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Modulation of Atherothrombotic Factors: Novel Strategies for Plaque Stabilization

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Lentiviral ShRNA Silencing of Murine Bone Marrow Cell CCR2 Leads to Persistent Knockdown of CCR2 Function *in Vivo*

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Abstract

A major barrier in hematopoietic gene function studies is posed by the laborious and time-consuming generation of knockout mice with an appropriate genetic background. Here we present a novel lentivirus based strategy for the *in situ* generation of hematopoietic knockdowns. A short hairpin RNA (shRNA) was designed targeting murine CC-chemokine receptor 2 (CCR2), which was able to specifically blunt CCR2 expression at mRNA, protein and functional level *in vitro*. Reconstitution of irradiated recipient mice with autologous bone marrow, that had been *ex vivo* transduced with shRNA lentivirus, led to persistent down-regulation of CCR2 expression, which translated into a 70% reduction in CCR2 dependent recruitment of macrophages to an inflamed peritoneal cavity without noticeable side effects on related chemokine receptors or general inflammation status. These findings clearly demonstrate the potential of shRNA lentivirus infected bone marrow transplantation as a rapid and effective method to generate hematopoietic knockdowns for leukocyte gene function studies.

Introduction

Bone marrow transplantation studies have greatly facilitated the identification of macrophage genes that play a significant role in inflammatory disorders¹, e.g. atherogenesis²⁻⁴. However, this experimental setup essentially depends on the availability of transgenic mice or knockouts of the gene of interest for providing bone marrow. Thus, progress is considerably hampered by the laborious construction of in particular knockout mice and the subsequent backcrossing to an appropriate disease background for analysis of gene function. Moreover, the development of knockouts is even more difficult when deletion results in embryonic lethality as is the case for Tissue Factor (TF)⁵ or Vascular Endothelial Growth Factor (VEGF)⁶.

Efficient and stable gene transfer to hematopoietic stem cells has been a major challenge in gene therapy. For several years, a range of vectors of onco-retroviral origin, which integrate into the host cell genome, have been developed.⁷ A major drawback of these retroviral systems, mostly based on Moloney leukemia viruses, is their inability to integrate in the genome of non-mitotic cells. Since hematopoietic stem cells are rather quiescent, their retroviral transduction generally is inefficient. While transduction efficiency can be improved by cytokine supplementation⁸, by reducing cyclin-dependent kinase inhibitor levels⁹, or by fibronectin coating to induce stem cell proliferation^{10,11}, these manipulations may likely affect stem cell differentiation. Unlike onco-retroviral vectors, lentivirus offers the advantage of stably transducing not only mitogenic, but also quiescent cells¹²⁻¹⁵. Indeed, efficient lentiviral transduction of bone marrow stem cells using lentiviral vectors has been reported¹⁶. Such transduction is not only required to obtain required gene expression levels *in vivo*, but also obviates the use of time-consuming pre-selection strategies.

We argued that the high levels of lentiviral gene transfer to bone marrow may be exploited to knockdown hematopoietic genes *in vivo* via RNA interference (RNAi), defined as sequence specific post-transcriptional gene silencing¹⁷⁻²⁴. The advent of RNAi or small interfering RNA (siRNA) has instigated a true revolution in functional genomics and allowed the *in situ* knockdown of genes. Synthetic short double-strand DNA sequences of 21-25 nucleotides in length²⁵ were reported to be efficient in inducing selective mRNA degradation and to suppress gene expression^{26,27}. Recently, Brummelkamp *et al.*²⁸ have generated a mammalian expression vector, encoding a short hairpin dsRNA transcript under the control of the RNA polymerase-III dependent H1 gene promoter for sustained silencing of a transgene. At present, several groups reported successful lentiviral delivery of short hairpin RNA (shRNA) to eukaryotic cells *in vitro*. Tiscornia *et al.*²⁹ showed decreased Green Fluorescent Protein (GFP) levels after transduction with a lentiviral shRNA construct. Likewise, successful lentiviral delivery of shRNAs to human cells^{30,31} and even to human hematopoietic stem cells was reported³².

In this study we have addressed the potential of lentivirus-aided gene silencing of genes in bone marrow for the *in situ* generation of chimeras with blunted gene expression in the hematopoietic cell lineage *in vivo*. As model gene we chose murine CC-chemokine Receptor 2 (*CCR2*), which was previously shown to play a central role in the recruitment of monocytes to sites of inflammation and during atherogenesis³³⁻³⁵. A short hairpin RNA construct was developed against *CCR2* (sh*CCR2*), which proved highly efficient in downregulating *CCR2* expression both at an mRNA as well as at a protein level in *CCR2* transfected HEK 293 cells. Furthermore, we have efficiently transduced bone marrow cells and reconstituted irradiated C57Bl/6 mice with sh*CCR2*-transduced bone marrow cells, which considerably attenuated macrophage recruitment to the site of inflammation to a level comparable with that of mice reconstituted with *CCR2*^{-/-} bone marrow.

Methods

Cloning and expression of murine CCR2-GFP in HEK 293 cells

Total RNA was extracted from the murine monocytic cell line WEH1, reverse transcribed using M-MuLV reverse transcriptase (RevertAid, MBI Fermentas, Leon-Roth, Germany) and the cDNAs were amplified by polymerase chain reaction (PCR) to obtain full-length cDNA for murine *CCR2* using the following primers: forward: 5'-AGCCTCGAGATGGAAGACAATAATATGTT-3' and reverse: 5'-ATAAAGCTTTTACAACCCAACCGAGACCTCT-3'. Primers contained extra *Xho*I and *Hind*III restriction sites to facilitate cloning (underlined). Purified PCR products were subsequently cloned into pCR2.1 (Invitrogen, Breda, The Netherlands) and subcloned into pEGFPN-1 (Clontech, Palo Alto, CA). Murine *CCR2* and GFP were fused at the GFP N-terminus via removal of the stop codon, affording the pEGFPN-1/*CCR2* fusion vector. A *CCR2* expression vector was generated by cloning full length *CCR2* into pcDNA3.1(-) (Invitrogen), designated as pcDNA3.1(-)/*CCR2*. All constructs were sequenced to confirm the identity of murine *CCR2*.

Design and cloning of shRNA directed against murine CCR2

A pair of 64-nucleotide oligonucleotides encoding a 19-nucleotide *CCR2* shRNA (5'-GATCCCCCTGTGTGATTGACAAGCACTTCAAGAGAGTGCTTGTCATCA CACAGTTTTTGGAAA-3' and 5'-AGCTTTTCCAAAACTGTGTGATTGACAAGCACTCTCTTGAAGTGCTTG TCAATCACACAGGGG-3') were designed, which contained extra *Bgl*II and *Hind*III restriction sites to facilitate cloning. The position of the core 19-nucleotide sequence (underlined) targeted nucleotides 867 to 885 of the murine *CCR2*. Blast search was performed using the National Center for Biotechnology Information (NCBI) Expressed Sequence Tags (EST)

database to ensure that the shRNA construct only targeted murine CCR2. The 64-nucleotide oligonucleotides were annealed and cloned into the *Bgl*II and *Hind*III sites of the pSUPER vector (kindly provided by Dr. Agami, The Netherlands Cancer Institute, Amsterdam, The Netherlands) and the resulting vector, designated pSUPER-H1.shCCR2, was subsequently sequenced to confirm identity.

Silencing effect of pSUPER-H1.shCCR2

HEK 293 cells, grown at 37°C and 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS), 100 µg/mL streptomycin, 100 U/mL penicillin and 2 mM L-glutamine (all from Cambrex Bio Science, Verviers, Belgium), were transiently cotransfected with a constant amount of pEGFPN-1/CCR2 (0.5 µg) and 0.1 µg, 0.5 µg and 1.5 µg of pSUPER-H1.shCCR2, supplemented to 1.5 µg with pSUPER-H1.Empty vector using Lipofectamine 2000 (Invitrogen). As controls, the effects of a shRNA designed against luciferase (pSUPER-H1.shLuc, CGTACGCGGAATACTTCGA³⁶) and a nonsense sequence (pSUPER-H1.shInert, AGGCTGCTTGCACGATCTA) cloned into pSUPER on CCR2 expression levels were determined. After 48 hours, total RNA was extracted from the HEK 293 cells and reverse transcribed as described above. Quantitative analysis of CCR2 expression was performed using an ABI PRISM 7700 Taqman apparatus (Applied Biosystems, Foster City, CA) as described previously³⁷ (Table 1). The amplicon encompassed the shCCR2 core sequence (nucleotides 851-991), enabling the analysis of sequence-specific degradation of murine CCR2 mRNA and excluding artifacts due to the presence of shRNA. Human hypoxanthine phosphoribosyltransferase (hHPRT) was used as standard housekeeping gene and non-reverse transcribed RNA samples were used as control to determine genomic DNA contamination. Murine CCR2 mRNA expression was calculated relatively to that of hHPRT on the basis of Δ Ct values.

Table 1. Primer set sequences used for PCR analysis

Gene	Forward (5'-3')	Reverse (5'-3')
<i>hHPRT</i>	TGACTGGCAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
<i>mHPRT</i>	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
<i>CCR2</i>	CTTGGGAATGAGTAACTGTGTGA	GGAGAGATACCTTCGGAACTTCT
<i>CCR3</i>	TGCAGGTGACTGAGGTGATTG	CGGAACCTCTCACCACAAAG
<i>CCR5</i>	GACTGTCAGCAGGAAGTGAGCAT	CTTGACGCCAGCTGAGCAA
<i>CXCR3</i>	GCTGCTGTCCAGTGGGTTTT	AGTTGATGTTGAACAAGGCGC
<i>IFNγ</i>	ATAACTATTTAACTCAAGTGGCATAGATGT	TCTGGCTCTGCAGGATTTTCA
<i>IFNβ</i>	GCTCCTGGAGCAGCTGAATG	TCCGTCATCTCCATAGGGATCTT
<i>SRY</i>	GTGTGGTCCCTGGGTGAGA	TGTAAGGCTTTTCCACCTGCAT

After transfection with pEGFPN-1/CCR2 and pSUPER-H1.Empty, pSUPER-H1.shLuc, pSUPER-H1.shInert or different amounts of pSUPER-H1.shCCR2, the cells were harvested, resuspended in PBS and analyzed using Fluorescence Activated Cell Sorting (FACS, FACScalibur, BD Biosciences) for GFP expression. For western blotting, transfected cells were washed gently with PBS, scraped off and subsequently lysed in sodium dodecyl sulphate (SDS) sample buffer supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany). Equal amounts of protein were applied to a polyacrylamide gel (12.5%) and transferred to nitrocellulose. Visualization of GFP with a mouse-anti-GFP monoclonal antibody and a peroxidase-conjugated rat-anti-mouse IgG as a secondary antibody (1:2,000 and 1:1,000 dilution, respectively, Santa Cruz Biotechnology Inc., CA) was performed via the enhanced chemiluminescence (ECL) technique (Amersham Pharmacia Biotech., Piscataway, NJ). Blots were simultaneously probed with a mouse anti- β -tubulin antibody (Sigma, Zwijndrecht, The Netherlands) to verify equal loading of the samples.

Functional loss of CCR2 by shCCR2

The effect of the shRNA vector on CCR2 function was determined by analyzing calcium influx in response to the endogenous ligand for murine CCR2, CC-chemokine ligand 2 (CCL2) or JE. HEK 293 cells, expressing murine CCR2, were transfected with 0.1 μ g of pSUPER-H1.shCCR2 or with pSUPER-H1.Empty control vector. After 48 hours, cells were seeded onto poly-lysine coated glass coverslips to measure transient intracellular calcium influx. Cells were loaded with 20 μ M of Fura-2-AM (Invitrogen) for 60 minutes and after washing with Hank's balanced salt solution, the coverslips were mounted in an incubation chamber. JE (Santa Cruz Biotechnology Inc.) was added to a final concentration of 1 nM and real-time digital fluorescence imaging was performed using a fluorescence microscope (excitation: 340 and 380 nm, emission: 495 nm) for 20 minutes. The mean fluorescence intensity was determined to calculate intracellular calcium concentrations.

Lentivirus vector construction and production

The lentiviral expression vectors pRRI-cPPt-H1.PreSIN (H1.Empty) and pRRI-cPPt-H1.shCCR2-PreSIN (H1.shCCR2) were constructed by removing the cytomegalovirus (CMV) promoter from the expression vector pRRI-cPPt-CMV-PreSIN¹⁵ using *Cla*I and *Pst*I digestion, followed by insertion of the H1 promoter or the complete H1.shCCR2 construct. Virus was produced as described³⁸ using transient calcium phosphate cotransfection of 293T cells with the H1.Empty or H1.shCCR2 vector together with pMDL/RRE, pRSV-REV and pVSV-G. Viral supernatants were passed through a 0.45 μ m filter and concentrated by ultracentrifugation (20,000 rpm, 2 hours, 4°C). Viral titers were determined essentially as described by Sastry *et al.*³⁹ In short, HEK 293 cells were transduced with serially diluted viral supernatant, 48 hours after transduction total genomic DNA was isolated from these cells

and the number of vector DNA copies was determined using PCR analysis with pRRI-cPPt-H1.PreSIN vector as calibration standard (forward primer: GTGCAGCAGCAGAACAATTTG, reverse primer: CCCCAGACTGTGAGTTGCAA).

Animals

All animal work was approved by the regulatory authority of Leiden and performed in compliance with the Dutch government guidelines. C57Bl/6 mice were obtained from Charles River (Maastricht, the Netherlands). *CCR2*^{-/-} mice were kindly provided by Dr. Maeda from the University of North Carolina (Chaphil, USA). Bone marrow transplanted mice were housed in sterile filter-top cages and fed a sterile regular chow diet (RM3, Special Diet Services, Witham, UK). Drinking water was infused with antibiotics (83 mg/L ciprofloxacin and 67 mg/L polymyxin B sulfate) and 6.5 g/L sugar and was provided ad libitum.

In vitro transduction protocol for bone marrow cells

Bone marrow cell suspensions were isolated from C57Bl/6 mice by flushing the femurs and tibias with PBS. Single cell suspensions were prepared by passing the cells through a cell strainer and 10^5 cells/well were plated in a 12 well plate. Viral transductions were performed by incubating the bone marrow cells with different titers of pRRI-cPPt-PGK-GFP-PreSIN in complete DMEM supplemented with 10 µg/mL diethylaminoethyl (DEAE)-dextran at 37°C. After 24 hours, cells were washed with PBS and fixed in 4% paraformaldehyde in PBS for FACS analysis to determine GFP expression levels. Viral copy number inserted in the bone marrow cells after 24 hours was determined by isolation of total genomic DNA (from 10^6 bone marrow cells) and PCR analysis as described above.

Irradiation and bone marrow transplantation

To induce bone marrow aplasia, female C57Bl/6 mice (12-14 weeks of age) were exposed to a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA) total body irradiation, using an Andrex Smart 225 Röntgen source (YXLON International, Copenhagen, Denmark) with a 6 mm aluminium filter, one day before transplantation. Bone marrow cell suspensions were isolated from C57Bl/6 mice as described above. Subsequently, bone marrow cells (1.0×10^7) were transduced with either H1.Empty or H1.shCCR2 lentivirus in the presence of 10 µg/mL DEAE-dextran (multiplicity of infection [m.o.i.] = 15). After 24 hours, the cells were injected into the tail vein of the irradiated recipients (n=4 per group). As a positive control, irradiated C57Bl/6 mice (n=5) received 1.0×10^7 bone marrow cells isolated from male *CCR2*^{-/-} mice. In a similar set-up, C57Bl/6 mice (n=2) were injected with bone marrow cells transduced with GFP lentivirus. Of these mice, GFP expression levels in blood cells were determined 6 weeks after transplantation by preparing cytopins of white blood cells (300 rpm, 5 minutes) after lysis of the

erythrocytes. The images were taken with a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems).

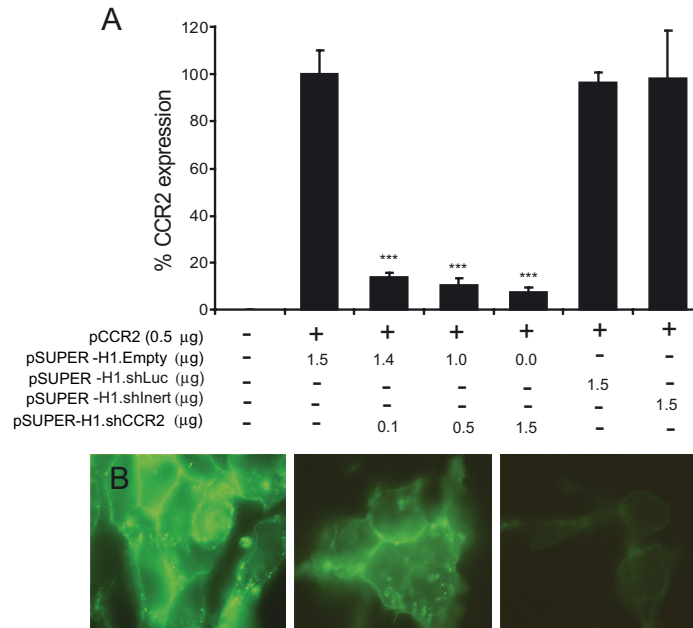


Figure 1. Silencing of CCR2 expression by pSUPER-H1.shCCR2. (A) Expression of murine CCR2 in HEK 293 cells after cotransfection with pEGFPN-1/CCR2 and pSUPER-H1.Empty (1.5 µg), relative to that of HPRT, was not affected by pSUPER-H1.shLuc or pSUPER-H1.shInert, while CCR2 mRNA levels were up to 94% reduced after cotransfection with increasing amounts of pSUPER-H1.shCCR2 (**P<0.001). Error bars represent SEM. (B) HEK 293 cells cotransfected with either pEGFPN-1/CCR2 and pSUPER-H1.Empty (1.5 µg; left panel) or pEGFPN-1/CCR2 and pSUPER-H1.shCCR2 (0.5 or 1.5 µg; middle and right panel, respectively), which show increased quenching of GFP protein expression on the cell surface, indicating almost complete loss of CCR2 surface protein after transfection with pSUPER-H1.shCCR2. Cells were viewed with a Biorad confocal laser scanning microscope.

Macrophage recruitment

Six weeks after bone marrow transplantation, the mice receiving H1.Empty or H1.shCCR2 lentivirus transduced bone marrow and the mice reconstituted with *CCR2*^{-/-} bone marrow were injected intraperitoneally with sterile 3% (w/v) Brewer's thioglycollate solution (Difco, Detroit, USA). Peritoneal macrophages were isolated 5 days later by lavage of the peritoneal cavity with PBS (10 mL) and counted after lysis of erythrocytes. Also, blood smears were prepared, stained with Wright's Giemsa (Sigma) and scored manually. Total RNA was isolated from peritoneal macrophages and gene expression was determined by RT-PCR compared to murine *HPRT* (Table 1). Also, bone marrow was isolated from each individual mouse as described above and after lysis of the bone marrow cells, genotyped for the presence of the *CCR2* gene in a single reaction (primers:

CCR2 forward: 5'-GATGATGGTGAGCCTTGTC-3', CCR2 reverse: 5'-CACAGCATGAACAATAGCCA-3', and a pgk-*neo* specific primer: Neo 5'-TTAAGGGCC-AGCTCATTCCCT-3'). In wild-type mice, a 360 base pair sequence is amplified corresponding with intact CCR2, whereas in the CCR2^{-/-} mice a 290 base pair sequence is generated. Also, viral copy number in the bone marrow was determined. In addition, the percentage repopulation of male bone marrow in the female recipients at that time point was determined by means of Taqman PCR analysis on the *SRY* gene, located on the Y-chromosome. To quantify the percentage male bone marrow, a calibration curve was used containing increasing amounts of male bone marrow supplemented with female bone marrow to standardize the amount of genomic DNA per well.

Statistical analysis

Data are expressed as mean \pm SEM. A 2-tailed Student's t-test was used to compare individual groups. To determine the significance of the relative mRNA expression levels, statistical analysis was performed on Δ Ct values.

Results

Silencing CCR2 expression by shRNA vectors

To determine the silencing capacity of a shRNA against CCR2 (pSUPER-H1.shCCR2) on murine CCR2 expression, HEK 293 cells were cotransfected with pEGFPN-1/CCR2 fusion vector and increasing amounts of pSUPER-H1.shCCR2. As controls, the effects of a shRNA sequence against luciferase and a nonsense shRNA sequence on CCR2 expression were also determined. CCR2 mRNA levels were already found to be significantly reduced after co-transfection with only 0.1 μ g of pSUPER-H1.shCCR2 (Figure 1A, $P < 0.001$ compared to the shRNA controls at all concentrations) with a maximum reduction of 94% at 1.5 μ g of pSUPER-H1.shCCR2, while the shRNA controls and pSUPER-H1.Empty had no effect. In agreement, expression analysis by fluorescence microscopy revealed an almost complete quenching of GFP-tagged murine CCR2 protein expression in HEK 293 cells cotransfected with pSUPER-H1.shCCR2 compared to that of the pSUPER-H1.Empty cotransfected control (Figure 1B). Subsequent FACS analysis (Figure 2A) confirmed that CCR2/GFP fusion protein expression was dose-dependently blunted after cotransfection with increasing amounts of pSUPER-H1.shCCR2, while the shRNA controls (i.e. pSUPER-H1.Empty, pSUPER-H1.shLuc and the nonsense shRNA sequence) were ineffective. Also, the fluorescence intensity of the GFP-positive cells was reduced to the level of untransfected HEK 293 cell controls (on average 380) after cotransfection with pSUPER-H1.shCCR2 (Figure 2B). These FACS data were confirmed by western blot analysis (Figure 2C)

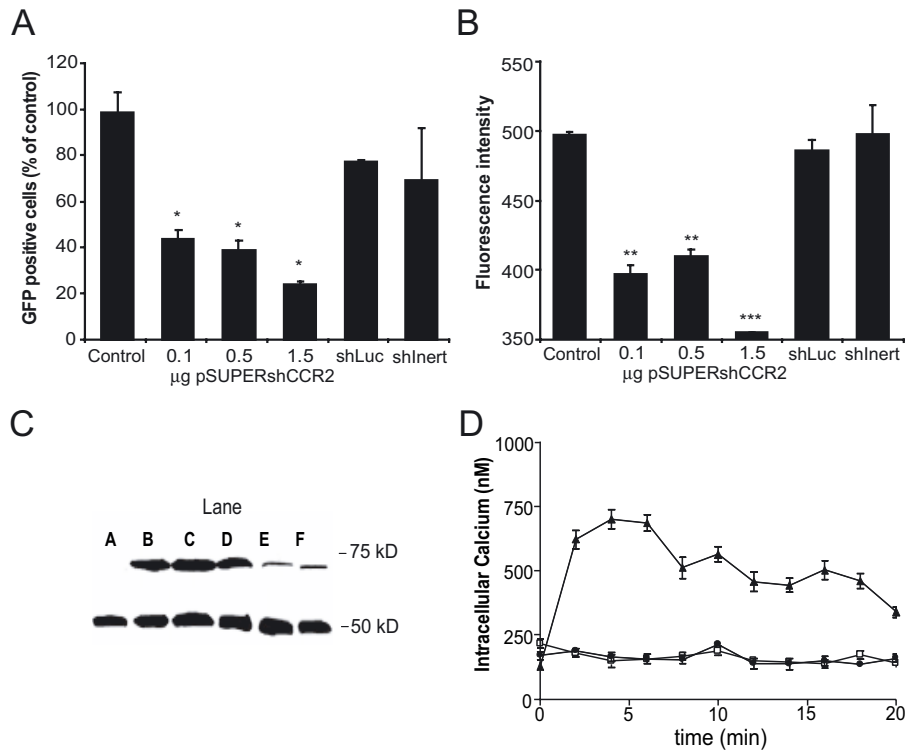


Figure 2. Silencing of CCR2 protein by pSUPER-H1.shCCR2. (A) FACS analysis of HEK 293 cells after cotransfection with pEGFPN-1/CCR2 and pSUPER-H1.Empty (1.5 μ g, control), which was not significantly affected by cotransfection with pSUPER-H1.shLuc or pSUPER-H1.shInert. Cotransfection with increasing amounts of pSUPER-H1.shCCR2 lead to a significant and dose-dependent silencing of CCR2 protein level up to 75% (* $P \leq 0.05$). (B) The average fluorescence intensity of the GFP-positive cells was even more markedly reduced after cotransfection with pSUPER-H1.shCCR2 compared to untransfected HEK 293 control level (approximately 390). The non-specific controls had no effect on GFP fluorescence intensity compared to pSUPER-H1.Empty control levels (** $P \leq 0.01$, *** $P \leq 0.001$) (C) Western Blot analysis of HEK 293 cell lysates for GFP protein expression using β -tubulin (50 kD) as internal standard, showing a dose-dependent and specific reduction in GFP protein expression in cells co-transfected with increasing amounts of pSUPER-H1.shCCR2 (lanes d, e, and f) compared to only pEGFPN-1/CCR2 transfected (lane b) or pSUPER-H1.Empty co-transfected cells (lane c). Lane a represents a sample of untransfected HEK 293 cells. (D) Measurements of JE-induced calcium influx in HEK 293 cells expressing murine CCR2 and the H1.Empty (\blacktriangle) or the H1.shCCR2 sequence (\square). As a control, unstimulated HEK 293 cells expressing CCR2 were measured (\bullet). In contrast to pSUPER-H1.Empty transfected cells, no calcium influx was observed in pSUPER-H1.shCCR2 transfected cells after stimulation with JE, indicating that CCR2 signal transduction is silenced by shCCR2 treatment. Error bars represent SEM.

Intracellular calcium levels were measured in HEK 293 cells expressing CCR2 and the H1.shCCR2 or H1.Empty sequence in response to the murine CCR2 ligand JE. Upon incubation with JE (1 nM), transient calcium influx was observed after 2 minutes, which peaked at 4 minutes, after which

intracellular calcium levels gradually declined to base values. Transfection of these cells with pSUPER-H1.shCCR2 completely abolished the JE induced calcium influx to levels comparable to that of non-stimulated cells (Figure 2D), indicating that the observed reduction in CCR2 mRNA and protein levels establishes a complete loss of CCR2 function in response to JE.

Transduction of CCR2 transfected HEK 293 cells with H1.shCCR2 lentivirus resulted in a significantly reduced expression of CCR2 relative to hHPRT (5.95 ± 0.67 versus 14.4 ± 3.6 in cells transduced with H1.Empty virus, $P=0.02$).

Transduction of bone marrow cells

In the next stage, the transduction efficiency of lentivirus in bone marrow cells was optimized. FACS analysis of bone marrow cells showed that transduction was not satisfactory at low multiplicities of infection (m.o.i., $6.5 \pm 0.6\%$ and $9.8 \pm 3.3\%$ at m.o.i.'s of 1 and 2, respectively). The use of higher titers and DEAE-dextran ($10 \mu\text{g/mL}$) led to a strong increase in infection efficiency from $3.1 \pm 0.5\%$ in control cells via $67.1 \pm 6.0\%$ (m.o.i.=5) and $76.2 \pm 2.0\%$ (m.o.i.=10) to $86.1 \pm 2.3\%$ (m.o.i.=20) 24 hours post infection (Figure 3A). Viral incorporation analysis revealed that bone marrow cells contained on average 2 copies of virus per *HPRT* gene 24 hours after transduction of the cells with GFP, H1.shCCR2 or H1.Empty control lentivirus (figure 3B). The high transduction, obtained at m.o.i.'s of 10-20 in the presence of $10 \mu\text{g/mL}$ DEAE-dextran, suffices for *in vivo* transplantation purposes and circumvents the need of a laborious pre-selection step. Six weeks after injection of GFP lentivirus transduced bone marrow into irradiated C57Bl/6 mice, cytopsin analysis showed that approximately 80-90% of all white blood cells were GFP positive (Figures 3C to F).

Transplantation of H1.shCCR2 transduced bone marrow

To generate chimeras with silenced CCR2 expression, bone marrow from C57Bl/6 mice was transduced with either H1.shCCR2 or H1.Empty control lentivirus in the presence of $10 \mu\text{g/mL}$ DEAE-dextran and i.v. injected into irradiated C57Bl/6 recipient mice, while a third group received *CCR2*^{-/-} bone marrow. No differences were observed in either weight gain during the experiment or in mononuclear cell and neutrophil content of the total white blood cell population at seven weeks after transplantation (data not shown). At that point, macrophages were recruited to the peritoneal cavity by an intraperitoneal thioglycollate challenge. Assessment of the number of peritoneal macrophages, 5 days after challenge, revealed a significant 70% reduction in macrophage recruitment in mice which had received H1.shCCR2 lentivirus transduced bone marrow compared to the H1.Empty lentivirus controls ($8 \pm 4 \times 10^6$ cells versus $27 \pm 6 \times 10^6$ cells, respectively; $P=0.03$, Figure 4A).

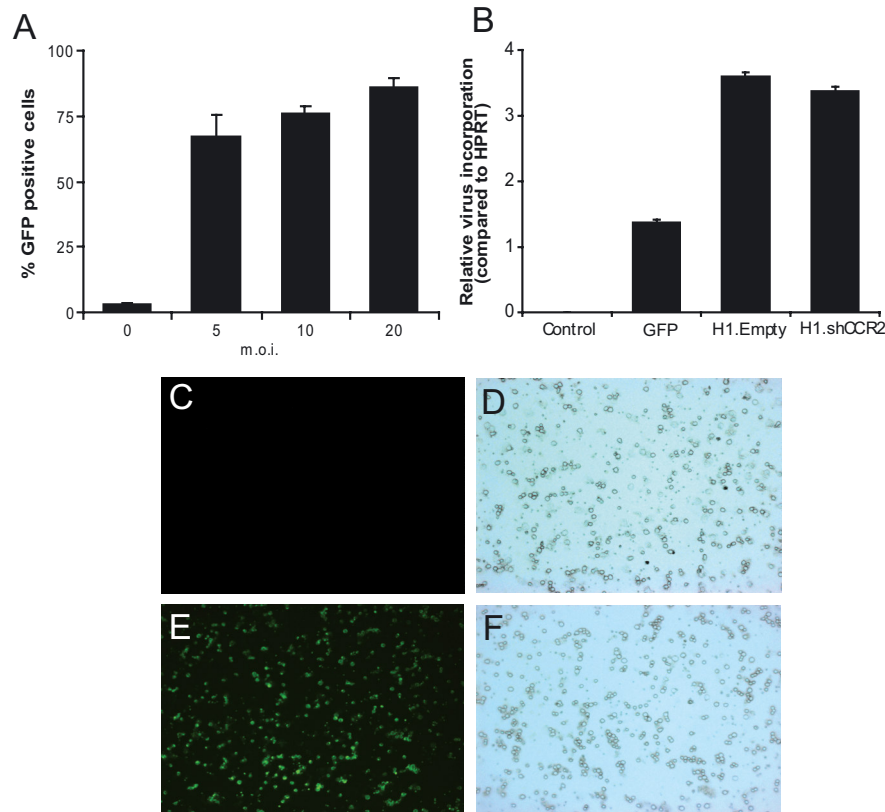


Figure 3. Efficient transduction of bone marrow cells using GFP lentivirus. (A) Transduction of whole bone marrow with GFP lentivirus led to a titer-dependent increase in percentage of GFP expressing cells. (B) Virus particle incorporation in bone marrow cells compared to HPRT of untransduced control cells and 24 hours after transduction with GFP-, H1.Empty- or H1.shCCR2-lentivirus. Error bars represent SEM. (C, D) Fluorescence and light microscopic high power field, respectively, of a white blood cell cytospin from control mice 6 weeks after transplantation of non-transduced bone marrow (200x). (E, F) White blood cells from mice transplanted with lentiviral GFP transduced bone marrow show approximately 80-90% GFP positive cells (panel E, 200x), indicating that transplantation of lentivirus transduced bone marrow results in sustained transgene expression by blood cells. Images were viewed with a Leica DR-ME microscope and using Leica Qwin software.

Interestingly, macrophage recruitment was comparable to that of mice reconstituted with *CCR2*^{-/-} bone marrow ($8 \pm 1 \times 10^6$ cells; $P=0.9$ versus the H1.shCCR2 lentivirus group). Macrophage recruitment levels of mice reconstituted with H1.Empty transduced bone marrow matched with those found in control mice that underwent transplantation (female C57Bl/6 recipient mice that had received untransduced male C57Bl/6 bone marrow, data not shown), which indicates that lentiviral transduction of bone marrow per se does not elicit an inflammatory response.

Analysis of CCR2 expression by peritoneal macrophages revealed a highly significant reduction in CCR2 mRNA levels in mice transplanted with H1.shCCR2 versus H1.Empty lentivirus transduced bone marrow (expression relative to murine HPRT: 0.4 ± 0.1 and 1.3 ± 0.2 , respectively; $P=0.002$). For comparison, mice that had received $CCR2^{-/-}$ bone marrow showed a relative CCR2 expression of 0.09 ± 0.02 (Figure 4B).

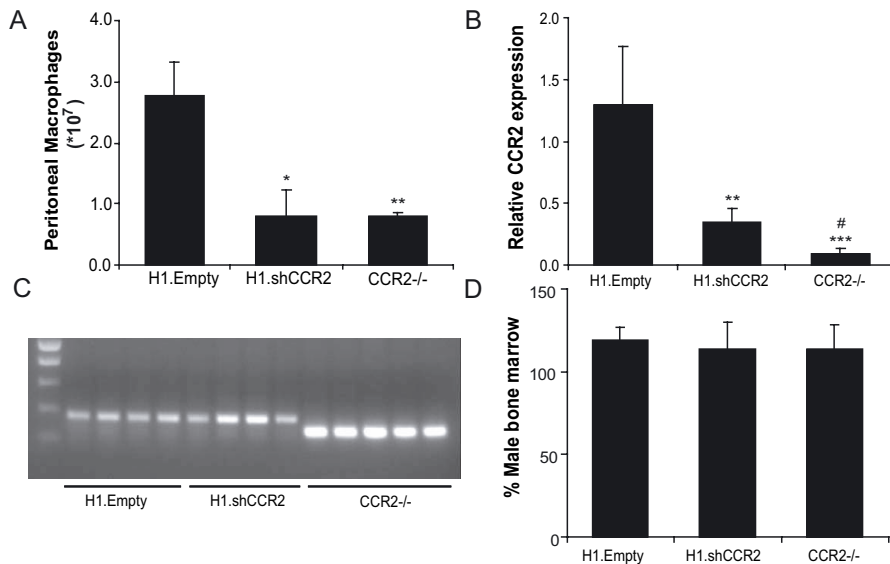


Figure 4. Lentiviral shCCR2 results in reduced macrophage influx due to reduced CCR2 expression. (A) Macrophage recruitment to the peritoneum in mice transplanted with H1.shCCR2 lentivirus transduced bone marrow is significantly reduced compared to those that received H1.Empty lentivirus transduced bone marrow. Recruitment levels were found to be similar to $CCR2^{-/-}$ bone marrow transplanted control mice ($*P<0.05$, $**P<0.01$). (B) CCR2 mRNA expression level in peritoneal macrophages of H1.shCCR2 transduced bone marrow transplanted mice was reduced 7 weeks after transplantation ($**P<0.01$, $***P<0.001$ compared to H1.Empty, $\#P<0.01$ compared to H1.shCCR2). (C) $CCR2$ genotyping of transplanted C57Bl/6 mice, revealing a 360 base pair amplicon of wild-type $CCR2$ in mice transplanted with H1.Empty or H1.shCCR2 lentivirus transduced bone marrow and the 290 base pair amplicon was observed in the $CCR2^{-/-}$ bone marrow transplanted mice. (D) % Male bone marrow in bone marrow cell lysates from recipient mice, determined by PCR analysis on the SRY gene, located on the Y-chromosome. Error bars represent SEM.

As expected, genotyping showed that C57Bl/6 mice transplanted with H1.Empty or H1.shCCR2 lentivirus transduced bone marrow, both displayed the 360 base pair amplicon, indicative of wild-type $CCR2$ (Figure 4C). C57Bl/6 mice that had received $CCR2^{-/-}$ bone marrow showed the $CCR2^{-/-}$ phenotype (290 base pair sequence). Reconstitution with male bone marrow in the female recipient mice was quantitative and persistent for at least 7 weeks in all three groups, as judged from PCR analysis of the SRY gene (Figure 4D). PCR analysis of genomic DNA from bone marrow revealed that

unpurified bone marrow from H1.shCCR2 transplanted mice contained on average 2 virus integrants per *SRY* copy in both the H1.shCCR2 (2.30) and the H1.Empty group (1.98), while as expected in bone marrow from *CCR2*^{-/-} transplanted mice no virus particles were detected.

Furthermore, no differences were observed in peritoneal macrophage expression levels of other chemokine receptors, such as CCR3, CCR5 and CXCR3 (Figures 5A to C, P=NS), or of inflammatory mediators like interferon β (IFN β) and IFN γ (data not shown), indicating that the shRNA against CCR2 was highly specific and did not display significant off-target effects on the expression levels of other relevant genes. Intriguingly, macrophages of *CCR2*^{-/-} transplanted mice showed an almost 50% reduction in relative CCR5 mRNA expression levels (P=0.04).

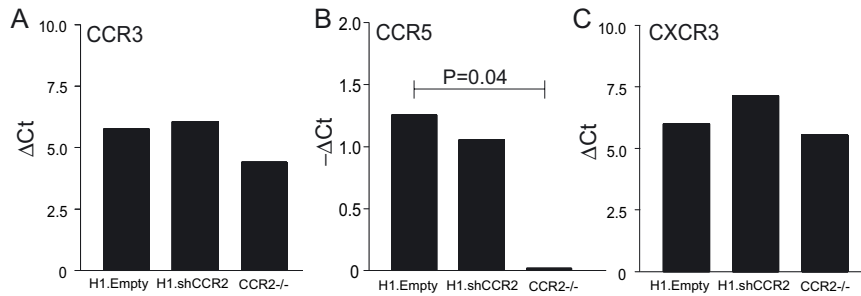


Figure 5. Absence of non-specific effects on related chemokine receptors. No non-specific effects were observed on peritoneal macrophage mRNA levels of other chemokine receptors, as CCR3 (A), CCR5 (B) and CXCR3 (C) in mice transplanted with H1.shCCR2 lentivirus transduced bone marrow compared to the H1.Empty controls. *CCR2*^{-/-} bone marrow transplantation led to a significant decrease in macrophage CCR5 expression (P=0.04).

Discussion

Transplantation of transgenic or knockout bone marrow to wild-type recipients is a widely used technique to determine the role of a particular leukocyte gene in several inflammatory disorders, such as arthritis¹ and atherosclerosis²⁻⁴. Unfortunately, the time-consuming generation of transgenic or knockout mice and the subsequent backcrossing to an appropriate disease background for analysis of gene function considerably delays progression in gene function research. In this study, we demonstrate the high potential of a novel lentiviral gene silencing approach on bone marrow cells for the *in situ* generation of leukocyte knockdowns of the aimed target gene. Targeted gene silencing by means of shRNA has, since its discovery, proven to be a powerful tool in the analysis of gene function *in vitro* as well as *in vivo* as it can result in prolonged selective inhibition of target gene expression even in eukaryotic cells²¹⁻²⁵. To deliver shRNA and warrant prolonged shRNA expression, we made use of 3rd generation self-inactivating lentiviruses^{12,13}, which have been demonstrated to efficiently

infect and integrate their genome into a range of mitotic and non-mitotic cells^{14,15,32}. Although rather efficient retroviral transduction of stem cells of up to 64% has been reported⁸⁻¹¹, this required extensive manipulation of bone marrow stem cells to increase their mitotic capacity, possibly compromising the hematopoietic potential⁴⁰. Efficient lentiviral infection requires only a short *in vitro* incubation period and preventing undesired differentiation of the hematopoietic stem cells.

In this study, the transduction of bone marrow cells with GFP lentivirus was almost quantitative (80-90%) when using titers of 10-20 m.o.i. Transduction levels in our study exceeded reported transduction efficiencies for human bone marrow derived CD34⁺ stem cells⁴¹⁻⁴³ and even were sufficiently high to perform bone marrow transplantation without prior selection of infected cells. Generally, two or more consecutive transduction steps were required to achieve similarly high levels, although e.g. Banerjee *et al.* communicated an efficient gene transfer in a single step protocol⁴⁴. Our protocol is a compromise of a limited culture period of bone marrow cells in growth factor-free medium (24 hours) on the one hand, preventing undesired differentiation or maturation of the bone marrow cells, and lowest possible virus titer on the other. Furthermore, a single transduction protocol will also minimize the risk of multiple, potentially harmful, viral integrants per cell due to, amongst others, insertional mutagenesis⁴⁵. However, it should be noted that in the case of shRNAs, multiple integrations per host cell may even be beneficial as multiple shRNA integrations will be accompanied by higher transcript expression leading to more efficient silencing of the target gene. In our study, the initial infection levels of the bone marrow cells by the H1.Empty and the H1.shCCR2 lentivirus were shown to be similar to that of GFP lentivirus transduced cells.

As model gene to illustrate the potential of lentiviral gene silencing *in vivo*, we have chosen the CC-Chemokine Receptor 2 (CCR2). CCR2 is critically involved in monocyte recruitment to sites of inflammation in response to an inflammatory stimulus⁴⁶ during atherosclerosis, renal fibrosis⁴⁷ or pulmonary inflammation.⁴⁸ A shRNA sequence, targeting nucleotides 867 to 885 of the murine CCR2 gene, reduced CCR2 mRNA levels by 94% and protein levels in HEK 293 cells to an almost similar extent. Furthermore, the shCCR2 construct completely prevented CCR2 signal transduction in response to its natural ligand JE, as revealed by intracellular calcium release measurements.

Interestingly, transplantation of bone marrow cells, transduced with a lentivirus carrying the shCCR2 construct strongly attenuated thioglycollate elicited CCR2 dependent migration of macrophages into the peritoneal cavity. The amount of recruited peritoneal macrophages in mice transplanted with H1.shCCR2 lentivirus transduced bone marrow was comparable to that of CCR2^{-/-} bone marrow transplanted mice. Macrophage recruitment levels of the H1.Empty and H1.shCCR2 lentivirus group were essentially similar to values described by Kurihara *et al.*⁴⁶ for wild-type and CCR2^{-/-} mice, respectively, indicating that local macrophage recruitment and proliferation

was not affected by lentiviral transduction of the bone marrow per se. Peritoneal macrophages derived from the H1.shCCR2 transduced bone marrow cells displayed a 70% reduction in CCR2 mRNA content compared to the H1.Empty group, which apparently translated in the observed CCR2 knockout phenotype. In the CCR2^{-/-} bone marrow transplanted mice, only approximately 7% of circulating blood cells originated from the recipient mice, which accounted for the residual CCR2 mRNA expression in blood cells.

The bone marrow transplantation led to a quantitative and persistent repopulation of donor bone marrow as determined by genomic DNA analysis of the Y-chromosomal SRY gene. Moreover, insertion of the target gene apparently did not result in a shifted differentiation pattern of hematopoietic stem cells. At the end-point of the study, viral copy number in the bone marrow from recipient mice was essentially similar to that of the initial transduction level, implying that the lentivirus-infected cells are not outcompeted by residual or non-transduced cells and that the expansive capacity of bone marrow cells is not affected by lentiviral transduction. We realize that these data were all acquired at seven weeks after transplantation and that more long-term studies are required on the effects and persistence of lentivirus transduced bone marrow, however we are confident that the results will not be very different.

In line with the *in vitro* data, the obtained reduction in CCR2 expression *in vivo* was sufficient for a complete and specific knockdown of CCR2 function. Importantly, IFN β and IFN γ mRNA levels did not differ, indicating that the shRNA lentivirus-transduced bone marrow did not elicit an inflammatory response after transplantation⁴⁹. While CCR2 silencing by shCCR2 was not accompanied by an altered expression pattern of other chemokine receptors, the peritoneal macrophages isolated from CCR2^{-/-} transplanted mice displayed a significant 50% reduction in CCR5 mRNA levels, pointing toward transcriptional modulation of the flanking CCR5 gene by insertion of the PGK-*neo* cassette into the CCR2 gene on mouse chromosome 9. Downregulation of genes downstream of the site of PGK-*neo* cassette insertion has been reported previously^{50,51} and can be circumvented when using the shRNA approach.

In conclusion, here we show effective delivery of lentiviral shRNA to bone marrow cells and subsequent transplantation into irradiated recipient mice as a strategy to generate hematopoietic knockdowns with silenced CCR2 expression. ShRNA mediated silencing of gene expression persisted for at least seven weeks after transplantation and led to a complete loss of CCR2 function without any noticeable side effects. The speed and efficiency renders this strategy very valuable for addressing the role of other hematopoietic, and in particular leukocyte, genes in inflammatory disorders.

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