HHS Public Access

Author manuscript

Am J Transplant. Author manuscript; available in PMC 2020 March 01.

Published in final edited form as:

Am J Transplant. 2019 March ; 19(3): 633–645. doi:10.1111/ajt.15072.

C5aR1 regulates migration of suppressive myeloid cells required for costimulatory blockade-induced murine allograft survival

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Abstract

Costimulatory blockade-induced murine cardiac allograft survival requires intragraft accumulation of $CD11b^{+}Ly6C^{lo}Ly6G^{-}$ regulatory myeloid cells (M_{REG}) that expand regulatory T cells (T_{REG}) and suppress effector T cells (T_{EFF}). We previously showed that C5aR1 signaling on T cells activates T_{EFF} and inhibits T_{REG}, but whether/how C5aR1 impacts M_{REG} required for transplant survival is unknown. Whereas BALB/c hearts survived >60 days in anti-CD154-(MR1)-treated or CTLA4-Ig-treated wild type (WT) recipients, they were rejected at ~30 days in MR1-treated or CTLA4-Ig-treated recipients selectively-deficient in C5aR1 restricted to myeloid cells (*C5ar1*$^{fl/fl}$*xLysM-Cre*). This accelerated rejection was associated with ~2-fold more donor-reactive T cells and ~40% less expansion of donor-reactive T_{REG}. Analysis of graft-infiltrating mononuclear cells on post-transplant day 6 revealed fewer $Ly6C^{lo}$ monocytes in *C5ar1*$^{fl/fl}$*xLysM-Cre* recipients. Expression profiling of intragraft $Ly6C^{lo}$ monocytes showed C5aR1 deficiency downregulated genes related to migration/locomotion without changes in genes associated with suppressive function. Co-transfer of *C5ar1*$^{fl/fl}$ and *C5ar1*$^{fl/fl}$*xLysM-Cre* myeloid cells into MR1-treated allograft recipients resulted in less accumulation of *C5ar1*$^{-/-}$ cells within the allografts and i*n vitro* assays confirmed that $Ly6C^{hi}$ myeloid cells migrate to C5a/C5aR1-initiated signals. Together our results newly link myeloid cell-expressed C5aR1 to intragraft accumulation of myeloid cells required for prolongation of heart transplant survival induced by costimulatory blockade.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

INTRODUCTION

Blocking CD40/CD154 and/or CD28/CD80/CD86 interactions promotes murine allograft tolerance (1–4). It prolongs transplant survival, and at the same time allows reduction of immunosuppressant dosing in nonhuman primates (5) and human transplant recipients (6–9).

The pro-tolerogenic immune mechanisms initiated by costimulatory blockade are incompletely understood but experimental evidence supports induction and maintenance of donor-reactive regulatory T cells (T_{REG}) as crucial (3, 10, 11). Studies published since 2008 have additionally implicated a subset of regulatory myeloid cells (M_{REG}) as important contributors to costimulatory blockade-induced transplant survival (2, 4, 12). Myeloid cells capable of suppressing T cell immunity, sometimes referred to as myeloid derived suppressor cells (MDSC), were initially observed in tumor systems (13) and were shown to inhibit anti-tumor T cell immunity. Tumor-associated MDSC produce inducible nitric acid synthase, L-arginase, and IL-10 (among other molecules), can directly inhibit effector T cells (T_{EFF}), and importantly facilitate proliferation and accumulation of T_{REG} at the tumor site (14). In transplantation, M_{REG} were first observed in a rat model of kidney allograft tolerance following costimulatory blockade with anti-CD28 (15). In 2010, the Ochando lab demonstrated that $CD11b^+CD115^+Gr1^+$ myeloid cells accumulate in heart allografts of MR1-treated recipients and that these M_{REG} are required for MR1-induced long-term allograft survival (2). In further studies the Ochando group showed that the M_{REG} derives from a $CD11b^+Ly6C^{hi}$ bone marrow precursor that undergoes CSF1-dependent differentiation into a $CD11b^+Ly6C^{lo}Ly6G^-$ subset within the allograft of MR1-treated recipients (4). Functionally, the $Ly6C^{lo}$ M_{REG} require surface expression of DC-SIGN, directly inhibit T_{EFF} (in part by producing IL-10) and facilitate proliferation/expansion of protective T_{REG} (4).

The complement system has been traditionally considered a component of innate immunity. Our cumulative work since 2005 has delineated unanticipated roles for complement, including autocrine C5a/C5aR1 ligations in T cells and dendritic cells (DCs), as crucial signals that activate $CD4^+$ T_{EFF} and inhibit generation, function and stability of T_{REG}, together augmenting T cell immunity (16–25). Absence/blockade of these signals inhibits $CD4^+$ T_{EFF} and enhances generation, function and stability of T_{REG}, favoring immune tolerance. These concepts apply to T cells responding to model antigens, autoantigens, infectious pathogens and transplant antigens (18–20, 22, 23, 26–28). In contrast to the above-noted effects of autocrine C5aR1 signaling as a direct modulator of T cell immunity one 2008 study using a murine tumor system showed that pharmacological C5ar1 blockade enhanced tumor-reactive $CD8^+$ T cell responses and prevented tumor progression (29). Experiments in that system suggested that the dominant mechanism involved inhibition of MDSC function/accumulation which indirectly unleashed protective, tumor-reactive T cell immunity. Direct evidence that C5aR1 impacts M_{REG} is lacking, and whether/how analogous mechanisms apply to M_{REG} in transplantation has not been previously addressed.

Herein, we generated mice in which C5aR1 is conditionally deleted from myeloid cells (with T cell C5aR1 remaining intact). We used the animals to test the impact of disabled myeloid cell C5aR1 signaling on costimulatory blockade-induced allograft survival and to

delineate the mechanisms. Our findings demonstrate that myeloid cell C5aR1 is required for costimulatory blockade-induced cardiac allograft survival and newly link C5aR1 expression to M_{REG} accumulation within the allograft, together altering current thinking about how complement impacts alloimmunity and transplant outcomes.

MATERIALS AND METHODS

Mice

C57BL/6 (B6, *H-2*^{b}) and BALB/c (*H-2*^{d}) mice 8 weeks of age were purchased from Jackson Laboratory (Bar Harbor, ME) or bred from Jackson-derived animals at Mount Sinai. B6 *C5ar1*$^{fl/fl}$ mice were generated from ES cells with loxp sites surrounding exon 2 of the C5ar1 gene obtained from the EUCOMM consortium (Figure 1A). Offspring were initially crossed to *B6*$^{flp/flp}$ mice to delete the FRT sites surrounding the lacZ and neo genes required for selection following homologous recombination, and then crossed offspring to B6 *LysM-Cre* mice or a B6 *S100A8-Cre* (Jackson Laboratory). T cell receptor transgenic (Tg) TEa mice [CD4^{+} reactive to *I-A*^{b} + *I-E*$\alpha_{52–58}$] were obtained as a gift from A. Rudensky (Memorial Sloan Kettering Cancer Center, NY, nY) and bred at Mount Sinai. All animals were housed in the Center for Comparative Medicine and Surgery at the Icahn School of Medicine at Mount Sinai under Institutional Animal Care in accordance with guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (IACUC). Experiments were performed with age- and sex- matched mice and using animals that were littermates or were maintained in the same room and/or were co-housed within the same cages for >2 weeks to limit potential effects of microbiome differences.

Heterotopic Heart transplantation

Heterotopic Heart transplantation was performed as previously described (4, 19, 30, 31). In some experiments recipients were treated with either 250μg anti-CD40L mAb (clone MR1, BioXcell) i.v. on the day prior surgery or 250μg CTLA4-Ig (abatacept) on day +1. Rejection was defined as the day on which a palpable heartbeat was no longer detectable and was confirmed by histological examination.

Antibodies

Fluorochrome-conjugated monoclonal antibodies (mAbs) specific to mouse, CD11b (M1/70), CD11c (N418), CD45 (30-F11), CD8 (53–6.7), CD4 (GK1.5), Foxp3 (JFK16s), Ly6C (HK1.4), Vα2TCR (B20.1), IFNγ (XMG1.2) and isotype controls were purchased from eBioscience. Fluorochrome-conjugated anti-Ly6G (1A8) mAb and CD115 (CSF1-R; AFS98) were purchased from BioLegend. Two different antibodies were used for C5aR staining: anti-mouse CD88 (20/70, BioLegend and P12/1, Serotec). Fixed Viability Dye eFluor450 or eFluor780 (eBioscience) and PE-Annexin V (BD Biosciences) were used for dead/apoptotic cell analysis.

Cell staining and flow cytometry

Cell washes and monoclonal antibody (mAb) dilutions were performed in phosphate buffered saline (PBS) plus 1% fetal bovine serum at 4°C. For Foxp3 detection, cells were first stained for CD4, permeabilized, and stained for intracellular Foxp3 using the Fixation-

and-Permeabilization buffers FoxP3 Kit (eBioscience). Fc receptors were blocked with purified anti-CD16/CD32 (Fc Shield; 2.4G2; TONBO Biosciences).Flow cytometric analysis was performed on FACS CANTO II (BD Biosciences) and analyzed using Cytobank (Cytobank, Santa Clara, CA) or FlowJo software (Tree Star, Inc.).

Isolation and purification of graft infiltrating myeloid cells

Cell suspensions of mouse hearts allografts were prepared as published (19). Cell suspensions were stained with antibodies to CD45, CD11b, Ly6G and Ly6C. Cell sorting was performed at the Flow cytometry core facility at Mount Sinai. To assess suppressive function purified, 5×10^5 $CD11b^+Ly6C^{lo}$ myeloid cells obtained from graft infiltrating lymphocytes were mixed with purified, CFSE-labeled syngeneic T cells (2×10^5 T cells) purified to >95% by cell sorting (Sony SH800Z) obtained from naïve mice were co-cultures with anti-CD3 (1μg/mL, clone 145–2C11; Thermo Fisher Scientific) and 5×10^5 APCs in complete RPMI medium for 4 days. For splenic DC isolation, single-cell suspensions of BALB/c (H-2d) splenocytes were incubated with CD11c MicroBeads (Milteny Biotec), following manufacturer's protocol. T cell proliferation/suppression was measured by flow cytometric analysis of CFSE dilution gated on the $CD4^+$ T cells.

Analysis of donor-reactive IFNγ-producing $CD8^+$ T cells

Recipient spleen cells were stimulated with donor-derived spleen cells overnight, incubated Golgi Plug (BD) for the last 4h of the culture. The cells were then stained for surface CD8, fixed, permeabilized, and stained with fluorochrome-conjugated anti-IFNγ, and analyzed by flow cytometry. In some experiments IFNγ-producing spleen cells were quantified by cytokine ELISPOT assays as previously described (4, 19, 30, 31) and analyzed on ImmunoSpot Series 4 analyzer (CTL, Shaker Heights, OH).

In vivo T_{REG} expansion assays

CD4+ T cells were isolated (>97% purity) from naïve B6 TEa mice by negative selection (Easy Sep Mouse CD4+ T cell Isolation Kit, StemCell Technologies). Aliquots were stained for Foxp3 expression. 20×10^6 TEa cells were then injected i.v. into MR1-treated B6 recipients on the day prior to transplantation with a BALB/c heart. Spleen cells were harvested one week later and analyzed for Foxp3 expression within the $CD4^+V\alpha2^+TCR^+$ gate.

Myeloid cell transfers

Bone marrow monocytes were obtained from *C5ar1*$^{fl/fl}$ and *C5ar1*$^{fl/fl}$*xLysM-Cre*$^+$ mice as published (2, 4) and $CD45^+CD11b^+Ly6C^{hi}$ monocytes were purified to >95% by cell sorting (Sony SH800Z). The monocytes from each strain were differentially labeled with either 5μM CFSE or 2×10^{-6}M PKH26 (Sigma) and 1×10^6 cells from each labeled preparation were co-injected i.v. into MR1-treated *C5ar1*$^{fl/fl}$ recipients of BALB/c hearts 24h post-transplantation. Three days later the mice were sacrificed and graft infiltrating cells were analyzed by flow cytometry.

Microarrays and analysis

Graft infiltrating $CD45^{+}CD11b^{+}Ly6C^{lo}Ly6G^{-}$ cells were isolated by flow sorting (>95% purity) from MR1-treated *C5aR1*$^{fl/fl}$ and *C5aR1*$^{fl/fl}$*xLysM-cre* recipients of BALB/c hearts at day 6 post-transplantation and immediately placed in TRizol (Ambion, Life Technologies). Total RNA was isolated (>150 pg RNA per sample), and the samples were processed using standard WT Pico protocols and hybridized to Mouse Gene 2.0 ST Arrays. Chips were scanned using a GeneChip Scanner 7G (Affymetrix) at the State University of New York Albany Center for Functional Genomics. Analysis was performed at Mount Sinai. The intensity data at the probe set level were extracted and normalized with the RMA algorithm (32) and data quality was assessed using Expression Console (Affymetrix). The Affymetrix control probe sets as well as the probe sets with low intensity across all samples were excluded from downstream analysis. The limma test (33) was performed on normalized data between comparison groups, and the differentially expressed genes with $p<0.05$ were identified and visualized using a heat map. Gene ontology enrichment analysis (34, 35) using a Fisher exact test was performed on differentially expressed genes to investigate their associated biological functions or pathways. Iterative analyses were performed on subsets of genes determined by fold-change thresholds ranging from 1.5 to 2.5 to assess the robustness to noise (unspecific genes) and number of genes in the signal. Stable enrichment signals were only detected for the downregulated subset with a strongest signal-to-noise ratio found at a threshold of 2 (70 genes).

Serum donor-reactive alloantibody

Donor reactive alloantibodies were detected as published (36). Briefly, serum samples from recipient mice were diluted in PBS as indicated and incubated for 30 minutes at 4ºC with syngeneic, donor or third-party thymocytes as target cells. Following a wash step with PBS 1% albumin, the bound antibody was detected by incubation with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IgG (eBioscience) and quantified by flow cytometry.

Real time quantitative polymerase chain reaction (qRT-PCR)

RNA isolation, cDNA synthesis, RT, and real-time RT–polymerase chain reaction (PCR) were performed as described previously (37). PCR products were normalized to the 18S control gene and expressed as fold increase over the mean value of the control group using the DDCt method.

Cell migration assays

Enriched murine bone marrow derived myeloid cells from WT and *C5ar1*$^{-/-}$ mice were isolated by negative selection (Easy Sep Mouse Monocyte Isolation Kit, StemCell Technologies) and purity assessed by flow cytometry. The isolated monocytes were stimulated overnight in serum free HL1 medium (Lonza) with recombinant mouse C5a (100ng/mL; 5×10^{5} cells/well; R&D Systems) or recombinant murine CXCL9 (1μg/mL; R&D Systems) and CCL2 (2ng/mL; BioLegend) were used as a positive control. A quantitative determination of cell migration was tested using a CytoSelect™ 96-Well Cell Migration Assay Kit (5μm, Fluorometric format; Cell Biolabs) following manufacturer's

instructions. Fluorescence measurement was performed on SpectraMax M3 Fluorometer (Molecular Devices) with 485/538 nm filter set and 530 nm cutoff.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software version 5 (La Jolla, CA). Differences between graft survival rates were assessed by Mantel-Cox log rank test. Group comparisons of paired data were analyzed by paired t tests. One-way repeated measures ANOVA was used for multiple comparisons among treatment groups, with pairwise multiple comparison procedures performed using Bonferroni post hoc tests. All experiments were repeated at least twice. Data are presented as mean values with SD. Statistical significance is expressed as follows: *P < 0.05, NS not significant.

RESULTS

Monocyte-expressed C5aR1 is required for prolonged allograft survival induced by costimulatory blockade

In previous work we identified a subset of regulatory monocyte-derived macrophages that a) develop within the grafts of MR1-treated heart transplant recipients, b) are required for murine heart transplant tolerance (2, 4), and c) are phenotypically and functionally similar to myeloid derived suppressor cells that spontaneously develop in response to tumors. Building upon these findings that blocking C5aR1 limits myeloid derived suppressor cell function in tumor models and causes tumor rejection (29), we tested whether C5aR1 is required for M_{REG}-dependent allograft survival. We generated *C5ar1*$^{fl/fl}$ mice from EUCOMM embryonic stem cells and crossed them with animals expressing the Cre recombinase (Cre) under a LysM promoter (expresses in myeloid cells, Figure 1A). Comparative flow cytometric phenotyping of spleen cells showed similar frequencies of immune cell subsets between naïve *C5ar1*$^{fl/fl}$ and *C5ar1*$^{fl/fl}$x*LysM-cre* mice (Figure S1). *In vitro* cultures using spleen cells from naïve animals stimulated overnight with allogeneic BALB/c APCs showed no differences in baseline frequencies of BALB/c-reactive IFNγ-producing $CD4^+$ or $CD8^+$ T cells (Figure S2). We did not detect C5aR1 on splenic monocytes or neutrophils of the *C5ar1*$^{fl/fl}$x*LysM-cre* mice (Figure 1B) but C5aR1 was detected on splenic T cells, B cells and dendritic cells at levels comparable to those of *C5ar1*$^{fl/fl}$ controls (Figure 1C), documenting specific deletion of C5aR1 from myeloid cells in the *C5ar1*$^{fl/fl}$x*LysM-cre* mice.

We transplanted groups of MR1-treated *C5ar1*$^{fl/fl}$ mice and *C5ar1*$^{fl/fl}$x*LysM-cre* mice with fully MHC-disparate BALB/c hearts. As anticipated, allografts transplanted into MR1-treated *C5ar1*$^{fl/fl}$ recipients survived significantly longer than those transplanted into untreated *C5ar1*$^{fl/fl}$ recipients [median survival time (MST) of 60 vs 8 days, (Figure 1D). Allografts transplanted into MR1-treated *C5ar1*$^{fl/fl}$x*LysM-cre* recipients (in which C5aR1 is disabled in myeloid cells) survived for only 30 days ($p<0.05$ vs. MR1-treated *C5ar1*$^{fl/fl}$ recipients). Histological examination of the rejected allografts in all MR1-treated recipients confirmed mononuclear cell infiltration consistent with cellular rejection in all cases (data not shown). Untreated *C5ar1*$^{fl/fl}$ and *C5ar1*$^{fl/fl}$x*LysM-cre* recipients each rejected BALB/c hearts with a MST of 7 days (Figure 1D, p=ns). When we transplanted groups of *C5ar1*$^{fl/fl}$ and *C5ar1*$^{fl/fl}$x*LysM-cre* recipients with BALB/c hearts and treated the recipients with

CTLA4-Ig (abatacept) we also observed accelerated rejection in the *C5ar1*$^{fl/fl}$x*LysM-cre* recipients (Figure 1E), demonstrating that the effects of C5aR1-deficiency on myeloid cells apply to a distinct (and clinically relevant) costimulatory blockade strategy.

The *C5ar1*$^{fl/fl}$x*LysM-cre* mice lack C5aR1 in all myeloid cells, including neutrophils. We performed additional heart graft survival experiments in MR1-treated, *C5ar1*$^{fl/fl}$x*S100A8-cre* recipients (Figure 1D) that lack C5aR1 solely in neutrophils but otherwise are phenotypically similar to *C5ar1*$^{fl/fl}$ controls (Figures S2–S4) and functionally, have baseline frequencies of alloreactive T cells that are not different from the *C5ar1*$^{fl/fl}$ controls (Figure S2). These studies remarkably showed that the MST of heart allografts transplanted into MR1-treated *C5ar1*$^{fl/fl}$x*S100A8-cre* recipients (with C5aR1 deficiency restricted to neutrophils) did not differ from the MST of hearts transplanted into MR1-treated *C5ar1*$^{fl/fl}$ controls (and was significantly longer than the MST of grafts transplanted into MR1-treated *C5ar1*$^{fl/fl}$x*LysM-cre* mice). Together the findings support the conclusion that monocyte expression of C5aR1 is required for graft prolongation in this system.

Absence of myeloid cell C5aR1 augments donor-reactive T cell immune responses despite costimulatory blockade

To assess the impact of absent myeloid cell C5aR1 on the donor-reactive T cell response, we transplanted BALB/c hearts into groups of MR1-treated *C5ar1*$^{fl/fl}$ and *C5ar1*$^{fl/fl}$x*LysM-cre* recipients and quantified donor-reactive IFNγ-producing cells in the recipient spleens by ELISPOT (Figure 2A, day 14, all allografts beating). These assays showed ~2-fold higher frequencies of donor-reactive IFNγ-producers in the *C5ar1*$^{fl/fl}$x*LysM-cre* recipients vs. the *C5ar1*$^{fl/fl}$ controls (~250 vs 100 per 5×10^5 splenocytes, $p<0.05$).

To document that the above detected IFNγ derived from recipient T cells we performed mixed lymphocyte responses (MLR) in which recipient spleen cells from day 14 post-transplant recipients were stimulated with BALB/c APCs and analyzed for intracellular IFNγ production within $CD8^+$ T cells by flow cytometry (Figure 2B–C). These assays confirmed higher frequencies of splenic donor-reactive IFNγ-producing $CD8^+$ T cells in the MR1-treated *C5ar1*$^{fl/fl}$x*LysM-cre* recipients. Frequencies of donor-reactive IFNγ-producing $CD4^+$ T cells were low (~1%) and did not differ between groups at the 14 day post-transplant time-point tested (n=7/group, 2 different experiments, data not shown). We did detect donor-reactive alloantibodies on day 14 post-transplant in the sera MR1-treated *C5ar1*$^{fl/fl}$x*LysM-cre* recipients (titer >1:250) but not in sera from MR1-treated *C5ar1*$^{fl/fl}$ control recipients (Figure 2D).

Absence of myeloid cell C5aR1 limits T_{REG} expansion

In our published work we previously showed that allograft-derived myeloid cells in MR1-treated recipients mediate their suppressive function by driving expansion of donor-reactive T_{REG} (4). When we quantified frequencies and total numbers of splenic $CD4^+Foxp3^+T_{REG}$ in the recipients on day 14 post-transplant we observed ~50% higher frequencies of T_{REG} in *C5ar1*$^{fl/fl}$x*LysM-cre* recipients (Figure 2E). However, calculated ratios of absolute numbers of T_{REG}:donor reactive IFNγ-producing $CD8^+$ effector T cells in each animal trended lower in the *C5ar1*$^{fl/fl}$x*LysM-cre* recipients (Figure 2F).

To test whether the absence of myeloid cell expressed C5aR1 inhibits induction/expansion of donor-reactive T_{REG} *in vivo*, we used TEa TCR Vα2$^+$ transgenic CD4$^+$ cells specific for *I-A^b*+ the donor-derived peptide, I-E^{d}α$_{52–68}$ (3). We isolated splenic CD4$^+$ T cells from TEa mice (~0.9% expressed Foxp3, Figure 3A), adoptively transferred them into *C5ar1$^{fl/fl}$* and *C5ar1$^{fl/fl}$*x*LysM-cre* hosts, treated the recipients with MR1, and 24 h later transplanted them with BALB/c hearts. On day 7 post-transplant, we quantified the percentage and total numbers of splenic CD4$^+$Vα2$^+$ TEa TCR$^+$ FoxP3$^+$ T cells in the recipient spleens (Figure 3B–D). These analyses showed significantly fewer Foxp3$^+$ TEa cells in *C5ar1$^{fl/fl}$*x*LysM-cre* recipients (percentage and absolute numbers), indicating that C5aR1 expression on myeloid cells facilitates T_{REG} expansion/induction in MR1-treated allograft recipients.

Myeloid cell expression of C5aR1 modulates intragraft accumulation of suppressive, Ly6C^{lo} myeloid cells in MR1-treated recipients

We previously showed that MR1 induces intragraft accumulation of Ly6C^{lo}Ly6G$^-$ myeloid cells that are a) derived from Ly6C^{hi} precursors and b) are required for prolonged allograft survival (2, 4). To assess the impact of myeloid cell C5aR1 on intragraft myeloid cell accumulation we analyzed graft-infiltrating myeloid cell subsets from MR1-treated *C5ar1$^{fl/fl}$* and *C5ar1$^{fl/fl}$*x*LysM-cre* graft recipients (day 6) by flow cytometry (Figure 4A, schematic). Gating on live CD45$^+$CD11b$^+$ cells and consistent with previous work (2, 4), we discerned three populations based on differential expression patterns of Ly6C and Ly6G: Ly6C^{hi}Ly6G$^-$, LyC6^{int}Ly6G$^+$ (neutrophils) and Ly6C^{lo}Ly6G$^-$ (contains the suppressive myeloid cells, Figure 4B). Whereas in the *C5ar1$^{fl/fl}$* mice MR1 induced an increase in intragraft accumulation of Ly6C^{lo}Ly6G$^-$ myeloid cells compared to untreated controls, we detected significantly fewer intragraft Ly6C^{lo}Ly6G$^-$ myeloid cells in *C5ar1$^{fl/fl}$*x*LysM-cre* recipients ($p<0.05$, Figure 4B–C). We did not observe differences in the frequencies of Ly6G$^+$ cells between groups (Figure 4B–C). Control analyses showed that whereas all intra-graft myeloid cells from *C5ar1$^{fl/fl}$* recipients expressed C5aR1, no C5aR1 was detectable on any of the intragraft myeloid cell subsets from the *C5ar1$^{fl/fl}$*x*LysM-cre* recipients (Figure 4D–E).

C5aR1 regulates migration of myeloid cells to the allograft

Potential mechanisms to account for less intragraft accumulation of M_{REG} and accelerated rejection in the absence of C5aR1 include defects in myeloid cell migration, defects in intragraft induction/conversion into M_{REG} phenotype, and accelerated myeloid cell death. In an effort to discern these mechanisms (among others), we flow sorted intragraft Ly6C^{lo}Ly6G$^-$ myeloid cells from MR1-treated *C5ar1$^{fl/fl}$*x*LysM-cre* and *C5ar1$^{fl/fl}$* recipients on day 6 post-transplant, isolated RNA and hybridized the RNA to microarrays. Analysis showed >1.5-fold upregulation of 277 genes and downregulation of 412 genes in the graft-infiltrating myeloid cells of the *C5ar1$^{fl/fl}$*x*LysM-cre* recipients (Figure 5A, full dataset available on GEO database). Enrichment analysis of the upregulated genes showed no significant signal (not shown). Enrichment analysis of the downregulated genes implicated gene ontology (GO) term pathways predominantly involved in cell migration, cell motility, locomotion, and localization (Figure 5B). The genes comprising these GO terms include *C5ar1* (a receptor for C5a, a known chemoattractant) as well as multiple genes associated with tumor metastasis [*Cmtm3, Nr4a1, Sparc, Tnf, Gpnb* (38–41)] and with chemotaxis, adhesion and cytoskeletal interactions [*Fermt3, Rhod, Cd151, Sema4a, Plxnd1* (42–50)].

To test for functional evidence of migratory defects in the absence of C5aR1, we flow-sorted CD11b^{+}Ly6C^{hi} cells from bone marrow of *C5ar1$^{fl/fl}$* and *C5ar1$^{fl/fl}$*x*LysM-cre* mice, differentially labeled them with fluorescent dyes CFSE or PKH26 and co-transferred equal numbers into MR1-treated *C5ar1$^{fl/fl}$* recipients of BALB/c hearts (Figure 6A, schematic). We quantified the frequencies of labeled *C5ar1$^{fl/fl}$* vs *C5ar1$^{fl/fl}$xLysM-cre* cells within each graft 3 days later (Figure 6B–C). In 2 separate experiments, these analyses showed significantly fewer *C5ar1$^{fl/fl}$xLysM-cre* vs control *C5ar1$^{fl/fl}$* myeloid cells within the allografts, a result that was independent of the whether the cells were labeled with CFSE or PKH26 (noting that these 2 dyes may differentially result in nonspecific cellular toxicity). *In vitro* cell migration assays (Figure 6D) demonstrated that while WT murine myeloid cells migrated in response to C5a (and CXCL9/CCL2 as a positive control) C5aR1-deficient murine myeloid cells did not migrate to C5a ($p<0.05$ vs control) but did respond to CXCL9/CCL2.

To separately assess whether absence of myeloid cell C5aR1 impacts conversion of Ly6C^{hi} myeloid cells to Ly6C^{lo} suppressive myeloid cells (4), we phenotyped the above, adoptively transferred, CFSE-labeled and PHK26-labeled cells within each allograft based on Ly6C/G expression levels (Figure 6E–F). These analyses showed a predominance of Ly6C^{lo} cells with similar elevated ratios of Ly6C^{lo}:Ly6C^{hi} myeloid cells *C5ar1$^{fl/fl}$* WT and *C5ar1$^{fl/fl}$xLysM-cre* Ly6C^{lo} cells within the graft-infiltrating myeloid cells, indicating conversion is unaffected by C5aR1 expression. Moreover, while neutrophil-produced CSF1 binding to CSF1-receptor (CSF1-R) on Ly6C^{hi} myeloid cells is required for conversion to the Ly6C^{lo} regulatory phenotype (4, 12) we did not detect differences in either CSF1 gene expression (Figure 6G) or CSF1-R surface expression on graft infiltrating mononuclear cells between allografts in control *C5ar1$^{fl/f}$* vs. *C5ar1$^{fl/fl}$*x*LysM-cre* allograft recipients (Figure 6H–I).

In contrast to the above observed downregulated expression of genes related to migration/locomotion in intragraft *C5ar1$^{fl/fl}$xLysM-cre* myeloid cells C5aR1 shown in Figure 5, our gene expression profiling revealed no differences in expression of genes/proteins associated with myeloid cell suppressive function [*Cx3cr1*, *Siglec1* (CD169), CD68, *Sirpa* (CD172), and MHC-II (Figure 7A)] (4). Functionally, intragraft Ly6C^{lo} myeloid cells isolated on day 6 post-transplant from *C5ar1$^{fl/f}$* and from *C5ar1$^{fl/fl}$*x*LysM-cre* allograft recipients were each able to suppress T cell *in vitro* stimulated CD4^{+} T cells (Figure 7B).

When we compared expression levels of genes related to cell death/survival (including *Caps8, Bcl2, Fas, Fasl*), which we previously showed is one mechanism through which C5aR1 impacts T cell immunity (17, 18), the analyses revealed no differences (Figure 7C). Surface staining of graft infiltrating lymphocytes for annexin-V as a measure of apoptotic cells showed no differences between groups (Figure 7D–E).

DISCUSSION

Our data newly demonstrate that C5aR1 expression on recipient myeloid cells is crucial for costimulatory blockade-induced survival of fully MHC-disparate cardiac allografts. In the absence of myeloid cell-expressed C5aR1, CD11b^{+} myeloid cells do not optimally migrate

to the allograft to become M_{REG}, and as a consequence, do not fully promote T_{REG} expansion, together resulting in enhanced $CD8^+$, IFNγ-producing T_{EFF} expansion in the spleen and accelerated graft rejection.

The gene array analyses performed on RNA derived from graft infiltrating mononuclear cells add to the literature by associating numerous genes previously shown to participate in chemotaxis, angiogenesis, adhesion, and cancer metastasis (38–50) with C5aR1. Additional studies to be performed by our laboratory among others are required to test the specific hypothesis that these gene products, individually or together, are requisite intermediaries of C5a/C5aR1-initiated M_{REG} chemotaxis.

The chemotaxis receptor function of myeloid cell-expressed C5aR1 (in response to C5a ligation) in costimulatory blockade-induced allograft survival has not been previously reported, but C5aR1 signaling has been linked to chemotaxis functions (51–53) and several groups have shown that C5a/C5aR1 ligations on tumor-associated MDSC facilitate MDSC accumulation into the tumor, promote angiogenesis and prevent T cell mediated tumor rejection by augmenting T_{REG} and inhibiting T_{EFF} (29, 54–58). The observations that C5aR1 is required for M_{REG} migration/accumulation into tumors and into transplants, and as a consequence to mediate tumor and transplant survival illustrates commonality of mechanism despite distinct pathophysiological contexts. Complement component C3 has been separately shown to be important in the differentiation of MDSC (59, 60), an effect that has not been attributed to C5aR1. While we have not formally documented the source of C5a in this system, we speculate based on our previous work that it derives from recipient plasma and/or recipient immune cell-produced complement (19, 37, 61).

The findings described herein, in which we show that myeloid cell deficiency of C5aR1 augments alloreactive T_{EFF} and limits T_{REG} expansion following costimulatory blockade are distinct from our previous work in which we demonstrated that global absence/blockade of C5a/C5aR1 interactions inhibits T_{EFF}, promotes T_{REG} induction and function, and inhibits T cell-mediated diseases including transplant rejection (17, 18, 21, 22, 25, 26, 62, 63). This apparent paradox can be explained by cell type-specific differences in C5a/C5aR1-initiated cellular responses. While we showed herein that C5aR1 ligation directly stimulates M_{REG} chemotaxis to the allograft (resulting in immunoregulation), C5a/C5aR1 signaling directly on T cells activates PI-3Kγ-dependent AKT phosphorylation which stimulates T cell proliferation and inhibits apoptosis (17, 18), and simultaneously prevents Foxo1-dependent Foxp3 production to limit T_{REG} induction and function (22, 23, 25). C5a/C5aR1 interactions activate DCs, manifested by increased expression of costimulatory molecule and innate cytokine production, which also contribute to T_{EFF} differentiation and expansion while inhibiting T_{REG} (24, 26). Differential effects of complement/C5aR1 signaling on various cell types likely explains why absence of complement components prevents graft tolerance under some experimental conditions (64, 65) yet promotes tolerance to alloantigens in other circumstances (22, 66–70). While we did not quantify IL-17 or IL-4 production by alloreactive T cells in these experiments, our previous work showed that complement activation augments the strength of the effector response (and inhibits the regulatory response) without altering cytokine profiles (17, 18, 21, 22, 25, 26, 62, 63).

Our observed cell-specific effects of C5aR1 signaling on graft outcome have translational implications for human transplant recipients. $CD11b^{+}CD33^{+}HLA\text{-}DR^{-}$ myeloid cells isolated from peripheral blood of kidney transplant recipients have been shown to suppress proliferation of $CD4^{+}$ T cells and to expand T_{REG} *in vitro*, and their time-dependent accumulation in transplant recipients directly correlated with increased peripheral blood T_{REG} frequencies, together consistent with an M_{REG}/MDSC phenotype (71). As human $CD11b^{+}CD33^{+}HLA\text{-}DR^{-}$ myeloid cells also express C5aR1 (Figure S5), it is important to consider the possibility that pharmacological complement inhibition aimed at blocking antibody-initiated effector functions or T cell-dependent allograft injury could have unanticipated adverse effects on M_{REG} and thereby negatively impact, rather than improve, transplant outcomes.

In conclusion, the studies described herein add to our understanding of the role of complement receptor C5aR1 in transplantation by linking myeloid cell-expressed C5aR1 to intragraft accumulation of M_{REG} required for suppressing pathogenic T cells and prolonging transplant survival. Understanding cell-specific effects of C5aR1 and devising approaches to better target the complement inhibitors to desired cell types will be important considerations as clinical complement inhibition moves into the transplant field.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work was supported by NIH grant R01 AI071185 awarded to PSH and MEM. IL is a recipient of a postdoctoral fellowship grant from the American Society of Transplantation. MF is supported by NIH grant U19AI117873. The authors thank Weijia Zhang and Zhengzhi Li (Icahn School of Medicine at Mount Sinai) for assistance with the gene array and the microsurgery core facility (P Boros, Y. Li, J Liu), and the Mouse Genetics Shared Resource facility (K Kelly, Director) at the Icahn School of Medicine at Mount Sinai for their contributions to the studies. Special thanks to N Chun and J Horwitz (Mount Sinai) for their technical assistance with selected experiments.

Abbreviations:

CFSE	Carboxyfluorescein succinimidyl ester
C5aR1	C5a receptor
CSF1	colony stimulating factor 1
CSF1-R	colony stimulatory factor 1 receptor
DC	dendritic cell
IFNγ	interferon gamma
mAb	monoclonal antibody
MDSC	myeloid derived suppressor cells
MHC	major histocompatibility complex

MLR	mixed lymphocyte response
M_{REG}	regulatory myeloid cell
tg	transgenic
MST	median survival time
PBS	phosphate buffered saline
T_{REG}	regulatory T cell
T_{EFF}	effector T cell

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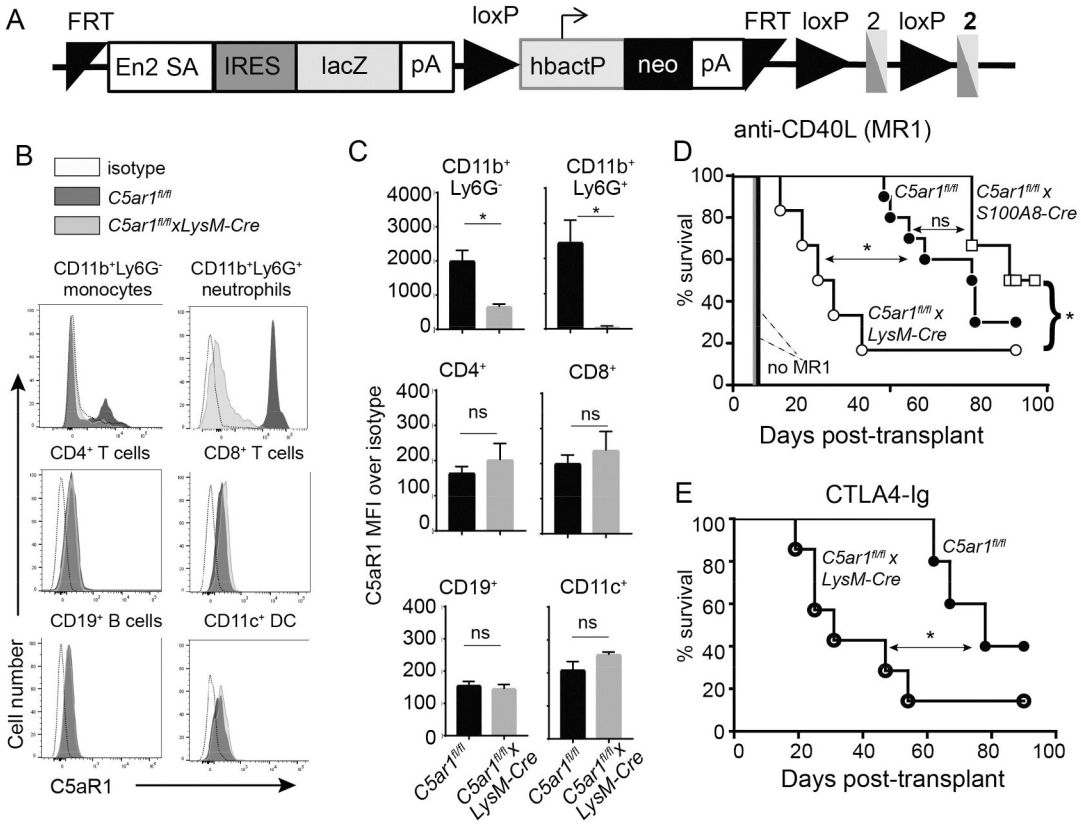

Figure 1.

Myeloid cell deficiency of C5ar1 abrogates costimulatory blockade induced prolonged cardiac allograft survival. A. Schematic representation of the targeting insert to conditionally delete C5ar1. Animals transmitting the insert were crossed with flp/flp mice to remove the genes between the 2 FRT sites. The resultant *C5ar1*$^{fl/fl}$ mice were crossed to a LysM-Cre transgenic to remove a portion of C5ar1 exon 2 from myeloid cells or to an *S100A8*-Cre transgenic to remove a portion of C5ar1 exon 2 from neutrophils. B-C. Representative flow cytometry plots (B) and quantified surface expression (MFI) of C5aR1 (C) on monocytes and neutrophils (top row) CD4$^+$ and CD8$^+$ T cells (middle row), and B cells and DCs (bottom row). D. Survival of BALB/c hearts transplanted into *C5ar1*$^{fl/fl}$, *C5ar*$^{fl/fl}$*xLysM-Cre* or *C5ar*$^{fl/fl}$*xS100A8-Cre* recipients treated with anti-CD40L mAb MR1 250μg on day −1 (n=6–10/group). Survival of BALB/c hearts in untreated *C5ar1*$^{fl/fl}$ (solid black line, no symbol) and *C5ar*$^{fl/fl}$*xLysM-Cre* (solid gray line, no symbol, n=4/group) recipients. E. Survival of BALB/c hearts transplanted into *C5ar1*$^{fl/fl}$ or *C5ar*$^{fl/fl}$*xLysM-Cre* and treated with CTLA4-Ig (n=5–7/group) (E). *p<0.05 by t-test. ns: nonsignificant. *p<0.05, **p<0.05 by survival analysis (Mantel-Cox log rank test). ns=not significant.

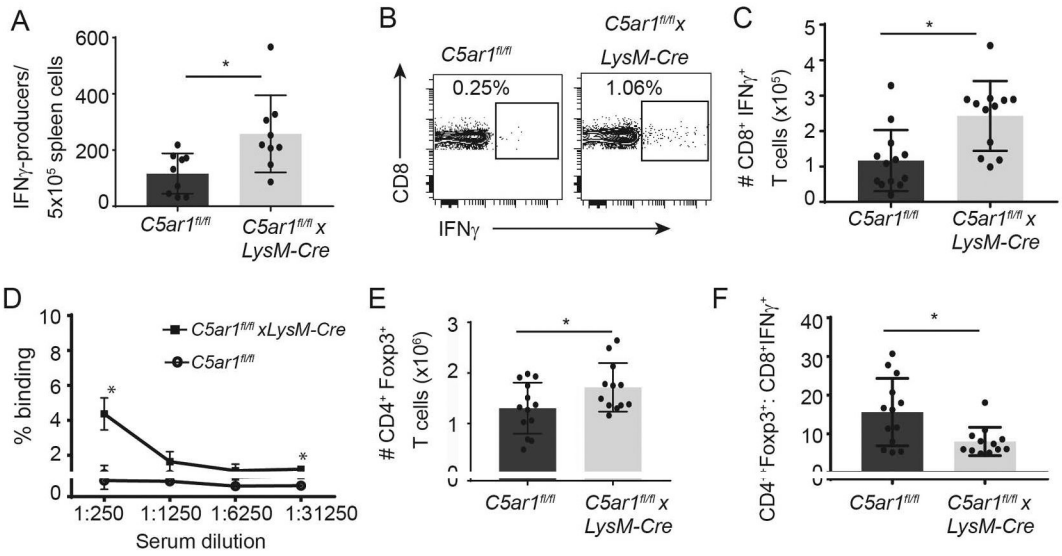

Figure 2.

Absence of myeloid cell expressed C5ar1 augments donor-reactive T cell immune responses in MR1-treated allograft recipients. A. Donor-reactive IFNγ ELISPOTs from *C5ar1*$^{fl/fl}$ and *C5ar1*$^{fl/fl}$*xLysM-Cre* recipients of BALB/c hearts 14 days post-transplant. n=9/group. Frequencies of donor-reactive IFNγ-producers in spleens of untreated *C5ar1*$^{fl/fl}$ and *C5ar1*$^{fl/fl}$*xLysM-Cre* recipients of BALB/c hearts at rejection (day 7) were ~1300/5×10^5 spleen cells and were not different between groups (not shown). B-C. Representative flow cytometry plots (B) and total numbers per spleen of donor-reactive IFNγ-producing CD8$^+$ T cells (C) from *C5ar1*$^{fl/fl}$ and *C5ar1*$^{fl/fl}$*xLysM-Cre* recipients of BALB/c hearts 14 days post-transplant, n=12/group. D. Serum donor-reactive alloantibodies in *C5ar1*$^{fl/fl}$ and *C5ar1*$^{fl/fl}$*xLysM-Cre* recipients of BALB/c hearts 14 days post-transplant, n=3/group E-F. Total numbers of Foxp3$^+$CD4$^+$ T cells per spleen (E) and calculated ratios of CD4$^+$Foxp3$^+$ cells/donor reactive IFNγ-producers per mouse (T_{REG}:T_{EFF} ratios), n=12/group (F). * p<0.05

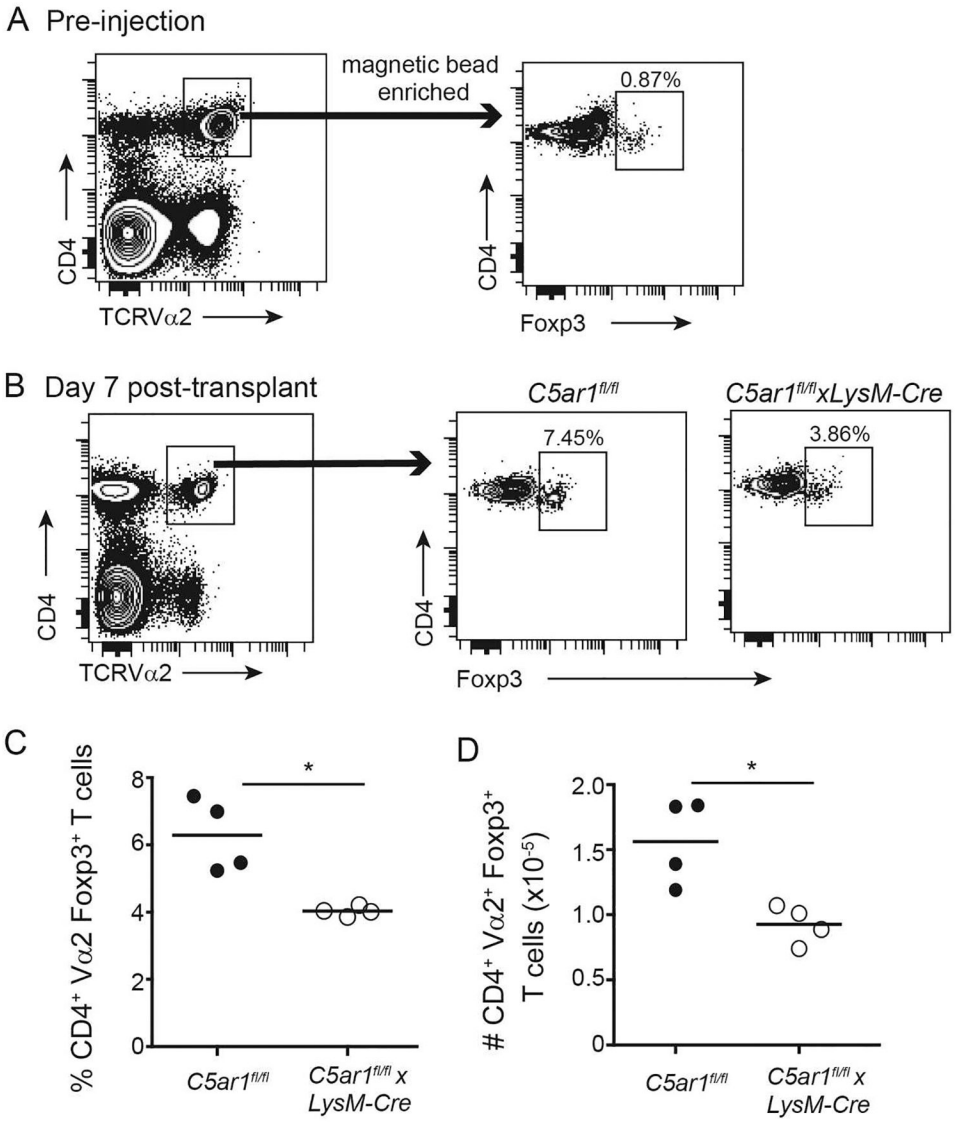

Figure 3.

Absence of myeloid cell-expressed C5ar1 limits in expansion of donor-reactive T_{REG} in MRI-treated allograft recipients. A. Gating strategy for enriching $CD4^+V\alpha2^+$ TEa T cells from spleens of TEa TCR tg mice (left), confirmation of purity of isolated population (right) demonstrating ~0.9% $Foxp3^+CD4^+$ T cells within the sorted TEa^+ population. The purified cells were adoptively transferred into *C5ar1*$^{fl/fl}$ and *C5ar1*$^{fl/fl}$*xLysM-Cre* and treated with MR1 (day 0), 24 h later transplanted with BALB/c hearts. B-D. Gating strategy, representative flow cytometry plots (B) and quantified results showing percentages (C) and total numbers (D) of adoptively transferred TEa cells analyzed 7 days post-transplant demonstrating fewer $Foxp3^+$ TEa cells in the *C5ar1*$^{fl/fl}$*xLysM-Cre* recipients, n=4/group (from 2 separate experiments). $p<0.05$

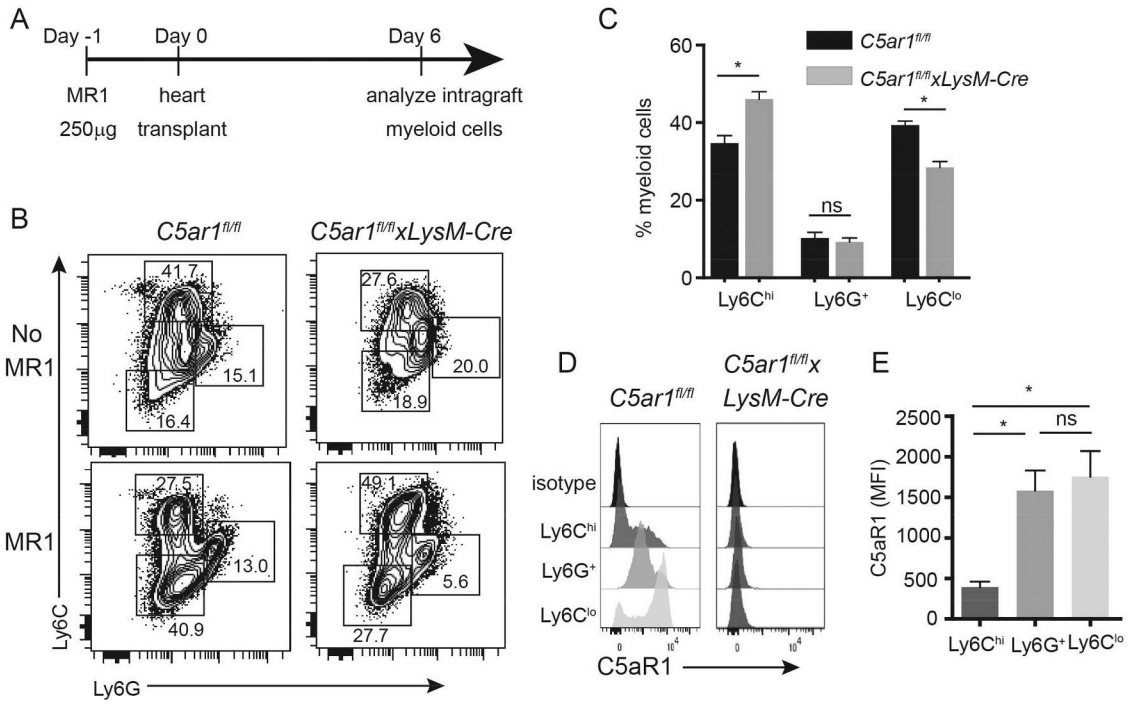

Figure 4.

Myeloid cell-expressed C5ar1 regulates accumulation of Ly6C^{lo} myeloid cells in allografts following recipient treatment with MR1. A. Schematic of experimental design. B. Representative flow cytometry plots of graft-infiltrating anti-Ly6C/Ly6G-stained cells gated on the live CD45^{+}CD11b^{+} subset from untreated or MR1-treated *C5ar1$^{fl/fl}$* and *C5ar1$^{fl/fl}$xLysM-Cre* recipients of BALB/c hearts. C. Quantified results of MR1 treated recipients (n=8/group). D-E. Representative flow cytometry histograms (D) and quantified MFI results (E) showing absence of C5aR1 expression on graft infiltrating CD11b^{+} Ly6G/Ly6C subsets from BALB/c allografts transplanted into MR1-treated *C5ar1$^{fl/fl}$* and *C5ar1$^{fl/fl}$xLysM-Cre* (day 6 post-transplant). *p<0.05, ns=not significant.

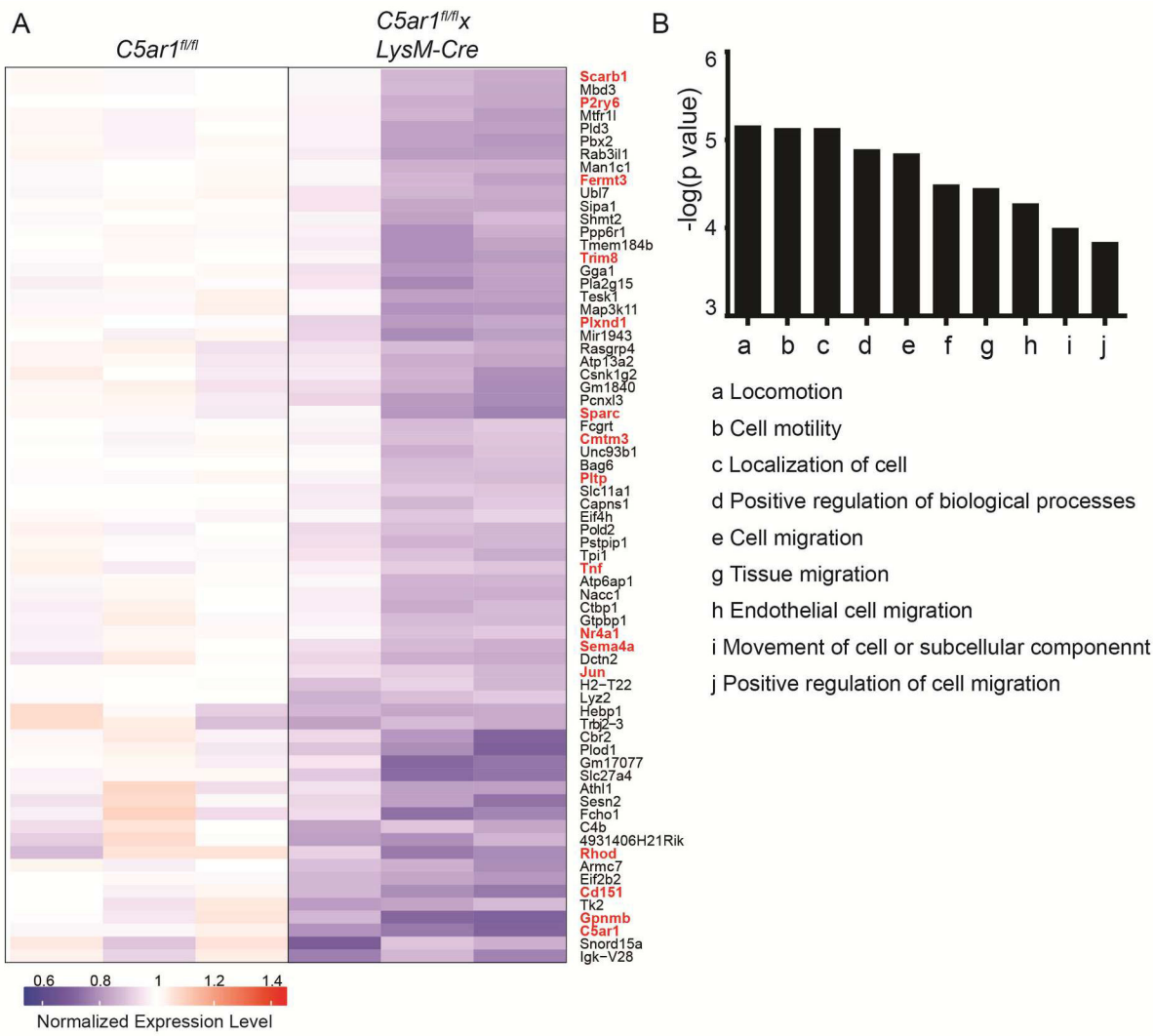

Figure 5.

Microarray analysis indicates absence myeloid cell-expressed C5aR1 specifically reduces the expression of genes involved in cell migration and locomotion. Heatmap (A) and enrichment analysis (B) of downregulated genes in microarrays of RNA obtained from flow-sorted intragraft $CD11b^{+}Ly6C^{lo}Ly6G^{-}$ cells on day 6 after BALB/c hearts were transplanted into MR1-treated *C5ar1*$^{fl/fl}$ (n=3) and *C5ar1*$^{fl/fl}$*xLysM-Cre* (n=4) recipients of BALB/c hearts. The genes that comprise these pathways are depicted in red font in A.

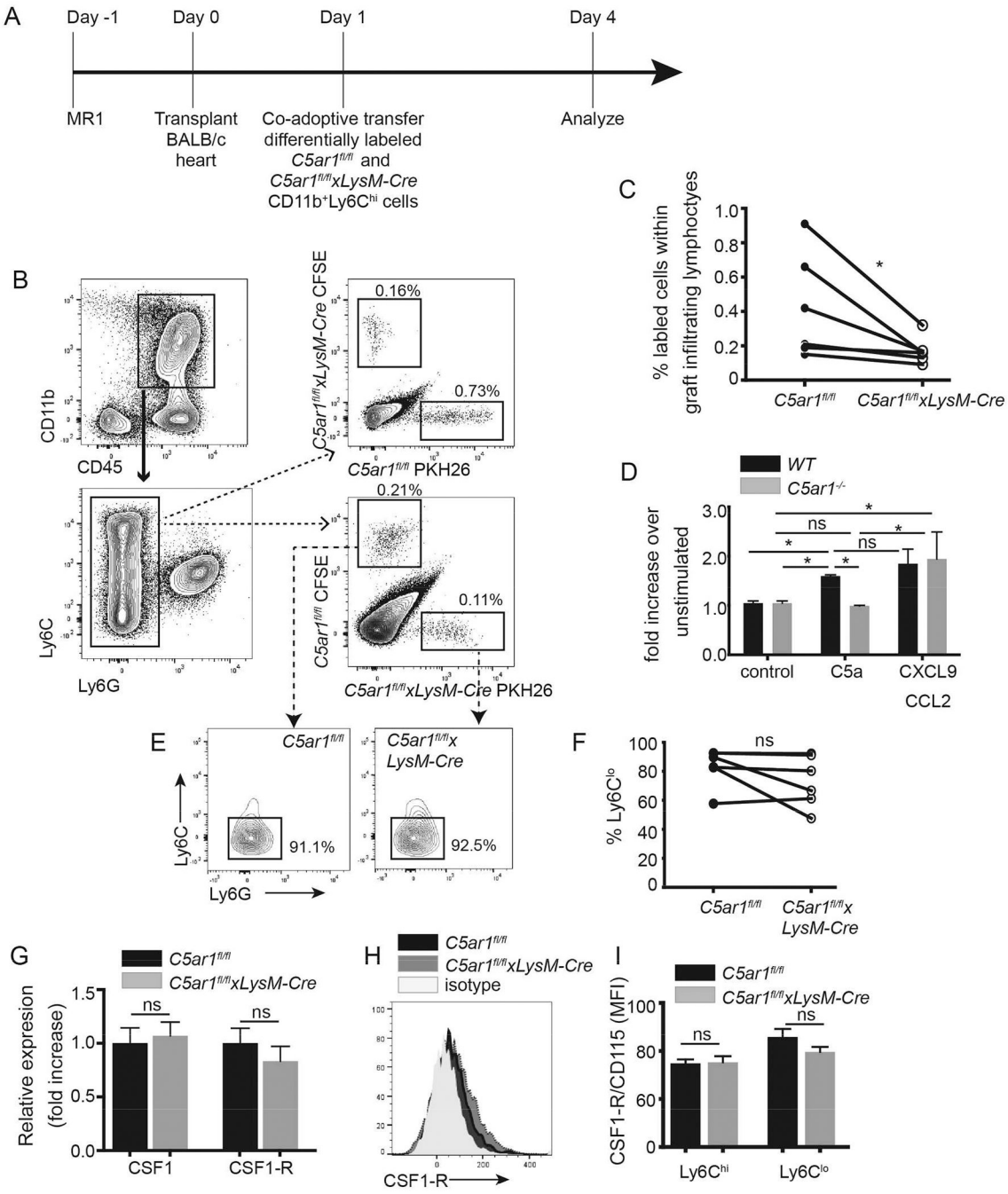

Figure 6.

Myeloid cell-expressed C5ar1 is required for optimal migration of myeloid cells to allografts in MR1-treated recipients. A. Schematic of experimental design (see text for details). B Representative flow cytometry plots showing gating strategy and revealing lower frequencies of intragraft, adoptively transferred *C5ar1$^{fl/fl}$xLysM-Cre* Ly6G^{lo} myeloid cells regardless of label with CFSE or PKH26. C. Quantified frequencies of intragraft myeloid cells in day 4 post-transplant allografts. n=6/group, 2 separate experiments. D. Chemotaxis assays of CD11b^{+}Ly6C^{hi} monocyte WT or *C5ar1$^{-/-}$* mice in response to C5a (100 ng/ml) and CXCL9

(1μg/mL) + CCL2 (2ng/mL). E-F. Representative flow cytometry histograms (E) and quantified results (F) depicting percentages of $Ly6C^{lo}Ly6G^{-}$ myeloid cells within the adoptively transferred intragraft populations. G-I. Quantitative RT-PCR for CSF1 and CSF1-R expression (G), and representative flow plot (H) and quantified surface expression (I) of CSF1-R on intragraft myeloid cells from MR1-treated *C5ar1*$^{fl/fl}$ and *C5ar1*$^{fl/fl}$*xLysM-Cre* recipients of BALB/c hearts. *p<0.05, ns=not significant

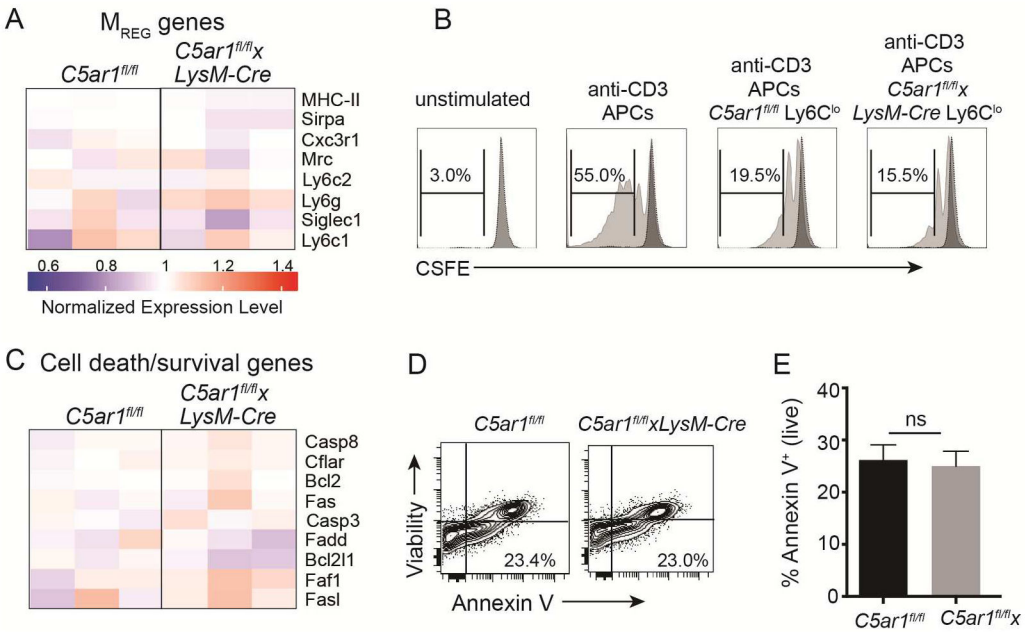

Figure 7.

Graft infiltrating *C5ar1*$^{fl/fl}$ and *C5ar1*$^{fl/fl}$*xLysM-Cre* Ly6C^{lo} myeloid cells exhibit equivalent suppressive capacities and rates of apoptosis. A. Heatmap depicting expression levels for M_{REG}-produced genes involved in suppressive function (see Figure 5 for details, n=3–4/group). B. Representative flow cytometry plots of unstimulated or anti-CD3 stimulated WT T cells ± flow sorted CD11b^{+}Ly6C^{lo}Ly6G^{-} myeloid cells (200,000 T cells, 50,000 CD11c^{+} APCs and 50,000 myeloid cells) isolated from allografts on day 6 of MR1-treated *C5ar1*$^{fl/fl}$ (controls) or *C5ar1*$^{fl/fl}$*xLySM-Cre* recipients, illustrating suppressive ability of myeloid cells from both genotypes. Representative of results from 2 independent experiments. C. Heatmap depicting expression levels genes involved in cell death/survival (see Figure 5 for details, n=3–4/group). D-E. Representative flow cytometry plot (D) and quantified results (E) depicting frequency of apoptotic (AnnexinV^{+}) CD11b+ Ly6C^{lo} graft-infiltrating cells. *p<0.05, ns=not significant.