hr HCC) in comparison to a counterpart animal infused with 3-hr MNC/LTC cells (column labelled 3-hr MNC/LTC). Cells were first gated according to FSC and human CD45 expression in conjunction with isotype-negative control. Forward (FSC) and side scatter (SSC) properties of human CD45⁺ cells were displayed and further gated into lymphoid R2, blasts/monocyte R3 and granulocyte R4 gates. T lymphocytes (CD3) are found in gate R2, myelocytes (CD33⁺CD61⁺) are found in gates R3 and R4. Haematopoietic stem cells (CD34) are found in gates R2, R3 and R4 and monocytes in gate R3. (B) Human T lymphocytes (CD3⁺) in spleen and (C) thymocytes (CD4⁺CD8⁻) in the thymus of animals infused with 3-hr MNC/HCC. In A-C the relevant isotype-negative controls are shown

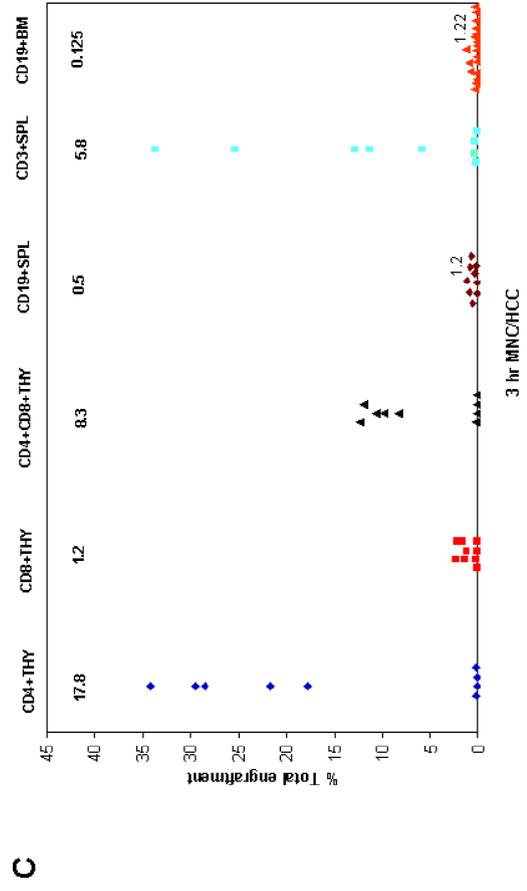
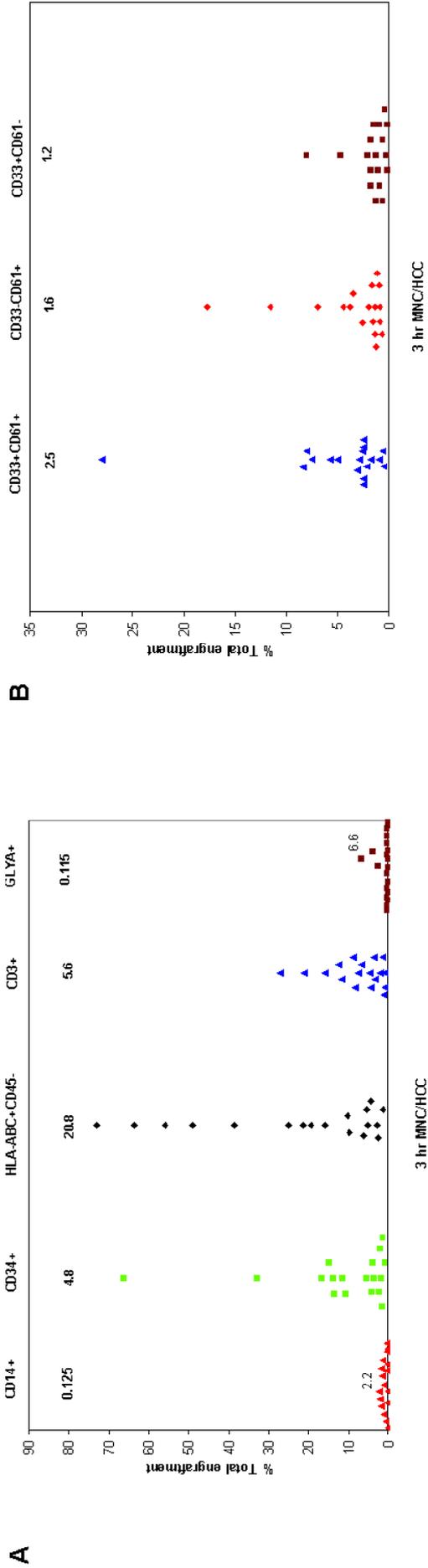


Figure 4. Analysis of human lymphohaematopoietic subsets in the bone marrow of engrafted NOD/SCID mice infused with 3-hr MNC/HCC. Percentage of human cell engraftment in the (A) bone marrow: CD14⁺ (monocyte/macrophage cell marker) (peak value 2.2% annotated); CD34⁺ (haematopoietic stem cell marker); HLA-ABC⁺CD45⁻ (human non-haematopoietic cell marker); CD3⁺ (T-lymphocyte marker); GLYA⁺ (erythroid marker) (peak value 6.6% annotated); (B) bone marrow: CD33⁺CD61⁺ (megakaryocyte cell marker); CD33⁺CD61⁻ (platelet marker); CD33⁺CD61⁻ (myeloid cell marker) and (C) thymus: CD4⁺ (helper T cell); CD8⁺ (cytotoxic T cell); CD4⁺CD8⁺ (thymocyte); CD19⁺ (B lymphocyte marker) (peak value 1.2% annotated); CD3⁺; bone marrow: CD19⁺ (peak value 1.22% annotated). Median values are shown in bold typeface above each plot

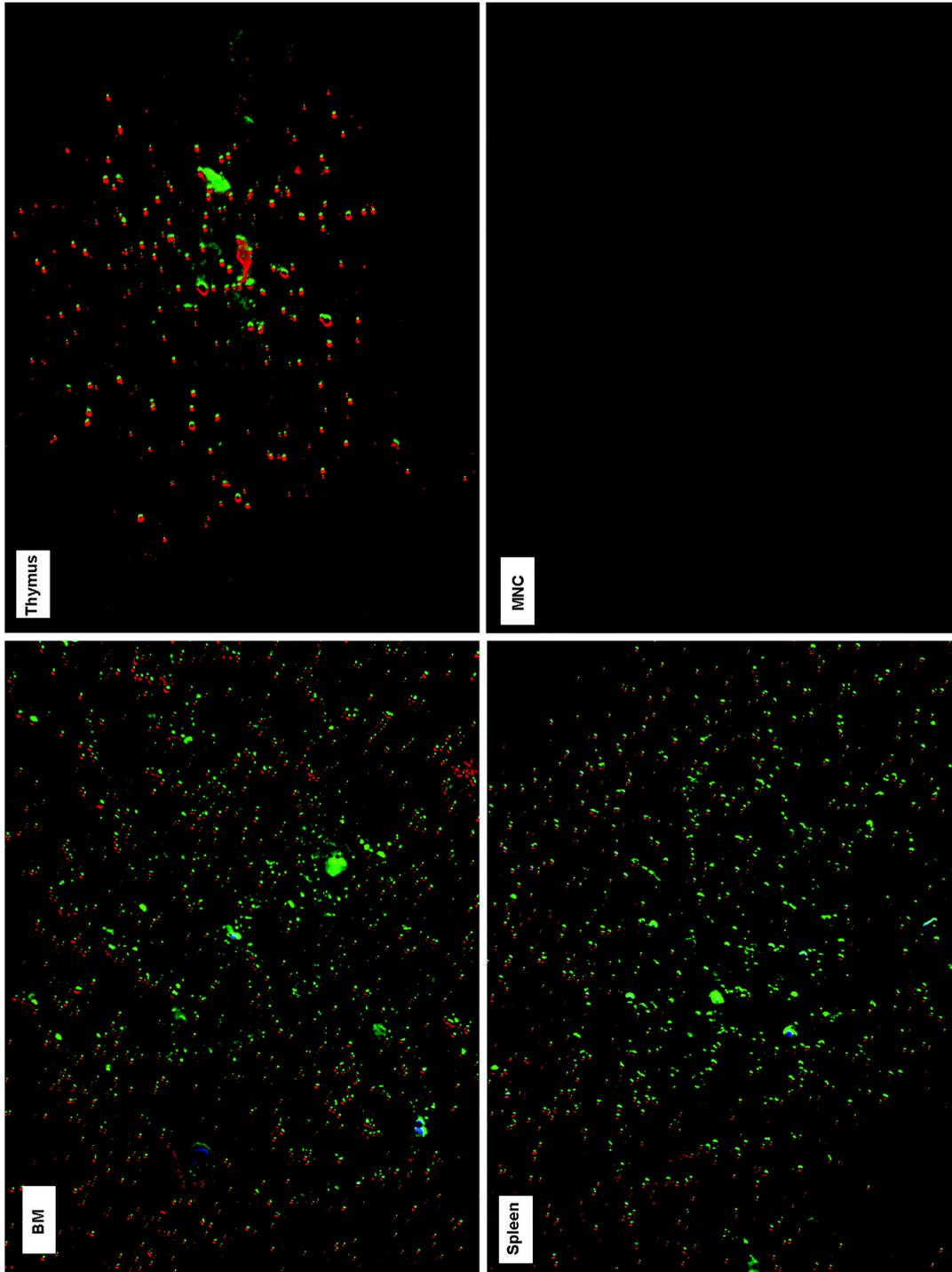


Figure 5. Genetic analysis of human cell engraftment in the bone marrow, thymus and the spleen of NOD/SCID animals infused with 3-hr MNC/HCC. (A) Human male XY chromosomes in the bone marrow, thymus and spleen of mice infused with 3-hr MNC/HCC when compared to bone marrow of animals infused with 3-hr MNC/LTC. The superimposed images of X (red) and Y chromosomes (green) present cells that are probed using TRITC and FITC conjugates, respectively. (Continued on page 96)

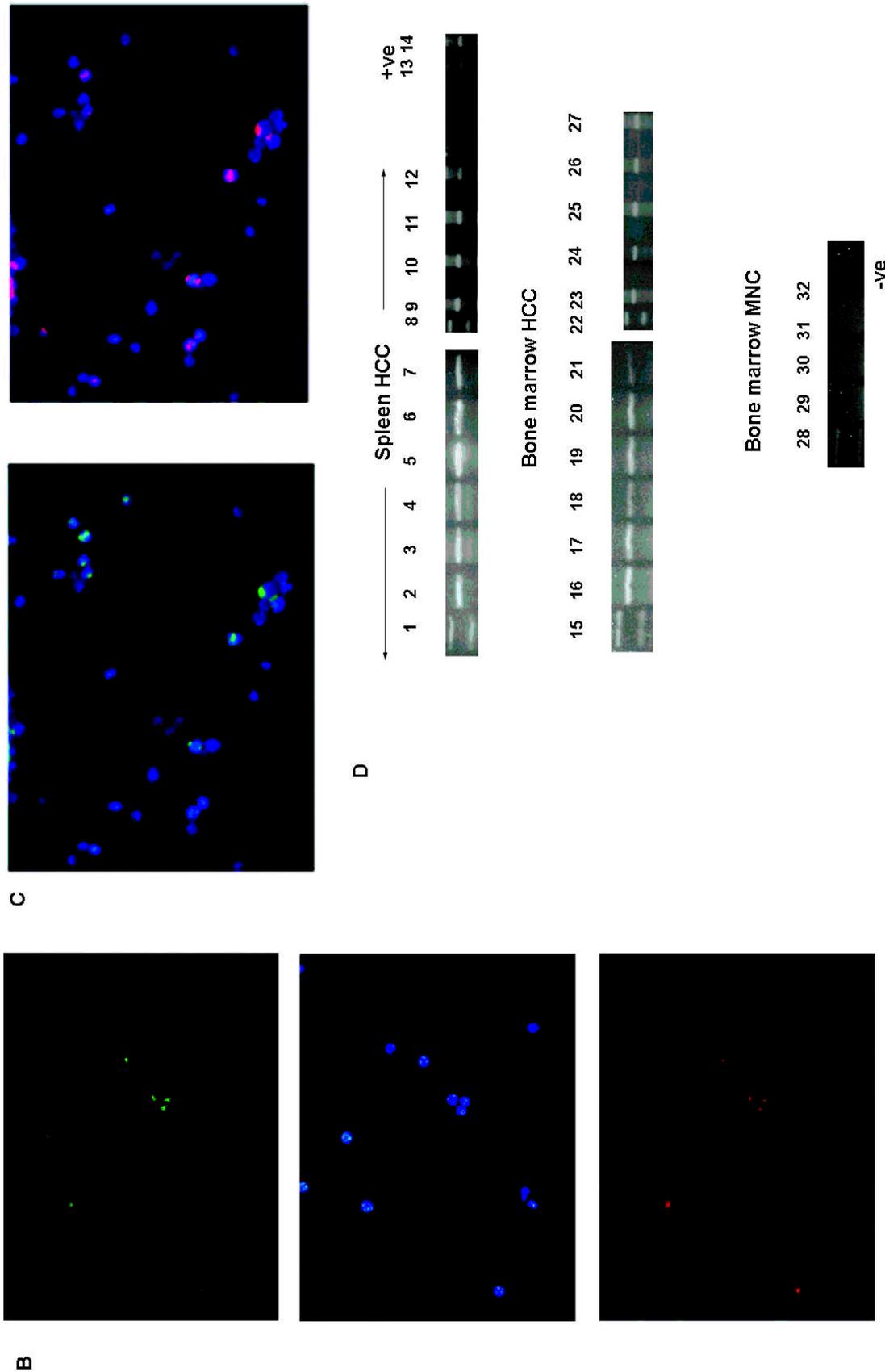


Figure 5. contd. (B) Same-field non-superimposed images of X (red) and Y (green) chromosomes in relation to DAPI stained nuclei (blue) of the bone marrow of an animal receiving 3-hr MNC/HCC. (C) Same-field superimposed images of DAPI with X or Y chromosomes of an animal receiving 3-hr MNC/HCC showing punctate staining. (D) PCR product of human Cart-1 gene amplification in the spleen (lanes 2–12); bone marrow (lanes 16–27) of animals infused with 3-hr MNC/HCC or bone marrow of mice receiving 3-hr MNC/LTC (lanes 29–31). In addition, human Cart-1 negative control is displayed (lane 32). The amount of template used for all test samples subjected to PCR analysis was 200 ng. For comparison, a total of 200 ng of genomic DNA from the bone marrow of a non-transplanted NOD/SCID animal was mixed with equal volumes of serially diluted 0.3 and 0.6 ng of human genomic DNA (lanes 13–14)¹⁰

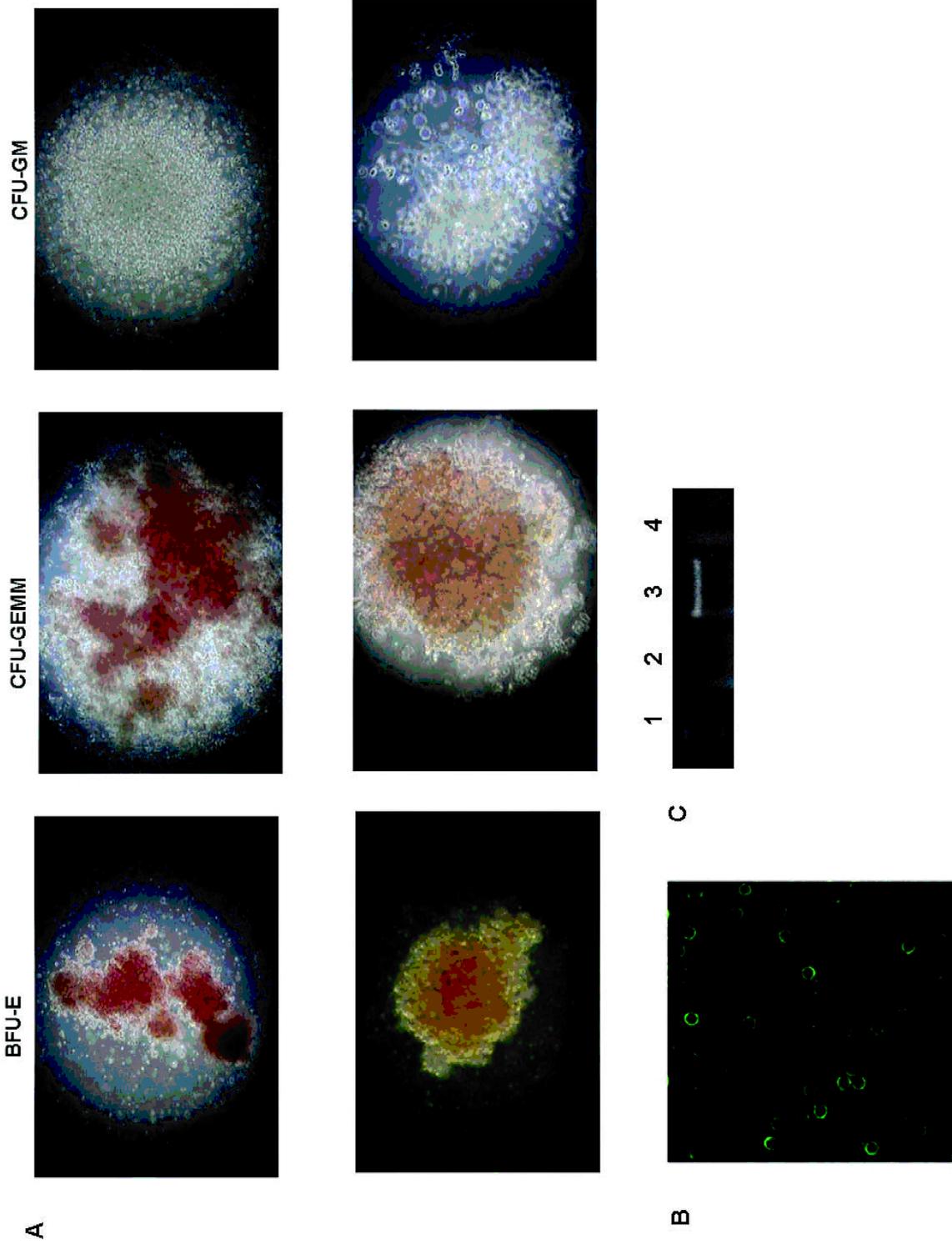


Figure 6. (A) Clonogenic human CD34⁺ cells purified from the bone marrow of an animal receiving 3-hr MNC/HCC. Supplementation of supernatant obtained from 3-hr MNC/HCC to MethoCult media gives rise to larger BFU-E, CFU-GEMM and CFU-GM haematopoietic colonies (upper images). (B) Human CD45 expression by haematopoietic colonies and (C) Human Cart-1 gene expression by haematopoietic colonies (lane 3). Lane 1, 100-kb ladder; lane 2, human Cart-1 gene negative control; lane 4, mouse DNA negative control

lethal dose of radiation (data not shown). Particularly noteworthy was the detected presence in the bone marrow and spleen of T lymphocytes bearing the CD3 surface marker (Figures 3A,B, 4A,C). Significantly, analysis of thymic lymphoid organs confirmed the presence of CD4⁺CD8⁺ double-positive human thymocytes, including CD8⁺ and CD4⁺ single-positive cytotoxic and helper T cells, respectively, collectively indicative of T-cell development (Figures 3C, 4C). The presence of a human non-haematopoietic HLA-ABC⁺CD45⁻ stromal-like component described herein could well be the reason behind the observed high level of human haematopoietic cell repopulation in the bone marrow, thymus and spleen of engrafted animals¹⁹.

Fluorescence *in Situ* Hybridisation (FISH) Analysis of Human Cell Engraftment

Further confirmation of human cell engraftment was sought by measuring the presence of human-specific X and Y chromosomes in bone marrow ($n = 18$), thymus ($n = 9$) and spleen ($n = 9$) cell extracts of 3-hr MNC/HCC-infused animals. This was achieved through the FDA-approved human-specific CEP X SpectrumOrange/Y SpectrumGreen DNA probe kit (Vysis Inc.). FISH analysis confirmed the presence of the human X and Y chromosomes at the aforementioned anatomical sites (Figure 5A–C) of NOD/SCID mice receiving 3-hr MNC/HCC; their levels correlating with human cell engraftment when determined by flow cytometry. However, human X and Y chromosomes were not detected in the bone marrow of six out of six animals receiving 3-hr MNC/LTC (Figure 5A–C) or animals that did not receive human cells (data not shown).

PCR Analysis of Human Cart-1 in Engrafted Animals

Engraftment was further evaluated through extraction of DNA from cells of the bone marrow and spleen compartments and their assay for the human-specific Cart-1 gene by PCR. DNA extracted from 3-hr MNC/HCC-infused recipients showed the presence of a 156-bp human Cart 1-specific product, thus confirming the presence of human cells by the PCR method (Figure 5D). In comparison, both animals that received 3-hr MNC/LTC and animals that did not receive human cells did not possess the human Cart-1 gene sequence by this assay.

Human Clonogenic Potential of CD34⁺ Subpopulation *in Vitro*

As a further means of determining haematopoietic colony-forming potential, human CD34⁺ cells isolated

from the bone marrow of two highly engrafted animals were assessed for proliferation and differentiation in response to culturing in methylcellulose containing human growth factors. Firstly, the bone marrow of such animals was subjected to positive selection using antihuman CD34⁺-coated magnetic beads (Miltenyi Biotec) to purify the human CD34⁺ cells. The purified cells obtained from two animals with high CD34 counts were clonogenic at an efficiency of approximately 1 in 10 (data not shown) when seeded in MethoCult cell culture containing human recombinant growth factors. The colonies consisted of blast forming unit-erythroid (BFU-E), colony forming unit-granulocytic, erythroid, monocyte, macrophage, megakaryocytic (CFU-GEMM) and colony forming unit granulocytic, monocyte, macrophage (CFU-GM) (Figure 6A). Moreover, at day 14, myeloid-containing colonies expressed human CD45 as determined using antihuman CD45-FITC conjugate and confocal microscopy (Figure 6B). Human Cart-1 gene transcripts were also detected in such colonies, thus further confirming the presence of human stem cell activity in the bone marrow graft (Figure 6C).

Discussion

The NOD/SCID mouse has provided researchers with an important preclinical model with which to assess repopulation in a living system. Transplanted populations of human haematopoietic stem cells derived from bone marrow, cord blood and mobilised peripheral blood have been shown to possess SRC in this model¹⁻⁴. In such an animal system CD34⁺CD38⁺, CD34⁺CD38⁻ and Lin⁻CD34⁺CD38⁻ subpopulations have been characterised to possess SRC potential, with varying developmental potentials¹³⁻¹⁵.

Recently, an alternative method for the production of pluripotent human progenitor stem cells from unmobilised peripheral blood has been documented¹⁶. This method includes the *ex vivo* production of haematopoietic stem cells from MNC under HCC (MNC/HCC) that are CD34⁺CD45^{lo}CD38[±]CD133[±] and rendered clonogenic in *in vitro* surrogate assays. Herein, we have shown that unfractionated 3-hr MNC/HCC containing both CD34⁺CD38⁺ and CD34⁺CD38⁻ progenitors have SRC characteristics as determined by their repopulation and wider developmental potential in the NOD/SCID model when compared to 3-hr MNC/LTC infusate. The frequency of repopulation potential of 3-hr MNC/HCC is high, although the level of engraftment of the various human leukocytes subsets appear to vary from one recipient mouse to another, despite the fact that donor cells separately obtained from two male blood donors

were distributed equally among control and test animals. This intraspecies variability is commonly observed when assaying for conventional SRC derived from bone marrow or mobilised and cord blood^{10,12-15,18}. The elevated frequency and levels of repopulation potential observed herein could be due to the presence of high levels of CD34⁺CD38⁻ cells¹³⁻¹⁵ in the infusate and/or the further presence of unidentified cellular component(s) capable of giving rise *in vivo* to the supportive environment essential for human stem cell maintenance, proliferation and differentiation. This latter notion is supported by the significant levels of engraftment of human non-haematopoietic HLA-ABC⁺/CD45⁻ cells in the bone marrow, spleen and the thymus of animals receiving 3-hr MNC/HCC.

As to which stem cell compartment found in the 3-hr MNC/HCC infusate is responsible for SRC characteristics remains to be determined. The high levels of CD34⁺CD38⁺ and CD34⁺CD38⁻ cells present in the infusate could have resulted in SRC having short and long-term human lymphohaematopoietic repopulation and differentiation potentials, respectively¹². In this regard, engraftment of T cells in the bone marrow and spleen of this immunodeficient animal host is a result of their maturation in the thymus and could have emanated from the presence of primitive CD34⁺ cells not expressing CD38. To our knowledge of the literature, we have reported for the first time detectable levels of T-cell reconstitution in the bone marrow, thymus and the spleen in the absence of growth factor co-administration or thymic explant administration²⁰. T-cell reconstitution has been previously documented under a different experimental regimen, whereby the NOD/SCID animal host was infused with unpurified human bone marrow MNC²¹. Furthermore, herein bone marrow cells of engrafted NOD/SCID animals purified for the human haematopoietic stem cell marker, CD34, were clonogenic in a semisolid colony-forming assay containing recombinant human growth factors. In contrast, infusion of 3-hr MNC/LTC derived from the same donor MNC population did not result in engraftment. The engrafted cells of the female NOD/SCID mice carried the human male X and Y chromosomes as determined by FISH and, moreover, the human *Cart-1* gene as detected by PCR; both assays genetically confirming that the engrafted cells are of human origin. Human *Cart-1* gene and CD45 expression were also detected in myeloid-containing colonies produced by CD34 cells purified from the bone marrow of engrafted animals.

The induction of CD34 expression in MNC in response to short-term *ex vivo* culturing with CR3/43 mAb¹⁶ is shown here to have SRC potential as determined by the NOD/SCID repopulation assay. The

described reconstitution of the T-cell compartment points towards the use of MNC/HCC in understanding and treating T-cell disorders, including the fighting of viral infections, cancer immunotherapy and the modulation of a variety of autoimmune diseases. In addition, the fact that 3-hr MNC/HCC are capable of reconstituting the erythroid and the megakaryocytic lineages in a xenogeneic host alludes to their potential use in treating a variety of congenital disorders afflicting such systems. Moreover, the erythroid and megakaryocytic lineages are integral components in transfusion medicine. Encouragingly, the speed at which MNC/HCC acquired SRC properties in response to *ex vivo* culturing with CR3/43 mAb may hold promise as an attractive option for extrapolation to the human situation with particular reference to diseases where time and identifying histocompatible donors are limiting factors.

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