Host Factors that Impact the Biodistribution and Persistence of Multipotent Adult Progenitor Cells

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ABSTRACT

Multipotent Adult Progenitor Cells (MAPCs) are marrow-derived pluripotent stem cells with a broad differentiation potential. We sought to identify factors that affect adoptively-transferred MAPCs. In vitro, MAPCs expressed low levels of MHC antigens, failed to stimulate CD4$^+$ and CD8$^+$ T cell alloreponses, and were targets of NK cytolysis. To study in vivo biodistribution, MAPCs were labeled with luciferase for sequential quantification of bioluminescence and DsRed2 for immunohistochemical analysis. C57BL/6 MAPCs were infused intravenously into C57BL/6, Rag-2$^{-/-}$ (T- and B-cell deficient) and Rag-2$^{-/-}$/IL-2R$\gamma_c^{-/-}$ (T-, B- and NK-deficient) mice. In C57BL/6 mice, MAPCs were transiently detected only in the chest compared to long-term persistence in T- and B- cell deficient mice. NK depletion reduced MAPC elimination. Since the lungs were the major uptake site after intravenous injection, intra-arterial injections were tested and found to result in a more widespread biodistribution. Widespread MAPC biodistribution and long-term persistence was seen in irradiated recipients given allogeneic marrow and MAPCs; such MAPCs expressed MHC class I antigens in tissues. Our data indicate that the biodistribution and persistence of reporter gene-labeled MAPCs is maximized after intra-arterial delivery or host irradiation and indicate that T- and/or B-cells as well as NK cells contribute to in vivo MAPC rejection.
INTRODUCTION

MAPCs are a novel class of stem cells derived from adult tissues, including bone marrow, muscle and brain\textsuperscript{1-5}. MAPCs are pluripotent and a single MAPC injected into a blastocyst contributes to all tissues, including skeletal muscle, cardiac muscle, liver, lung, intestine, central nervous system, skin, spleen, blood and marrow\textsuperscript{2}. Clonal MAPCs are able to differentiate into various lineages of mesodermal, ectodermal and endodermal origin in vitro, and contribute to terminally differentiated tissues when grafted postnatally\textsuperscript{1-5}. This capacity is enhanced in the setting of injury\textsuperscript{2}, suggesting a possible role of MAPCs in regeneration of tissues injured by chemotherapy or radiation used for conditioning for hematopoietic stem cell transplantation.

For any clinical application of MAPCs it is critical to understand the mechanisms that can lead to immune rejection of the infused cells. Since undifferentiated MAPCs express low levels of major histocompatibility complex (MHC) class I, MAPCs may be ideal targets for NK cell-mediated killing. If so, NK cell resistance may serve as a barrier to the infusion of undifferentiated MAPCs in vivo. As a part of innate immune defense, NK cells can kill infected or transformed tissues prior to sensitization and without restriction by MHC antigens\textsuperscript{6-8}. As NK function can influence the outcome of hematopoietic cell transplantation\textsuperscript{9,10}, we reasoned that NK activity may have a role in MAPC homing and rejection.

Other important considerations in the use of MAPCs include understanding the host factors that might affect in vivo MAPC biodistribution and persistence. Therefore, we analyzed the role of MAPCs as targets of immune response as a function of engraftment and persistence of infused MAPCs using a two reporter gene system with red
fluorescent protein derived from *Discosoma* coral, DsRed2\(^1\), and firefly luciferase\(^2\). Donor MAPC-derived optical signatures of fluorescent (DsRed2) and bioluminescent (luciferase) signals were chosen to complement each other in post mortem tissue analysis and in noninvasive imaging in live animals, respectively.

The success of stem cell therapies largely depends on immune tolerance of the infused cells in the host and on the proliferation and differentiation potential of the cellular graft. Using mice with various degrees of immune competence, we show for the first time that depletion of NK cells in the recipient results in higher levels of donor MAPC engraftment and persistence in real time in vivo. When compared to intra-venous delivery of the same dose of MAPCs, intra-arterial infusion resulted in more diverse homing of MAPCs and in higher levels of persistent engraftment. Additionally, we observed that conditioning for bone marrow transplantation using high-dose total body irradiation overcomes MAPC rejection. Therefore, immune competence of the recipient, route of delivery and conditioning regimen are likely to modulate the therapeutic impact of the MAPC infusion.
METHODS

Mouse strains

C57BL/6 (termed B6) (H2b) and B10.BR (H2k) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). BALB/c (H2d) mice were obtained from the National Institutes of Health (Hartford, CT). B6 recombinase activating gene-2 deficient (Rag-2\(^{-/-}\)) mice were obtained from Taconic Farms (Germantown, NY). B6 mice carrying mutations in the recombinase activating gene 2 and the common cytokine receptor (Rag2/IL-2R\(\gamma_c^{-/-}\)) were a gift from Dr. Stephen Jameson (University of Minnesota). All mice were housed under specific-pathogen free conditions, fed ad libitum according to University of Minnesota Research Animal Resources guidelines, and used at 6-12 weeks of age. All protocols involving mice were approved by the Institutional Animal Care and Use Committee (IACUC).

NK depletion

To deplete NK cells in vivo some mice were injected with anti-NK1.1 monoclonal antibody (hybridoma PK136, rat IgG2a; provided by Dr. Koo, Rahway, NJ) 3 days before MAPC infusion and then twice a week for 30 days.

Bone marrow transplantation

B6 mice (were lethally irradiated with 8.0 Gy by x-ray on the day prior to transplantation with 20 x 10\(^6\) B10.BR bone marrow cells (BM) with or without 10\(^6\) of C57BL/6 luciferase- and DsRed2-expressing MAPCs (MAPC DL). BM was T cell depleted by incubation with anti-Thy1.2 antibody (30-H-12; National Cell Culture Center, Minneapolis, MN) and rabbit complement (Nieffengger Company, Woodland, CA).
MAPC culture, labeling and injection

MAPCs were isolated from adult 129 x C57BL/6J BM (H2^b, transgenic for lacZ and NeoR genes), cultured at low density in fibronectin (Sigma Chemical Corporation, St Louis, MO) coated flasks, and induced to differentiate in vitro into neurons, hepatocytes and endothelium as described previously^2. A single MAPC-derived clone stably expressing DsRed2 and firefly luciferase was prepared using Sleeping Beauty transposons^13 as we described^14.

Intra-venous and intra-arterial infusion of MAPCs

For intravenous injections MAPCs were infused via tail vein. Intra-arterial injections were performed as follows: Under general anesthesia small midline upper abdominal incision was performed and the caudal aspect of diaphragm was exposed. After direct visualization of heart apex, 10^6 MAPCs (in 10 µL of PBS) was slowly injected intra-cardiac across the diaphragm and left cardiac ventricular wall.

Flow cytometry

Single cell suspensions of MAPCs were prepared in buffer (PBS + 2% bovine serum + 0.15% sodium azide). Pelleted cells were incubated for 15 minutes at 4°C with 0.4 µg of anti-Fc receptor monoclonal antibody (mAb; clone 2.4G2, rat IgG2b) to prevent Fc binding. Flow cytometry was performed using directly conjugated (fluorescein isothiocyanate, FITC, or phycoerythrin, PE) mAbs to assess cell surface antigen expression of MAPC before and after 24 hour stimulation with 1,000 units of IFNγ/mL (R&D Systems Inc., Minneapolis, MN). Optimal concentrations of directly conjugated mAbs were added to a total volume of 100 to 130 µL and incubated for 1 hour at 4°C. The following mAbs obtained from Pharmingen (San Diego, CA) included: anti-H2^b
specific mAb (clone EH-144, mouse IgG2a), anti-IA\(^b\) specific mAb (clone AF6-120.1, mouse IgG2a), anti-CD80 specific mAb (clone 16-10A1, Hamster IgG2), anti-CD86 specific mAb (clone GL1, rat IgG2a), anti-ICAM-1 specific mAb (clone 3E2, Hamster IgG1), and anti-CD40 specific mAb (clone HM40-3, Hamster IgM). All samples were analyzed on a FACScalibur (Becton Dickinson, Palo Alto, CA) using Cell Quest software. Forward and 90 degree side-scatter were used to identify and gate live MAPC population. A minimum of 10,000 events was examined.

**Mixed lymphocyte reaction (MLR) culture**

To measure the potential of MAPCs to stimulate allogeneic T cell responses, purified CD4\(^+\) T cells or whole T cells were prepared from single cell suspensions of axillary, inguinal, and mesenteric lymph node cells isolated from BALB/c mice. Lymph node cells were depleted of NK cells for all cell preparations and CD8\(^+\) T cells (hybridoma 2.43, rat IgG2b; provided by Dr. Sachs, Charlestown, MA) for CD4\(^+\) cell preparations by coating with monoclonal antibodies and passage through a goat anti-mouse and goat anti-rat-Ig-coated column (Cedarlane Laboratories, Hornby, ON, Canada). 10\(^5\) purified T cells and 10\(^3\)-10\(^5\) irradiated (3000 cGy by \(^{137}\)Cs irradiation) C57BL/6 MAPC or MAPC DL were cultured in 96-well round bottom plates at 37\(^\circ\) C and 10\% CO\(_2\) for the indicated time in DMEM (BioWhittaker, Walkersville, MD) containing 10\% FCS (Hyclone, Logan, UT), 50 mM 2-mercaptoethanol (2-ME; Sigma), 10 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) buffer, 1 mM sodium pyruvate (Life Technologies, Grand Island, NY), amino acid supplements (1.5mM L-glutamine, L-arginine L-asparagine) (Sigma), and antibiotics (100 U/mL penicillin; 100mg/mL streptomycin) (Sigma). Some MAPC were pretreated with 1,000
IU of IFN-γ/mL for 48 hours before initiating assay. Irradiated, T cell depleted splenocytes were prepared from C57BL/6 mice as a positive control for T cell proliferation. For secondary cultures, T cells were recovered after 96 hours of culture, extensively washed, and restimulated with irradiated allogeneic B6 splenocytes, MAPC or MAPC DL. On the indicated day of culture, each well was pulsed with tritiated thymidine (1 μCi/well) (Amersham Life Sciences, Buckinghamshire, United Kingdom) for 18 hours prior to harvesting and counted in the absence of scintillation fluid on a β-plate reader (Packard Instrument Company, Meriden, CT). At least four wells were analyzed per group.

Susceptibility of MAPCs to NK-mediated lysis

To increase NK cell number and activity B6 mice were injected intraperitoneally with poly I:C (120 μg/mouse). After 48 hours splenocytes were harvested. Target cells (Yac-1 cells, positive control for NK lysis; or MAPCs) were loaded with ¹⁵⁵Cr 1 hour before the experiment and washed three times as described previously. Cr-labelled MAPCs or Yac-1 cells (5,000/well) were mixed with splenocytes from poly I:C injected mice at various ratios (200:1 to 0.8:1) in 96-well plate. Cells were incubated at 37°C for 4 hours and pelleted by centrifugation (5 minutes at 500 rpm). To test cytolytic potential of cells γ radioactivity was measured in the supernatant and expressed as counts per minute (cpm). A total of three assays were performed. Relative target cell lysis was calculated as: (sample cpm – spontaneous release)/(maximum release-spontaneous release) x 100%.

In vivo imaging of MAPCs

At 30 days after infusion of MAPCs mice were anesthetized with Nembutol (0.1 cc/10 mg body weight) and the abdomen and chest were shaved. Luciferin stock (30
mg/ml, Xenogen, Alameda, CA) was injected into the mice at 150 mg/kg intraperitoneally. A grayscale reference image was taken of the position of the mice prior to assessing luciferase activity. Bioluminescent signals were assessed at 5 min post luciferin injection at an integration time of 2 minutes using an in vivo imaging system that utilizes a cooled charge-coupled device (CCD) camera (IVIS100, Xenogen). Pseudocolor images representing the bioluminescent signal intensity (blue is the least intense and red is the most intense) were superimposed over the grayscale reference image. The scales for the pseudocolor intensity plots are displayed with the images.

**In vitro quantification of luciferase expression**

Tissue homogenates of lung specimens were harvested by centrifugation, mixed with 10 µL of luciferin stock (30 mg/mL, Xenogen), and assayed immediately for bioluminescence activity on a Chameleon 425-100 Multi-label Counter (Hidex, Turku, Finland). Average relative luminescence values were expressed as counts/second and normalized to total protein (Dojindo Molecular Technologies, Gaithersburg, MD).

**Localization and differentiation of MAPCs in tissues**

Tissue specimens of the recipient animals were cryopreserved in optimal cutting temperature (OCT) medium (Sakura Finetek, Torrance, CA) at -80°C. Six micrometer thick fresh frozen sections were mounted on glass slides, fixed in acetone for 10 min at room temperature and incubated in Normal Donkey Serum (Jackson ImmunoResearch, West Grove, PA) for 20 min. Cryosections were stained with nuclear stain 4’,6-diamidino-2-phenylindole, DAPI (Molecular Probes, Eugene, OR) and examined for native fluorescence of DsRed2 by confocal fluorescence microscopy (Olympus AX70, Olympus optical Co. LTD, Japan). To assess MHC class I expression in donor cells,
tissue sections were also stained with biotinylated primary H2b antibody at 1:100 concentration (BD Pharmingen) and incubated 1 hour at room temperature. Slides were washed twice in PBS and stained using immunodetection with fluorescein (Vector M.O.M., Vector Laboratories, Burlingame, CA) according to manufacturer’s instructions. Slides were examined using confocal fluorescence microscopy (Olympus AX70).

**Data analysis**

Differences between measurements were assessed using Fisher’s exact test, with p-value <0.05 considered significant.
RESULTS

MAPCs as targets of T cells and NK cell immune response as assessed in vitro

Immunophenotyping revealed that B6 MAPCs were low/negative for MHC class I and class II, costimulatory molecules (CD80, CD86, CD40) and the adhesion molecule ICAM-1 (CD54) (Figure 1A). Upon 24 hour stimulation with interferon γ (IFN-γ) MHC class I and ICAM-1 expression were upregulated from 9.3% to 99% and from 2.9% to 35.9%, respectively. Expression of MHC class II, CD80, CD86, and CD40 remained low. Low expression of molecules required for optimal T cell responses suggested that MAPCs would be poor targets for T cells and good targets for NK mediated elimination.

To determine whether MAPCs can stimulate T cells, MLR assays were performed using BALB/c CD4+ T cells or BALB/c CD4+ plus CD8+ T cells as responders and B6 MAPCs as stimulators. Neither untreated MAPCs nor MAPCs pretreated with IFN-γ for 48 hours stimulated CD4+ (Figure 1B) or whole T cell alloresponses (Figure 1C) in vitro. Proliferative T cell responses to allogeneic MAPC or MAPC DL remained low even when the responder:stimulator ratio was increased as high as 1:1 (Figure 1D) or following repeated stimulation of T cells with allogeneic MAPC or MAPC DL (Figure 1E). To determine whether MAPCs could serve as targets of NK mediated cytolysis, splenocytes from poly I:C (an inducer of NK activity) treated B6 mice were mixed with Yac-1 (H2a), an NK sensitive target, or MAPCs in a 4 hour chromium release assay. Effector to target cell ratios indicated that MAPCs were susceptible to NK lysis but less so than Yac-1 cells (Figure 1F).
Figure 1A

IFNγ -  +

Counts

H2b

Counts

I-Ab

Figure 1A continued

IFNγ -  +

Counts

CD80

Counts

CD86
Figure 1A continued

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Figure 1B

CD4

- T only
- T + B6 spleen
- MAPC
- T + MAPC
- MAPC IFNγ
- T + MAPC IFNγ

S H UPTAKE (CPM)

0 2000 4000 6000 8000 10000 12000 14000

<100 CPM
Figure 1C

CD4 + CD8

T only
T + B6 spleen
MAPC
T + MAPC
MAPC IFNg
T + MAPC IFNg

^3H UPTAKE (CPM)

Figure 1D

Responder:Stimulator Ratio

B6 Spleen
MAPC
MAPC DL

* *
Figure 1E

Figure 1F

In vivo immune resistance to MAPCs

To assess in vivo immune responses to MAPCs, MAPCs were infused into mice with varying degrees of immune competence. MAPCs were nucleoporated with Sleeping
Beauty transposon constructs to permit expression of DsRed2 and firefly luciferase, yielding a doubly transgenic MAPC DLs (DsRed2\(^+\), luciferase\(^+\)), as previously described\(^{14}\). To sequentially follow homing, migration and persistence of MAPCs in live animals in vivo, whole body imaging (WBI) was performed using the luciferase-mediated bioluminescent imaging (BLI). One million MAPC DLs were injected intravenously into adult immune competent B6 or T- and B-cell deficient B6 Rag2\(^{-/-}\) mice. Additional cohorts of B6 or B6 Rag2\(^{-/-}\) mice were given anti-NK1.1 mAb to deplete NK cells. These data were compared to B6 Rag2/IL-2R\(\gamma c^{-/-}\) mice that lack T-, B-, and NK-cells.

Sequential BLI analysis was performed in five mice per group per timepoint on days 4, 14 and 30 after MAPC DL infusion. In B6 mice, MAPC DLs were detected in the thorax and the injection site (likely around the tail vein) on day 4 but not day 14 or day 30 (data not shown). In Rag2\(^{-/-}\) mice, MAPC DLs were detected throughout the 30 day period (B6 versus Rag2\(^{-/-}\) at day 30, mean values: 34 versus 180 photons/sec/cm\(^2\), respectively; p-value 0.044, Figure 2). While NK depletion did not substantially increase MAPC DL number by BLI quantification in B6 mice (B6 without versus with NK depletion: 34 versus 40 photons/sec/cm\(^2\), respectively; p-value 0.198), it did in Rag2\(^{-/-}\) mice by day 30 (B6 with NK depletion versus Rag2\(^{-/-}\) with NK depletion: 40 versus 584 photons/sec/cm\(^2\), respectively; p-value 0.004; Figure 2). Rag2\(^{-/-}\) mice, which have higher NK activity than B6 mice\(^{16}\), showed low level of MAPC DL engraftment unless they were in vivo depleted of NK cells (Rag2\(^{-/-}\) without versus with NK depletion: 180 versus 584 photons/sec/cm\(^2\), respectively; p-value 0.014; Figure 2). In Rag2/IL-2R\(\gamma c^{-/-}\) mice, persistence of MAPC DLs was similar to that in Rag2\(^{-/-}\) with NK depletion (data not shown). Collectively, these data indicate that endogenous NK cells resist MAPC DLs.
Intraarterial infusion of MAPCs results in superior biodistribution

As most of the bioluminescence of infused MAPC DLs was detected over upper thorax, we reasoned that capture of MAPCs in pulmonary vasculature after intravenous (IV) delivery may decrease the actual MAPC cell dose delivered to other visceral organs. This may be due to adhesion properties of MAPCs or their size (MAPCs are 10-12 µM in diameter) in combination with anatomic location of lungs as the first capillary bed MAPCs encounter after IV infusion. To compare biodistribution of MAPCs after IV and intra-arterial (IA) delivery, MAPC DLs (10^6) were infused either via tail vein or via left cardiac ventricle into Rag2/IL-2Rγc^−/− mice (three mice per group per time point) that were permissive hosts for MAPC persistence. After IA delivery WBI showed BLI signals distributed throughout the whole body at day 4 (Figure 3A) and thoracoabdominal area, face and paws at day 30 (Figure 3B), in contrast to mostly thoracic BLI signals after IV...
delivery (Figure 2). Quantitatively, BLI signals were higher at both day 4 and day 30 after IA delivery in comparison to the BLI signals observed after IV infusion of the same dose of MAPC DLs (on day 4 after IA mean BLI was 27-fold higher than after IV, p-value 0.004; on day 30 after IA mean BLI was 4-fold higher than after IV, p-value 0.008; data not shown and Figure 3).

To obtain an assessment of tissue specific engraftment of donor MAPCs, tissue homogenates of lung, liver, kidney, brain, intestine, spleen, and bone marrow were examined for luciferase activity at day 30 after IV or IA MAPC DL infusion. Lung luminescence after IA infusion was above background but significantly lower than lung luminescence after IV delivery (mean value, [range]: 14,081, [3921-15,564] photons/sec versus 205, [131-261], p-value 0.032; Figure 3C). After IV administration, other tissues tested were below the limits of detection. In contrast, tissue luminescence was significantly higher after IA delivery than the same tissues after IV delivery: liver (197, [111-252] photons/sec, p-value 0.05), kidney (6,197, [1221-10449] photons/sec, p-value 0.044) and brain (344, [297-379] photons/sec, p-value 0.004; Figure 3C). IA delivery also was higher in intestine, spleen and BM, although these data did not reach statistical significance. These findings correlate with the WBI data from the same animals (Figure 3A and 3B) and show that, when compared to IV delivery, IA infusion results in much more diverse homing of MAPC DLs.
Total body irradiation overcomes MAPC resistance

As a long-term goal of these investigations is to use MAPCs clinically for treatment of tissue injury as occurs in the setting of allogeneic hematopoietic cell
transplantation, we sought to determine whether MAPC DLs can persist under these conditions. B6 mice were lethally irradiated and given B10.BR BM cells IV along with 10^6 of MAPC DLs infused IV or IA. Bioluminescence signals from MAPC were detected over chest after IV administration (Figure 4A) and over chest, abdomen, head, and extremities of recipient mice from day 4 through day 30 (Figure 4B). While on day 4 after IA MAPC DL infusion mean BLI signals were not significantly different from mean BLI after IV infusion, the BLI signals on day 30 after IA were 22-fold higher than after IV (p value < 0.001). In addition, after IA administration the BLI signals increased three-fold between days 4 and 30 (p value < 0.001, data not shown and Figure 4B).

Using measurements of luciferase activity in tissue homogenates we assessed engraftment of donor MAPCs in lung, liver, kidney, brain, intestine, spleen, and bone marrow at day 30 after IV or IA MAPC DL infusion. After IA delivery tissue luminescence was found to be significantly higher than background (and than samples of same tissues after IV delivery) in spleen (mean value, [range]: 2,527, [168-4,631] photons/sec, p-value 0.04), kidney (56,689, [177-108,402] photons/sec, p-value 0.03) and brain (2,443, [225-4,669] photons/sec, p-value 0.04; Figure 4C). After IA or IV delivery, luciferase activity was also detected in intestine, liver and BM (IA versus IV: 2,644 vs 154 photons/sec, 12,146 vs 131 photons/sec, and 683 vs 619 photons/sec in intestine, liver and BM, respectively), although these differences were not statistically different between IV and IA delivery. These data are similar to those described above for the Rag2/IL-2Rγc^-/- mice that were permissive for MAPC engraftment. Lung luminescence was above background but significantly lower after IV when compared to the lung luminescence after IA delivery (171,236 versus 1,650 photons/sec, p-value 0.0007;
Overall MAPC DL engraftment was higher with MAPC DLs and allogeneic BM infusion than after infusion of MAPC DLs alone using either route of delivery (Figure 3 and 4). This indicates that conditioning for hematopoietic stem cell transplantation using TBI may override both NK and T cell mediated resistance, and be advantageous in the long-term survival and widespread homing of MAPCs.

Figure 4A and 4B
Tissue localization of MAPC DLs in vivo

To assess donor engraftment on the level of individual organs, visceral tissues and brain were collected at the end of experiments, 30 days after infusion of MAPC DLs. Tissue immunohistochemistry revealed MAPC DL cells in all tissues examined in all animals but non-conditioned B6 wild type mice after IV delivery. Donor MAPC DL-derived cells in various tissues were similar in quality but they differed in quantity, which in turn correlated with WBI and in vitro luciferase activity measurements (Figures 2, 3 and 4). MAPC DL-derived cells were detected in the highest numbers in lung, kidney and liver of irradiated B6 recipients of allogeneic BM and IA MAPC DLs (Figure 5A-C). In addition, in all tissues examined, including lung, donor MAPC DLs expressed MHC class I antigen (Figure 5D-F). These observations are critical since they provide evidence that transgenic MAPC DLs persist in vivo and since they suggest that MAPC DLs may be functional in vivo.
DISCUSSION

We show that MAPCs which are MHC class I low/negative are targets of NK lysis both in vitro and in vivo, and elimination of NK cells improves the persistence of reporter gene-labeled cells in T- and B-cell deficient recipients. MAPCs that escape NK mediated elimination persisted long-term. In vitro, undifferentiated MAPCs were found to express low levels of costimulatory and adhesion molecules and were poor stimulators of an MLR response. In vivo, tissue localized MAPCs acquired MHC class I expression and a role for the adaptive immune system was found in resisting reporter gene-expressing MAPCs which could be overcome by TBI conditioning. Route of delivery was found to be critical in directing MAPCs to the lung (IV) or toward other organs (intra-arterial).

MAPCs (and other non-hematopoietic stem cells, e.g., Mesenchymal Stem Cells, MSCs\textsuperscript{17-19}) may be advantageous in tissue repair after injury by conditioning chemoradiotherapy for hematopoietic stem cell transplantation due to their ability to contribute to various tissues. In contrast to hematopoietic progenitors, which have been associated with limited stem cell plasticity\textsuperscript{20-23}, undifferentiated MAPCs can differentiate into most, if not all, tissue types\textsuperscript{2,5,24,25}. Similar to MSCs\textsuperscript{26}, MAPCs were poor stimulators of an in vitro allogeneic MLR response. Consistent with the low level of MHC class I expression in undifferentiated MAPCs, NK cells were able to eliminate a large fraction of MAPCs in vivo.

Interestingly, despite the low/absent MHC class I expression on undifferentiated MAPCs, in vivo undifferentiated MAPCs were also resisted by the adaptive immune system. In vitro, IFN\textgamma preincubation upregulated MHC class I but not MHC class II
expression. In vivo, tissue-localized MAPCs acquired MHC class I antigen expression even in settings in which TBI was not given. Such induction of MHC class I expression could be due to local proinflammatory responses (e.g., IFNγ production in vivo) as might occur in immune deficient mice exposed to environmental pathogens that are not promptly removed or as a consequence of MAPC differentiation into a given tissue type in vivo. Despite the low level of costimulatory molecule expression even with IFNγ preincubation, undifferentiated MAPCs were resisted by the adaptive immune system. We speculate that this happens as these MAPCs acquire MHC class I antigens and due to the fact that these MAPCs express four distinct foreign antigens (DsRed2, luciferase, neomycin phosphotransferase, β galactosidase). Other studies have demonstrated neomycin phosphostransferase induces a cytolytic T cell responses in vivo\textsuperscript{27} and our own unpublished data indicates that up to a 10-fold higher dose of tumor cells expressing DsRed2 is required to achieve equivalent lethality as the parental cell line, similar to results described for green fluorescent protein expressing tumor cells\textsuperscript{28,29}. Thus, undifferentiated MAPCs that are poorly immunogenic in vitro are converted to targets of the adaptive immune response in vivo under these conditions. MAPC homing and persistence may be increased in the setting of hematopoietic stem cell transplantation as a result of tissue injury, immune suppression (including reduction in T and NK cell numbers), upregulation of MHC class I expression or induction of cytokine and chemokine responses.

Since the production of autologous MAPCs as it currently stands can take several months their use in clinical setting will require diseases that do not need emergent treatment. Allogeneic MAPCs may represent a viable strategy either on their own or as
an adjunct to hematopoietic stem cell transplantation, provided they are not rejected by the recipient. Human MAPCs have similar characteristics as murine MAPCs\textsuperscript{2,5,24,25}, therefore, optimal conditions for MAPC infusions can be identified in the murine models, and a basis for the use of human MAPCs in a clinical setting can be established.

While IV delivery of MAPCs may be superior for their delivery to lung, IA administration may be applicable in delivery of MAPCs to extrapulmonary organs. Thus, to promote tissue repair in various visceral organs the site of infusion may have to be chosen accordingly to increase usefulness of MAPCs for the recipient.

These data are the first to illustrate the immune responses to MAPCs. We conclude that MAPCs express low levels of MHC class I antigens and are targets of NK mediated resistance, and that total body irradiation facilitates persistence of MAPCs. In combination, our data suggest that the infusion of MAPCs along with BM may represent a new approach for correction of genetic disorders and for reducing the conditioning regimen related side-effects of hematopoietic stem cell transplantation.
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FIGURE LEGENDS

Figure 1. MAPCs do not stimulate T cell responses and are susceptible to NK mediated lysis. (A) MAPCs are low/negative for MHC class I and class II, CD80 (B7-1), CD86 (B7-2), ICAM-1, and CD40. Upon 24 hour stimulation with interferon γ (1,000 IU/mL of IFN-γ) MHC class I and ICAM-1 expression was upregulated while the expression of MHC class II, CD80, CD86, and CD40 remained low. Representative histograms of experiments performed three times are shown. Dashed line, irrelevant antibody; continuous line, antibody to antigen indicated in each panel; IU, international unit. (B) 1 x 10^5 BALB/c CD4^+ T cells/well or (C) 1 x 10^5 BALB/c CD4^+ plus CD8^+ T cells/well and irradiated, untreated B6 MAPCs or irradiated MAPCs (10^4/well) that were pretreated with 1000 IU IFN-γ/mL for 48 hours were mixed in T cell proliferation assays. In some wells, T cells were cultured alone or with irradiated T cell-depleted B6 splenocytes (10^5/well). T cell proliferation was measured by ³H-thymidine uptake on day 5 (CPM, counts per minute) and is expressed as mean ± SEM. (D) 10^5 BALB/c CD4^+ plus CD8^+ T cells per well were cultured with the indicated number of allogeneic splenic stimulators, MAPC, or MAPC DL per well. Mean background counts for 10^5 irradiated B6 splenocytes, MAPC or MAPC DL were 37, 362, and 274 cpm, respectively (data not shown). (E) Following a 96 hour primary MLR culture with irradiated B6 splenic stimulators or MAPC, 10^5 BALB/c CD4^+ plus CD8^+ T cell responders/well were restimulated with 4 x 10^4 freshly prepared B6 splenic stimulators, MAPC, or MAPC DL per well. Mean background counts of 4 x 10^4 irradiated B6 splenocytes, MAPC, or MAPC DL were 82, 434, and 507 cpm, respectively (data not shown). (F) To determine whether MAPCs are susceptible targets for NK mediated killing, splenocytes from poly
I:C treated B6 mice were mixed with Yac-1 cells or with MAPCs in a chromium release assay. Effector to target cell ratios shows that MAPCs are a target of NK lysis in vitro.

**Figure 2. Immune resistance to MAPC in Rag2\(^{−/−}\) mice.** (A) Using whole body imaging of firefly luciferase bioluminescence the biodistribution of donor MAPC DLs was monitored in real time. In T- and B-cell deficient Rag2\(^{−/−}\) mice (N=5), MAPC DL were detected throughout the 30 day period, and even more so (B) in the mice which were given the anti-NK1.1 monoclonal antibody (N=5). MAPC DL-derived BLI signals were observed predominantly over the area of upper thorax.

**Figure 3. Intraarterial infusion of MAPCs results in superior biodistribution.** To assess biodistribution of MAPCs after intra-arterial (IA) delivery, MAPC DLs (10\(^6\)) were infused via left cardiac ventricle (N=3) into Rag2/IL-2R\(γc^{−/−}\) mice. WBI is shown at 4 days (A) and at 30 days (B) after MAPC DL infusion. WBI showed BLI signals distributed throughout the whole body at day 4 (A) and thoracoabdominal area, face and paws at day 30 (B), in contrast to mostly thoracic BLI signals after IV delivery (Figure 2B). (C) Tissue homogenates were examined for luciferase activity at day 30 after IV or IA MAPC DL infusion. Lung luminescence after IA infusion was above background but significantly less than lung luminescence after IV delivery (p-value 0.032). After IA delivery tissue luminescence was significantly higher than samples of same tissues after IV delivery in liver (p-value 0.05), kidney (p-value 0.044) and brain (p-value 0.004). These findings show that, when compared to IV delivery, IA infusion results in much more diverse homing of MAPC DLs.

**Figure 4. TBI overcomes MAPC resistance.** To determine whether MAPC can persist under conditions of allogeneic hematopoietic stem cell transplantation, B6 mice were
lethally irradiated and given B10.BR bone marrow IV with MAPC DLs infused IV (A) or IA (B). At day 30 after IV infusion BLI signals from donor MAPC DLs were detected over thorax (A). After IA administration bioluminescence was detected over chest, abdomen, head, and extremities (B). (C) To quantify engraftment of donor MAPCs luciferase activity in tissue homogenates was determined at day 30 after cell infusions. After IA delivery we have detected tissue luminescence significantly higher than samples of same tissues after IV delivery in spleen (p-value 0.04), kidney (p-value 0.03) and brain (p-value 0.04). Lung luminescence was above background but significantly lower after IV when compared to the lung luminescence after IA delivery (p-value 0.0007). This indicates that conditioning for hematopoietic stem cell transplantation using total body irradiation may be advantageous in the long-term survival and widespread homing of MAPCs.

**Figure 5. MAPC DLs persist in tissues.** Post mortem tissues analysis was performed on two to four representative animals from each cohort: IV delivery with and without NK depletion (B6, Rag2−/−, Rag2/IL-2Rγc−/−), IA delivery (Rag2/IL-2Rγc−/−) and IV or IA MAPC DLs infused with B10.BR bone marrow into lethally irradiated B6 mice. MAPC DL-derived cells were detected multiple tissues. Shown here are donor MAPC DL-derived cells in the lung (A), kidney (B), liver (C) of the B6 mouse with the highest BLI 30 days after IA infusion of MAPC and IV infusion of allogeneic bone marrow (Magnification 200x). Donor MAPC DL-derived cells (thin arrows) appear red as a result on native DsRed2 fluorescence and nuclei are stained blue with DAPI. Donor MAPC DLs expressed MHC class I in multiple tissues. Shown here are lung cells expressing
H2b (green; D), DsRed2 (red; E) and superimposition of the green and red pictures (donor MAPC DL cell, thick arrow; recipient’s cell, thin arrow; magnification 400x; F).
REFERENCES


Host factors that impact the biodistribution and persistence of multipotent adult progenitor cells

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