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DC-SIGN⁺ Macrophages Control the Induction of Transplantation Tolerance

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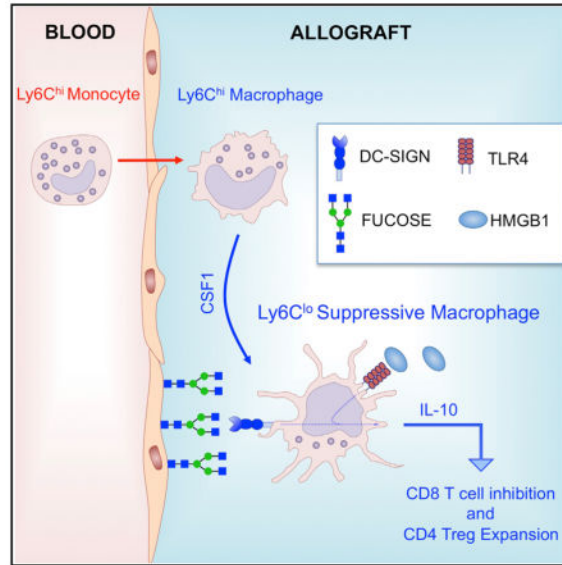
SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2015.05.009>.

SUMMARY

Tissue effector cells of the monocyte lineage can differentiate into different cell types with specific cell function depending on their environment. The phenotype, developmental requirements, and functional mechanisms of immune protective macrophages that mediate the induction of transplantation tolerance remain elusive. Here, we demonstrate that costimulatory blockade favored accumulation of DC-SIGN-expressing macrophages that inhibited CD8⁺ T cell immunity and promoted CD4⁺Foxp3⁺ Treg cell expansion in numbers. Mechanistically, that simultaneous DC-SIGN engagement by fucosylated ligands and TLR4 signaling was required for production of immunoregulatory IL-10 associated with prolonged allograft survival. Deletion of DC-SIGN-expressing macrophages *in vivo*, interfering with their CSF1-dependent development, or preventing the DC-SIGN signaling pathway abrogated tolerance. Together, the results provide new insights into the tolerogenic effects of costimulatory blockade and identify DC-SIGN⁺ suppressive macrophages as crucial mediators of immunological tolerance with the concomitant therapeutic implications in the clinic.

Graphical abstract



INTRODUCTION

Myeloid cells with suppressive activity inhibit graft-reactive T cell immunity and facilitate induction of regulatory T (Treg) cells, together enabling the induction of transplantation tolerance (Dugast et al., 2008; Garcia et al., 2010; Zhang et al., 2008). An emerging consensus is that myeloid cells with immune regulatory function are contained within a population of CD11b⁺ mononuclear cells that express the myeloid differentiation antigen Gr-1 (Bronte et al., 2000; Bronte et al., 1998). Given the wide range of myeloid cells that might be included in this category, identifying specific myeloid subsets capable of mediating suppression, understanding the molecular basis of their developmental requirements, and

deciphering the mechanisms that control their immune regulatory function represents a difficult task.

In previously published work, we demonstrated that monocytic cells that co-express CD11b, Gr-1, and the macrophage colony-stimulating factor 1 receptor (CSF1R) accumulate in cardiac allografts during tolerance induction, mediate T cell suppression in vitro, and are required for long-term graft survival induced by donor-specific transfusion plus anti-CD40L mAb (Garcia et al., 2010). Building upon these published observations, and the recognition that Gr-1 comprises the distinct and independently regulated surface-expressed glycoproteins Ly6C and Ly6G (Fleming et al., 1993), we demonstrate that myeloid suppressive cells expressing CD11b⁺CSF1R⁺Ly6C^{lo} Ly6G⁻CD169⁺ are responsible for transplantation tolerance. Transcriptome analysis revealed that graft infiltrating immune regulatory CD11b⁺CSF1R⁺Ly6C^{lo}Ly6G⁻CD169⁺ monocyte-derived cells correspond to suppressive macrophages.

Blockade of the CD40L-CD40 costimulatory pathway promotes the conversion of immunogenic CD11b⁺CSF1R⁺Ly6C^{hi} Ly6G⁻CD169⁻ into suppressive CD11b⁺CSF1R⁺Ly6C^{lo}Ly6G⁻ CD169⁺ macrophages through partial inhibition of interferon- γ (IFN- γ) production in the transplanted allograft. The conversion process requires CSF1, and interfering with this cytokine or its receptor (CSF1R) abrogates the induction of indefinite allograft survival. Mechanistically, we demonstrate that the dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN, CD209a) is upregulated in CD11b⁺CSF1R⁺Ly6C^{lo}Ly6G⁻CD169⁺-suppressive macrophages and that simultaneous DC-SIGN engagement by fucosylated ligands and TLR4 signaling is required for production of immunoregulatory interleukin-10 (IL-10) associated with immune regulation and prolonged allograft survival. In addition to delineating a unique set of phenotypic markers and offering new mechanistic insights into suppressive macrophage development and function during transplant tolerance, the data provide a foundation for developing robust protocols potentially capable of inducing immune regulatory macrophages for clinical use.

RESULTS

Suppressive Macrophages Accumulate during Tolerance Induction

To characterize myeloid cells that accumulate in allografts during tolerance induction, we transplanted BALB/c hearts (H-2^d) into fully mismatched C57BL6/MaFIA (H-2^b) recipient mice. These recipient animals constitutively express green fluorescent protein (GFP) under the CSF1R promoter, permitting us to identify recipient-derived graft-infiltrating myeloid cells that include monocytes, dendritic cells (DCs), macrophages, and neutrophils (Burnett et al., 2004). We treated groups of allograft recipients with anti-CD40L mAb (clone MR1) or with control anti-immuno-globulin G (IgG) mAb (Figure 1A), confirming previous work, which demonstrated that anti-CD40L mAb induced indefinite allograft survival, whereas rejection occurred by day 10 in the IgG-treated controls (Jiang et al., 2011). We harvested donor heart allografts on day 5 post-transplantation and analyzed graft-infiltrating leukocytes by flow cytometry. When we gated on live CD45⁺CD11b⁺CSF1R^{GFP+} recipient graft-infiltrating myeloid cells, we discerned three major populations based on differential expression patterns of Ly6C and Ly6G (Figure 1B). Quantitative analysis revealed a higher

frequency of CD11b⁺ CSF1R⁺Ly6C^{lo}Ly6G⁻ cells and a lower frequency of CD11b⁺ CSF1R⁺Ly6C^{hi}Ly6G⁻ cells in the allografts of anti-CD40L mAb-treated recipients compared to rejecting animals ($p < 0.01$). No differences in the frequencies of Ly6G cells were observed between groups.

We tested the ability of each myeloid cell subset to inhibit anti-CD3 and anti-CD28 stimulated CD8⁺ naive T cell proliferation (Figure 1C). The CD11b⁺CSF1R⁺Ly6C^{lo}Ly6G⁻ cell subset, but not the CD11b⁺CSF1R⁺Ly6C^{hi}Ly6G⁻ cell subset obtained from anti-CD40L mAb treated recipients, was potently suppressive. The CD11b⁺CSF1R⁺Ly6C^{int}Ly6G⁺ cell subset obtained from the anti-CD40L mAb-treated recipients also exhibited a modest suppressive activity. None of the myeloid cell subsets obtained from control IgG treated rejecting allografts exhibited in vitro suppression. We next tested the ability of each myeloid cell subset to induce expansion of CD4⁺Foxp3⁺ Treg cell in vitro (Figure 1D). Consistent with suppression assay results, only the CD11b⁺CSF1R⁺Ly6C^{lo}Ly6G⁻ cells obtained from anti-CD40L mAb-treated recipients, promoted the expansion of CD4⁺Foxp3 expressing Treg cell numbers. Thus, the graft-infiltrating CD11b⁺CSF1R⁺Ly6C^{lo}Ly6G⁻ cell subset that accumulates in anti-CD40L mAb-treated recipients possess many of the properties reported to be associated with monocytic myeloid suppressor cells, including their ability of inhibit CD8 T cell proliferation (Gallina et al., 2006) and to promote CD4⁺Foxp3⁺ Treg cell number expansion (Huang et al., 2006).

Further gene array characterization of graft-infiltrating myeloid CD11b⁺CSF1R⁺Ly6C^{lo}Ly6G⁻ cells that accumulate in tolerized recipients revealed that suppressive CD11b⁺CSF1R⁺Ly6C^{lo} Ly6G correspond to macrophages (Gautier et al., 2012), but not dendritic cells (Miller et al., 2012) (Figure S1A). Morphological examination of flow-sorted graft-infiltrating myeloid subsets (Figure S1B) confirmed that myeloid CD11b⁺CSF1R⁺Ly6C^{lo} Ly6G⁻ cells are of monocytic origin.

Suppressive Macrophages Are Required for Tolerance Induction

The transcriptional analyses of myeloid cell subsets revealed significantly higher transcript expression of CX3CR1, F4/80, CD206 (mannose macrophage receptor), CD68, CD172 (Sirp- α), CD169, and MHC-II in Ly6C^{lo} suppressive macrophages from anti-CD40L mAb treated mice (Figure 2A). Flow cytometry confirmed higher expression of these proteins on suppressive macrophages, and we exploited their differential CD169 expression (Figure 2A and Figures S2A and S2B) along with the availability of CD169 diphtheria toxin receptor (DTR) mice (Miyake et al., 2007) to evaluate the suppressive function of Ly6C^{lo}CD169⁺ macrophages in vivo. We transplanted BALB/c hearts into anti-CD40L mAb-treated WT or CSF1R^{GFP+}/CD169^{DTR} C57BL6 recipients and treated them with DT on the day of transplantation to deplete recipient CD169⁺ cells. Graft-infiltrating leukocytes by flow cytometry examined 5 days later (Figure 2B) showed specific depletion of recipient suppressive macrophages only in DT-treated animals. Depletion of Ly6C^{lo}CD169⁺ macrophages in the anti-CD40L mAb-treated recipients was associated with accumulation of memory or activated CD44^{hi}CD62L^{lo} CD8⁺ T cells on day 5 (Figures 2C and 2D) and a reduced percentage of graft infiltrating CD4⁺Foxp3⁺ Treg on day 21 posttransplant (Figure 2E). To verify that in vitro suppressive Ly6C^{lo}CD169⁺ macrophages also exhibit inhibitory

function in vivo, we adoptively transferred CFSE-labeled CD8 T cells into anti-CD40L mAb-treated CD169^{DTR} recipients and evaluated their ability to proliferate measured by CFSE dilution 5 days after DT treatment (Figure 2F). Whereas CD8 T cells transferred into tolerized recipients did not proliferate in vivo, CD8 T cells underwent proliferation in the allografts of tolerized recipients following depletion of CD169⁺ suppressive macrophages. Moreover, graft survival experiments showed that DT induced in vivo depletion of CD169⁺ suppressive macrophages resulted in graft rejection by day 30 despite tolerogenic treatment with anti-CD40L mAb ($p < 0.01$) (Figure 2G). Thus, Ly6C^{lo}CD169⁺-suppressive macrophages that accumulate in the allografts of anti-CD40L mAb-treated recipients inhibit T cell immune responses in vivo and are required for the induction of transplantation tolerance.

CD40L Blockade Inhibits Accumulation of Immunogenic Macrophages

Graft-infiltrating myeloid subsets express CD40, but not CD40L (Figures S3A and S3B), suggesting that tolerogenic properties of the anti-CD40L mAb treatment are not due to a direct effect on monocyte-derived cells because they do not express CD40L. To test whether anti-CD40L mAb therapy induces suppressive macrophages via inhibiting transmission of a CD40-dependent signal on the myeloid cells, we attempted to circumvent the effects of the anti-CD40L mAb blockade by co-administering an agonistic anti-CD40 antibody FGK45.5, an antibody that has been shown to transmit CD40-dependent signals to APC in the absence of CD40L (Bennett et al., 1998; Rolink et al., 1996; Schoenberger et al., 1998). Administration of the agonistic anti-CD40 mAb promoted the accumulation of immunogenic Ly6C^{hi} macrophages in the allograft (Figure 3A). CD40-mediated accumulation of Ly6C^{hi} macrophages might be mediated by increased IFN- γ expression in the allografts of tolerized recipients (Juttila et al., 1988). To test for a link between CD40L-CD40 ligation and IFN- γ -mediated Ly6C^{hi} macrophage activation in our transplant model, we measured IFN- γ in the allografts of untreated recipients, tolerized recipients, and tolerized recipients co-treated with agonistic anti-CD40 mAb (Figure 3B). These assays showed reduction of intra-graft IFN- γ in the tolerized allografts compared to the untreated controls as previously reported (Hancock et al., 1996) but restoration of intra-graft IFN- γ observed in the anti-CD40 mAb co-treated recipient. On the contrary, agonistic CD40 ligation-mediated accumulation of immunogenic Ly6C^{hi} macrophages and increased intra-graft IFN- γ expression was not observed in tolerized CD40-deficient (*Cd40*^{-/-}) recipients (Figures 3C). Agonistic CD40 mAb abrogated the induction of tolerance despite CD40L blockade in wild-type (WT), but not in *Cd40*^{-/-} recipients (Figure 3D). The data suggest that anti-CD40L mAb-induced tolerance can be abrogated by CD40 ligation, which favors the accumulation of Ly6C^{hi} immunogenic macrophages in the allograft through IFN- γ . To confirm this hypothesis, we treated tolerized recipients with recombinant IFN- γ and observed reduced intra-graft accumulation of Ly6C^{lo} macrophages on day 5 (Figure 3E) associated with allograft rejection (Figure 3F). Conversely, partial IFN- γ blockade restored accumulation of Ly6C^{lo} macrophages in the allografts (Figure 3G) and reestablished indefinite allograft survival (Figure 3H) in tolerized recipients despite agonistic anti-CD40 mAb treatment. Thus, costimulation blockade with anti-CD40L mAb prevents IFN- γ production and accumulation of immunogenic Ly6C^{hi} macrophages in the transplanted allografts.

CSF1 Mediates the Development of Suppressive Macrophages

The expression of CSF1R in CD11b⁺CSF1R⁺Ly6C^{lo}Ly6G⁻ CD169⁺-suppressive macrophages suggests an involvement of CSF1 in the development of these cells. We quantified CSF1 transcripts in transplanted mice by RT-PCR (Figure 4A) and observed significant upregulation of CSF1 in the allografts of anti-CD40L mAb-treated recipients. To test for a mechanistic link between CSF1 and development of Ly6C^{lo}-suppressive macrophages in anti-CD40L mAb-treated recipient mice, we transplanted BALB/c hearts into anti-CD40L mAb-treated tolerized C57BL6 recipients with or without neutralizing anti-CSF1 mAb (clone 5A1) at doses shown by others to inhibit their function in vivo (Gregory et al., 1992). Our results indicate that in vivo CSF1 blockade abrogated intra-graft accumulation of Ly6C^{lo} suppressive macrophages (Figure 4B). CSF1 blockade also prevented the in vivo expansion of CD4⁺ Foxp3⁺ Treg cell and abrogated the induction of transplantation tolerance (Figures 4C and 4D). In vivo blockade of CSF1R receptor (clone AFS98), at doses shown by others to inhibit their function in vivo (Hashimoto et al., 2011), also abrogated tolerance, which suggests that CSF1-CSF1R signaling is necessary for the development of suppressive macrophages. Ly6C^{hi} monocytes convert into Ly6C^{lo} macrophages (Arnold et al., 2007), the latter being able to function as suppressive cells in tumor models (Corzo et al., 2010). To test whether analogous mechanisms apply in transplant tolerance, we isolated CD11b⁺CSF1R⁺ Ly6C^{hi}Ly6G⁻ GFP⁺ bone marrow monocytes from C57BL6/ MaFIA mice and transferred them into C57BL6/WT recipients with or without anti-CD40L mAb and anti-CSF1 blocking mAb (Figure 4E). Whereas the Ly6C^{hi} monocytic precursors converted into Ly6C^{lo} macrophages in the allografts of anti-CD40L mAb-treated mice, Ly6C^{hi} monocytic precursors from anti-CSF1 mAb-treated recipient mice failed to convert and maintained a Ly6C^{hi} phenotype, similar to the untreated rejecting controls. Additional in vitro experiments confirmed that CSF1 mediates the conversion of Ly6C^{hi} monocytic precursors into Ly6C^{lo} myeloid cells that were functionally able to inhibit CD8⁺ T cell proliferation and promote Treg expansion (Figures 4F and 4G). Our in vitro human data is consistent with this hypothesis and suggests that CSF1, but not CSF2, promotes the development of CD14 monocytes into suppressive monocyte-derived cells that inhibit CD8 T cell proliferation and expand Foxp3-expressing Treg in vitro (Figure S4A) Thus, anti-CD40L mAb-induced tolerance requires prevention of IFN- γ production and upregulation of CSF1, the latter driving conversion of monocytic precursors into suppressive macrophages.

DC-SIGN Controls the Function of Suppressive Macrophages

CSF1 upregulates the expression of the dendritic-cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN, CD209a) (Choi et al., 2011; Domínguez-Soto et al., 2011). Our gene array, real-time PCR, flow cytometry, and immunofluorescence studies revealed higher expression of DC-SIGN in macrophages obtained from the allografts of anti-CD40L mAb-treated mice, non-rejecting human renal transplant recipients, or in vitro derived CSF1-dependent human macrophages (Figures 5A–5C, Figures S5A–S5D). To test whether DC-SIGN is required for anti-CD40L mAb-induced allograft survival, we transplanted BALB/c hearts into WT C57BL6 recipients under the cover of anti-CD40L mAb, together with either a blocking anti-DC-SIGN mAb or an iso-type IgG control. DC-SIGN blockade abrogated the induction of indefinite allograft survival in anti-CD40L mAb-

treated mice (Figure 5D). When we isolated and compared graft-infiltrating leukocytes from anti-DC-SIGN mAb-treated recipients with control mice (all treated with anti-CD40L mAb), we observed similar frequencies of graft-infiltrating Ly6C^{lo}Ly6G⁻ macrophages, suggesting that anti-DC-SIGN mAb treatment does not prevent intra-graft accumulation of Ly6C^{lo}Ly6G⁻ macrophages (Figure 5E). However, whereas flow-sorted graft-infiltrating CD11b⁺CSF1R⁺Ly6C^{lo}Ly6G⁻ macrophages from tolerized recipients suppressed CD8⁺ T cell proliferation and expanded CD4⁺Foxp3⁺ Treg in vitro, flow-sorted CD11b⁺CSF1R⁺Ly6C^{lo}Ly6G⁻ macrophages obtained from tolerized recipients treated with anti-DC-SIGN mAb did not exhibit either of these immune regulatory functions (Figure 5F). Finally, we flow-sorted CD11b⁺CSF1R⁺Ly6C^{lo}Ly6G⁻ suppressive macrophages from anti-CD40L mAb-treated recipients and assessed their ability to suppress CD8⁺ T cell proliferation and expanded CD4⁺Foxp3⁺ Treg following in vitro blockade of DC-SIGN by adding anti-DC-SIGN mAb to the cell cultures (Figure 5G). Thus, DC-SIGN expression is required for the immune regulatory function of suppressive macrophages that mediate indefinite allograft survival. Using DC-SIGN deficient (*CD209a*^{-/-}) and CD169^{DTR} tumor bearing mice our data revealed that depletion of CD169⁺ macrophages or absence of DC-SIGN significantly reduces in vivo tumor growth (Figure S6A). Together with data from renal cell carcinoma patients showing increased DC-SIGN expression at the tumor site (Figure S6B), this suggests that DC-SIGN⁺ macrophages also participate in the immune regulatory function that controls tumor progression.

Fucosylated DC-SIGN Ligands Are Required for Macrophage-Mediated Suppression and Tolerance

DC-SIGN binds to carbohydrates containing mannose or fucose residues, such as Lewis^X (van Liempt et al., 2006). We next investigated the role of fucosylated Lewis^X in the induction of transplantation tolerance using the α 1,3/4-fucosyltransferases (FucTs) IV-VII double-deficient (dKO) donor mice, which display impaired Lewis^X expression (Lowe, 2002). We next used FucT-IV and FucT-VII mice as donors to evaluate the effects of Lewis^X inhibition on suppressive Ly6C^{lo} macrophages and tolerance. Figure 6A indicates that the Lewis^X expression was significantly reduced in tolerized dKO donor allografts, which was associated with acute rejection despite tolerogenic treatment with anti-CD40L mAb (Figure 6B). We next compared recipient graft-infiltrating leukocytes from donor dKO and WT allografts treated with anti-CD40L mAb, and we observed similar frequencies of graft-infiltrating Ly6C^{lo}Ly6G⁻ macrophages (Figure 6C). These results suggest that Lewis^X deficiency does not prevent intra-graft accumulation of Ly6C^{lo} macrophages. However, whereas flow-sorted graft-infiltrating Ly6C^{lo} macrophages from WT donors suppressed CD8⁺ T cell proliferation and expanded CD4⁺Foxp3⁺ Treg cell in vitro, the flow-sorted Ly6C^{lo} macrophages obtained from dKO donor allografts did not exhibit neither of these immune regulatory functions (Figure 6D). We next investigated whether lacto-N-fucopentaose III (LNFPIII), a Lewis^X containing pentasaccharide that binds to DC-SIGN (Meyer et al., 2005), could overcome the fucosylated Lewis^X deficiency in dKO donor allograft recipients (Figure 6E). Our results indicate that unlike dextran (which does not bind to CD209a-mDC-SIGN [Takahara et al., 2004]), LNFPIII is able to restore tolerance in transplant recipients containing dKO donor allo-grafts. Flow-sorted graft-infiltrating Ly6C^{lo} macrophages from LNFPIII-treated dKO donor allograft tolerized recipients were able to

suppress CD8⁺ T cell proliferation and expanded CD4⁺ Foxp3⁺ (Figure 6F). Our results indicate that in vivo LNFPIII treatment restores the suppressive activity of Ly6C^{lo} macrophages. Thus, Lewis^X-mediated DC-SIGN ligation is necessary for the immune regulatory function of suppressive macrophages and for the induction of indefinite allograft survival.

IL-10 Is Essential for DC-SIGN-Mediated Suppression

Fucose-specific DC-SIGN signaling results in production of IL-10 (Caparrós et al., 2006; Gringhuis et al., 2014). We observed a significant IL-10 upregulation in the allografts of anti-CD40L mAb-treated WT recipients compared to untreated rejecting controls (Figure 7A). In contrast, IL-10 was essentially absent in anti-CD40L mAb-treated DC-SIGN-deficient (*CD209a*^{-/-}) recipient mice. Among graft-infiltrating leukocytes, we detected the highest IL-10 expression in Ly6C^{lo} macrophages obtained from anti-CD40L mAb treated recipients, but the same Ly6C^{lo} macrophages obtained from the allografts of *CD209a*^{-/-} recipients exhibited significant less IL-10 expression despite anti-CD40L mAb treatment (Figure 7A; Figure S7A). To specifically test whether IL-10 is required for regulatory macrophage function, we sorted intra-graft Ly6C^{lo} macrophages from anti-CD40L mAb treated IL-10-deficient (*Il10*^{-/-}) recipient mice and tested their ability to suppress CD8⁺ T cell proliferation and to expand CD4⁺Foxp3⁺ Treg cell in vitro (Figure 7B). In the absence of IL-10, Ly6C^{lo} macrophages did not exhibit either of these immune regulatory functions despite tolerogenic treatment with anti-CD40L mAb. Using *CD209a*^{-/-} recipient mice, we next investigated whether recombinant IL-10 could restore the suppressive function of Ly6C^{lo} graft-infiltrating macrophages (Figure 7C). Although intra-graft *CD209a*^{-/-} Ly6C^{lo} macrophages were unable to suppress CD8⁺ T cell proliferation and to expand CD4⁺Foxp3⁺ Treg in vitro, IL-10 addition rescued the immune regulatory function of *CD209a*^{-/-} Ly6C^{lo} macrophages. Thus, graft-infiltrating DC-SIGN⁺ Ly6C^{lo} macrophages exert their immune regulatory function in part through an IL-10-dependent mechanism. Because crosstalk between DC-SIGN and TLR4 signaling is required for fucose binding-mediated production of IL-10 (Gringhuis et al., 2007; Gringhuis et al., 2014), we explored the effects of TLR4 deficiency in suppressive macrophages using tolerized TLR4 recipients, and showed that in the absence of TLR4 stimulation, IL-10 production was reduced in Ly6C^{lo} macrophages (Figure 7D), and their in vitro suppressive function was defective (Figure 7E). To demonstrate that synergistic DC-SIGN and TLR4 signaling was necessary for IL-10 production, we cultured bone marrow cells from WT, *CD209a*^{-/-}, and TLR4-deficient (*Tlr4*^{-/-}) mice and stimulated them with the DC-SIGN ligand Lewis^X and the TLR4 ligand high mobility group box 1 (HMGB1) (Figure 7F). Simultaneous DC-SIGN and TLR4 signaling was necessary for optimal IL-10 production, and interfering with one of the signals resulted in impaired IL-10 production. We investigated whether ligation of DC-SIGN and/or TLR4 resulted in an increased inhibitory function (Figure 7G). Addition of both DC-SIGN and TLR4 agonists resulted in the highest suppressive function observed in monocyte-derived human cells in comparison with each of the ligands alone. Thus, DC-SIGN⁺ macrophages stimulated through DC-SIGN and TLR4 are negative regulators of the immune response and that their manipulation will open new avenues for therapeutic intervention either by inhibiting their function (i.e., in cancer patients) or by enhancing their suppressive effects and promoting their expansion (i.e., in transplant recipients).

DISCUSSION

We demonstrate here that DC-SIGN-expressing macrophages are required for the induction of transplantation tolerance. DC-SIGN is a type II transmembrane C-type lectin with a carbohydrate recognition domain, which is expressed in human DCs and macrophages (Geijtenbeek et al., 2000; Soilleux et al., 2002), and is involved in multiple aspects of the immunological response (van Kooyk and Geijtenbeek, 2003). Broxmeyer and colleagues reported that in vitro differentiation of monocytes in the presence of M-CSF and IL-4, which induces DC-SIGN expression (Martinez et al., 2006), are less efficient inducers of allogeneic mixed lymphocyte reactions (Li et al., 2004; Li et al., 2005). Here, we extend these findings to newly demonstrate that DC-SIGN⁺ macrophages inhibit T cell proliferation in vitro and in vivo in an experimental mouse model of solid organ transplantation.

Additionally, we demonstrate that human DC-SIGN expressing macrophages stimulated with M-CSF and IL-4 (Figure S5D) induced the expansion of Foxp3-expressing Treg from allogeneic naive CD4⁺ T cell precursors in vitro, whereas macrophages treated with GM-CSF and IL-4 did not drive Treg expansion (Figure S4A).

The ability of murine DC-SIGN⁺ macrophages to promote IL-10-mediated transplantation tolerance requires two synergistic signals: DC-SIGN engagement by fucosylated ligands and TLR4 signaling. The CDR domain of human DC-SIGN recognizes fucosylated Lewis glycans (van Liempt et al., 2006) expressed by self and non-self antigens (Geijtenbeek et al., 2004). In humans, DC-SIGN ligation potentiates the secretion of IL-10 (Geijtenbeek et al., 2003). Because DC-SIGN macrophages secrete IL-10 upon fucose ligand engagement (Gringhuis et al., 2014) and participate in the generation of regulatory T cells (Cai et al., 2013; Smits et al., 2005), DC-SIGN could actively contribute to the maintenance of an immunosuppressive tissue environment, as proposed by Yvette van Kooyk's laboratory (van Gisbergen et al., 2005). Indeed, DC-SIGN ligation by non-immune cells, such as pathogens and tumor tissue results in immune escape (Geijtenbeek and Gringhuis, 2009), suggesting that both tumor and pathogens have ways to escape immune activation by targeting DC-SIGN. Consistent with this hypothesis our data reveals that depletion of CD169⁺ macrophages or absence of DC-SIGN significantly reduces in vivo tumor growth (Figure S6A), suggesting that DC-SIGN⁺ macrophages might participate in the immune regulatory function that controls tumor progression (Figure S6B). Our transplant results indicate that fucosylated glycans are present in the donor allografts of tolerized recipients that serve as ligands of DC-SIGN expressing macrophages. Using fucosyltransferase-deficient donor heart allografts inhibits the expression of Lewis^X glycoproteins and prevents the induction of indefinite allograft survival despite tolerogenic treatment with anti-CD40L mAb treatment. This suggests common mechanisms of immune regulation following engagement of DC-SIGN by tumor and transplant microenvironment via Lewis^X recognition that lead to the production of IL-10 producing macrophages (Domínguez-Soto et al., 2011; Nonaka et al., 2008; van Gisbergen et al., 2005).

Induction of transplantation mediated by DC-SIGN⁺-suppressive macrophages depends on simultaneous TLR4 signaling. DC-SIGN signaling crosstalk with TLR4 has been demonstrated to mediate IL-10 production (Geijtenbeek et al., 2003; Gringhuis et al., 2007; Gringhuis et al., 2014). Here we report that DC-SIGN⁺ macrophages from TLR4-deficient

heart recipients produce significantly less IL-10 and do not exhibit suppressive function. Consistent with these results, a recent study indicates that during peripheral tolerance, DC-SIGN and TLR4 are required for IL-10 secretion and decreased T cell proliferation in mixed leukocyte reactions possibly caused by an increased frequency of Treg cell, which is associated with a high fucosyltransferase expression (García-Vallejo et al., 2014). In transplantation, while absence of innate MyD88 signaling prevents acute allograft rejection and promotes inducible allograft acceptance (Goldstein et al., 2003; Walker et al., 2006), it is possible that specific signaling molecules of the MyD88 pathway, such as TLR4, might have a critical role in the induction of tolerance mediated by suppressive myeloid cells. In this respect, the TLR4 agonist HMGB1, which is upregulated during tissue damage associated with ischemia reperfusion (Wu et al., 2007) and surgical transplantation (Huang et al., 2007), has been recently demonstrated to enhance the immune-suppressive capacity of myeloid-derived suppressor cells through the production of IL-10 (Parker et al., 2014).

In conclusion, we demonstrate that graft-infiltrating DC-SIGN⁺-suppressive macrophages mediate the induction of transplantation tolerance, revealing a previously unknown function of mouse DC-SIGN. Our delineation of a specific cell-surface phenotype for immunoregulatory, graft-protective suppressive macrophages in transplantation, as well as the mechanistic insights underlying the requirements for their differentiation *in vivo*, have important implications for understanding and potentially manipulating pathogenic immune responses. The C-type lectin DC-SIGN (CD209a) has a critical function in the induction of transplantation tolerance as demonstrated by its absence or *in vivo* blockade and might be used as phenotypic marker to define immune regulatory macrophages. The data provide a framework for developing CSF1-based *in vitro* protocols to induce therapeutic macrophages for clinical use to prevent transplant rejection and suggest that depleting or blocking suppressive macrophage development by targeting CSF1, which upregulates the expression of DC-SIGN, could be exploited to enhance anti-tumor immunity.

EXPERIMENTAL PROCEDURES

Mice

BALB/c, C57BL/6, C57BL/6-Foxp3tm1Flv/J, B6.129P2-Cd40tm1Kik/J, and B6.B10ScN-Tlr4lps-del/JthJ mice 8 weeks of age were purchased from The Jackson Laboratory. DC-SIGN-deficient mice (DC-Sign-KO, B6 [FVB]-Cd209atm1.1Cfg/Mmcd) were from the Mutant Mouse Regional Resource Centers, Consortium for Functional Glycomics (Scripps Res. Institute). The alpha(1,3)fucosyltransferases FucT-IV and FucT-VII double-deficient mice were from John Lowe (University of Michigan). The C57BL/6-Tg (Csf1r-EGFP-NGFR/FKBP1A/TNFRSF6) 2Bck/J MaFIA mice from D. Cohen (University of Kentucky) (Burnett et al., 2004). The C57BL/6 CD169^{DTR} mice have been previously described (Miyake et al., 2007). All experiments were performed with age- and sex-matched mice in accordance with Institutional Animal Care and Utilization Committee-approved protocols.

Vascularized Heart Transplantation

BALB/c hearts were transplanted as fully vascularized heterotopic grafts into C57BL/6 mice as previously described (Corry et al., 1973). Recipient mice were treated with 250 µg anti-CD40L mAb (clone MR1, BioXcell) for tolerance induction on days 0, 2, and 4 as previously described (Jiang et al., 2011). Graft function was monitored every other day by abdominal palpation. Untreated control mice received hamster IgG in PBS. Rejection was defined as complete cessation of a palpable beat and confirmed by direct visualization at laparotomy.

In Vivo Cell Depletion

For depletion of CD169 expressing CD11b⁺CSF1R⁺Ly6C^{lo}Ly6G⁻ regulatory macrophages heterozygous CD169-DTR recipients were injected intraperitoneally (i.p.) with 10 ng/g body weight of DT (Sigma-Aldrich) 24, 48, and 72 hr after transplantation (Miyake et al., 2007). Ly6G⁺ cell depletion was induced with anti-Ly6G mAb clone 1A8 (BioXcell) injected at 0.5 mg i.p. on days -3, -2, and -1 relative to transplantation as previously described (Daley et al., 2008; Garcia et al., 2010).

Antibody-Mediated In Vivo Treatment

Agonistic anti-CD40 mAb (clone FGK4.5 mAb) was produced by BioXcell. CD40-mediated priming independent of CD40L was achieved by intravenous (i.v.) injection of 100 µg of agonistic anti-CD40 mAb on days 0 and +1 relative to transplantation (Gorbachev and Fairchild, 2004). Blocking antibody to IFN-γ (Clone R4-6A2) was produced by BioXcell. Anti-IFN-γ mAb was injected at 500 µg on days 0 and +1 relative to transplantation. Blocking antibody to CSF1 (clone 5A1) (Lokeshwar and Lin, 1988) and CSF1R (clone AFS98) (Sudo et al., 1995) were produced by BioXcell. Anti-CSF1 mAb was injected at 150 µg i.p. on days -1, +1, +2, +3, and +4 relative to transplantation, which is known to neutralize the biological functions of CSF1 in vivo (Gregory et al., 1992). Anti-CSF1R mAb was injected at 2 mg/mouse on day -5 and 0.5 mg/mouse on days -4 and -3, which is known to neutralize the biological functions of CSF1R in vivo (Hashimoto et al., 2011). Blocking antibody to DC-SIGN (CD209a) (Cheong et al., 2010) was mAb was purified from culture supernatant, grown in a CELLLine Flask (BD) in serum-free medium (PFHM-II; Invitrogen) and injected at 250 µg i.p. on days +1, +2, +3, and +4 relative to transplantation.

Mouse Suppression Assay

Spleens of C57BL/6 or C57BL/6-Foxp3tm1Flv/J (H-2^b) mice were gently dissociated into single-cell suspensions, and red blood cells were removed using hypotonic ACK lysis buffer. Splenocytes were either stained with anti-CD4 mAb, or labeled with CFSE at 5 µM concentration (Molecular probes - Invitrogen) followed by staining with anti-CD8 mAb for 30 min on ice. Responder FoxP3⁺CD4⁺ and CFSE⁺CD8⁺ T cells were sorted using FACS Aria II (BD Biosciences) with a purity > 98%. Spleens of BALB/c (H-2^d) mice were gently dissociated into single-cell suspensions and were enriched for CD11c⁺ cells using the EasySep Mouse CD11c Positive Selection Kit (StemCell). Enriched CD11c⁺ splenocytes were stained with anti-mouse CD11c mAb and sorted using FACS Aria II (BD Biosciences) and were used together with anti-CD3 plus CD28 mAb (1 µg/ml) as stimulators. Stimulated

FoxP3⁺CD4⁺ or CFSE⁺CD8⁺ T cells were cultured with graft infiltrating CD11b⁺CSF1R⁺Ly6C^{hi}Ly6G⁻, CD11b⁺CSF1R⁺Ly6C^{lo}Ly6G⁻, and CD11b⁺CSF1R⁺Ly6C^{int}Ly6G⁺ myeloid cells for 4 days at 37°C in a 5% CO₂ incubator. T cell proliferation was measured by flow cytometric analysis of CFSE dilution on CD8⁺ T cells. Treg expansion was measured by flow cytometric analysis of Foxp3-RFP on CD4⁺ T cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- DC-SIGN⁺ macrophages inhibit CD8⁺ T cell proliferation and expand CD4⁺Foxp3⁺ Treg
- In vivo development of DC-SIGN⁺ macrophages is regulated by IFN- γ and CSF1
- IL-10 is essential for DC-SIGN⁺ macrophage-mediated suppression
- Simultaneous Fucose-DC-SIGN and HMGB1-TLR4 signaling is required for IL-10 production

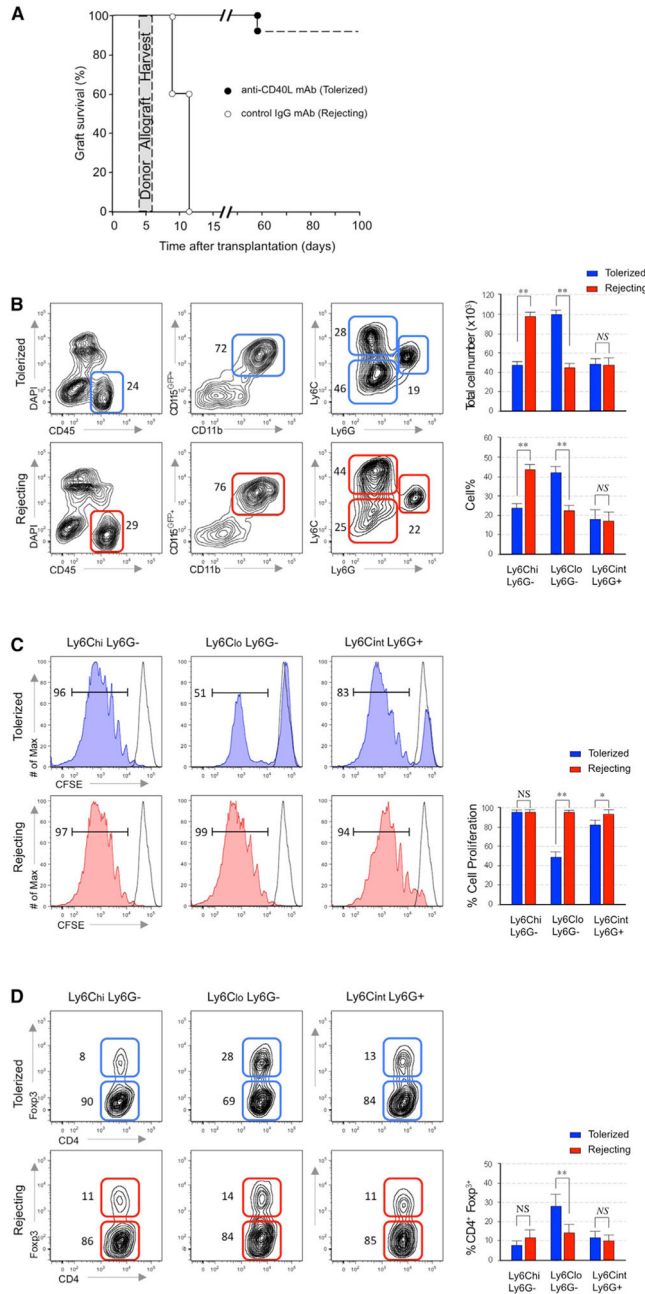


Figure 1. Suppressive Macrophages Accumulate during Tolerance Induction

(A) Graft survival of control IgG mAb (rejecting) and anti-CD40L mAb (tolerized) recipients of heterotopic cardiac allografts (n = 20 mice/group). The shaded area depicts heart allografts that were harvested at day 5 post-transplantation for subsequent analyses. (B) Representative and quantitative flow cytometry results for Ly6C and Ly6G expression in CD45⁺CD11b⁺ CSF1R^{GFP} myeloid cell subsets from the allografts of tolerized and rejecting recipients at day 5 post-transplantation. Results represent mean ± SEM (n = 8 mice per group).

(C) In vitro suppressive capacity of each myeloid subset for CD8⁺ T cells. Proliferation was measured by CFSE dilution after 96 hr by flow cytometry. Percentage of cell proliferation is presented as mean \pm SEM of five independent experiments.

(D) In vitro Treg expansion of each myeloid subset. Flow cytometric analysis indicates Foxp3 expression on CD4 T cells after co-culture for 96 hr with myeloid subsets. Percentage of Treg expansion is presented as mean \pm SEM of five independent experiments.

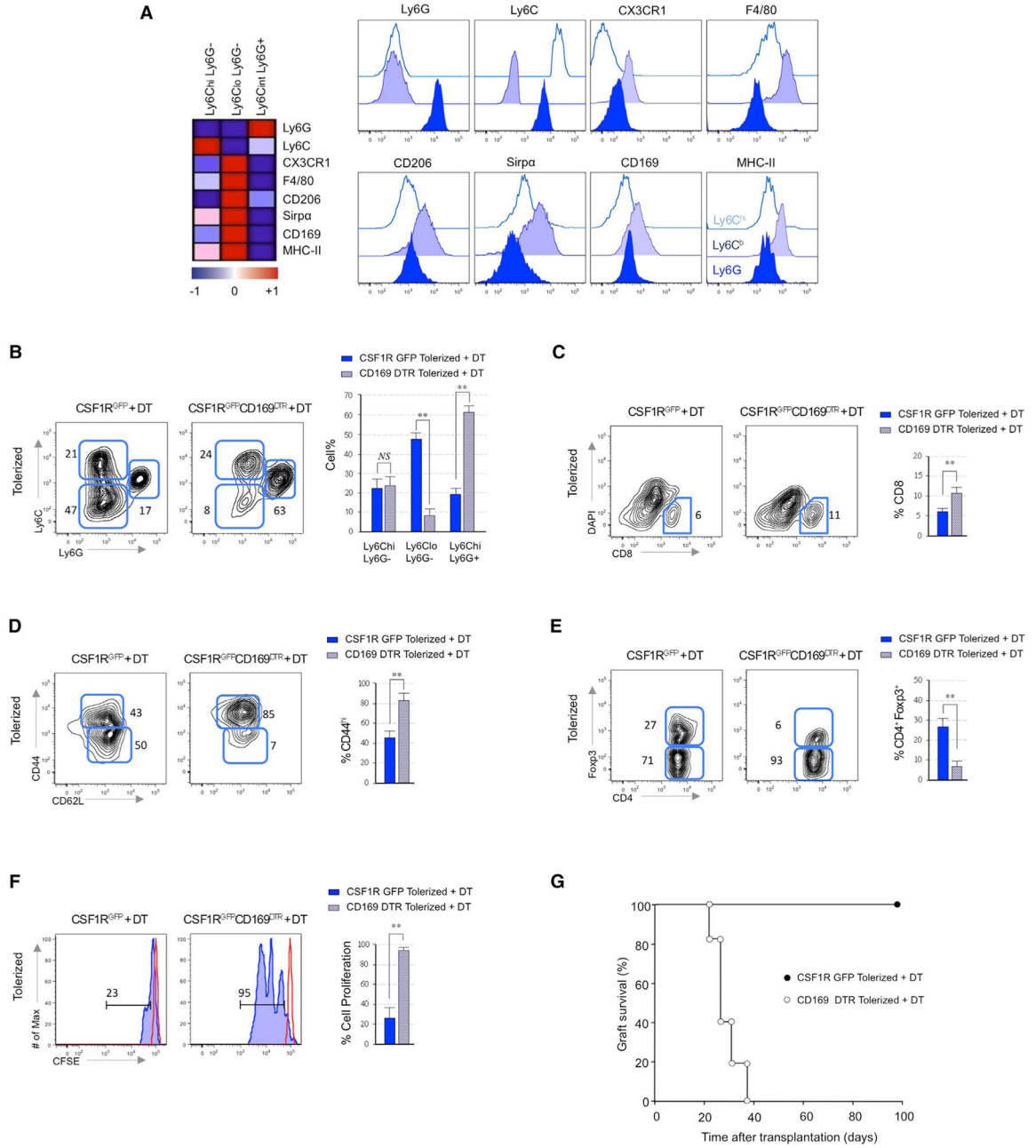


Figure 2. Suppressive Macrophages Are Required for Tolerance Induction
 (A) Heatmap derived from microarray data of selected myeloid markers that achieve $p < 0.05$ in myeloid subsets from the allografts of tolerized recipients at day 5 post-transplantation (means of $n = 3$ per group). Representative flow cytometry plots of the above myeloid markers on each myeloid subset. Data is representative of three independent experiments.

(B) Representative and quantitative flow cytometry results of recipient myeloid cell subsets in the allografts of tolerized CSF1R^{GFP} (wild-type) and CSF1R^{GFP} CD169^{DTR} recipients at

day 5 post-transplantation. Results represent mean \pm SEM (n = 12 mice per group of 3 independent experiments).

(C and D) Representative and quantitative flow cytometry results depicting percentages (C) and surface memory/naive CD44/CD62L phenotypes (D) of graft infiltrating CD8 T cells after CD169⁺ macrophage depletion. Results represent mean \pm SEM (n = 12 mice per group of 3 independent experiments).

(E) Representative flow cytometry results depicting percentages of Foxp3 expressing graft infiltrating CD4⁺ T cells on day 21 post-transplantation in tolerized recipients with or without CD169⁺ macrophage depletion. Results represent mean \pm SEM (n = 4 mice per group of 3 independent experiments).

(F) Effects of CD169⁺ macrophage depletion on in vivo T cell proliferation. CFSE-labeled CD8⁺ T cells (5×10^6) were injected into tolerized CSF1R^{GFP} and CSF1R^{GFP} CD169^{DTR} recipients. Proliferation was measured in the allograft by CSFE dilution after 120 hr by flow cytometry. Results represent mean \pm SEM (n = 4 mice per group of 3 independent experiments).

(G) Effects of CD169⁺ macrophage depletion on graft survival in tolerized CSF1R^{GFP} and CSF1R^{GFP} CD169^{DTR} recipients (n = 12 mice/group).

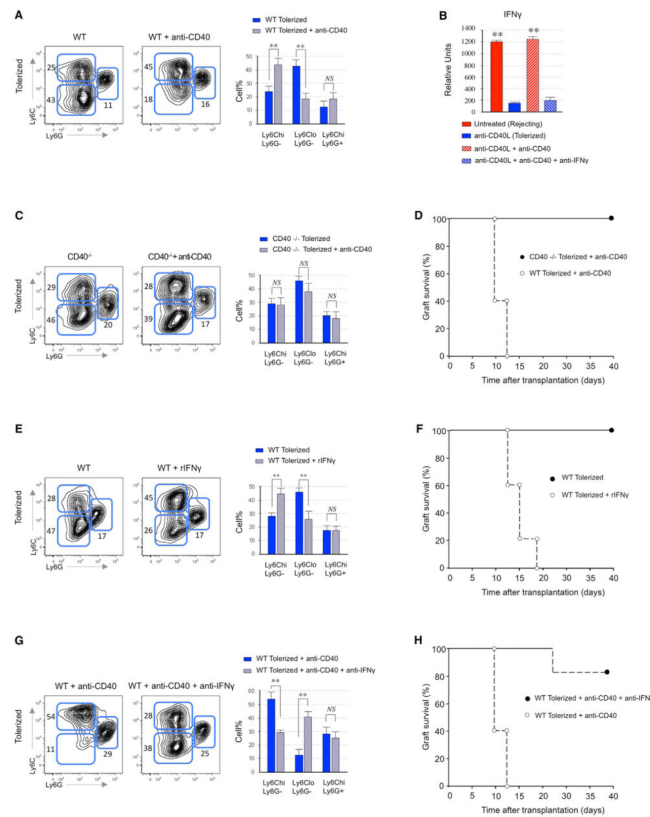


Figure 3. CD40L Blockade Inhibits Accumulation of Immunogenic Macrophages

- (A) Representative and quantitative flow cytometry results of recipient myeloid cell subsets in the allografts of tolerized WT recipients with or without co-treatment with agonistic CD40 mAb. Results represent mean ± SEM (n = 4 mice per group of 3 independent experiments).
- (B) IFN-γ expression in cardiac allografts. Cardiac allografts were harvested 5 days after transplantation from each group. Agonistic anti-CD40 mAb was injected at 100 μg/mouse on days 0 and +1 relative to transplantation. Recombinant mouse IFN-γ was injected at 4 × 10⁵ units/day for 10 days (n = 4 mice/group). Supernatants of single cell suspensions were analyzed for IFN-γ measured by ELISA. Bar graphs represent mean ± SEM of three independent experiments (**p < 0.01).
- (C) Representative and quantitative flow cytometry results of recipient myeloid cell subsets in the allografts of tolerized CD40 deficient recipients with or without agonistic CD40 mAb treatment. Results represent mean ± SEM (n = 4 mice per group of 3 independent experiments).
- (D) Effects of CD40 ligation on graft survival in tolerized WT and CD40 deficient recipients (n = 8 mice/group).
- (E) Representative and quantitative flow cytometry results of recipient myeloid cell subsets in the allografts of tolerized WT recipients with or without recombinant IFN-γ treatment (4 × 10⁵ units/day for 5 days). Results represent mean ± SEM (n = 4 mice per group of 3 independent experiments).

(F) Effects of recombinant IFN- γ on graft survival in tolerized WT recipients (n = 8 mice/group).

(G) Representative and quantitative flow cytometry results of recipient myeloid cell subsets in the allografts of tolerized WT recipients co-treated with agonistic CD40 mAb with or without anti-IFN- γ mAb treatment. Results represent mean \pm SEM (n = 4 mice per group of 3 independent experiments).

(H) Effects of partial IFN- γ blockade on graft survival in tolerized wild-type recipients co treated with agonistic CD40 mAb (n = 8 mice/group).

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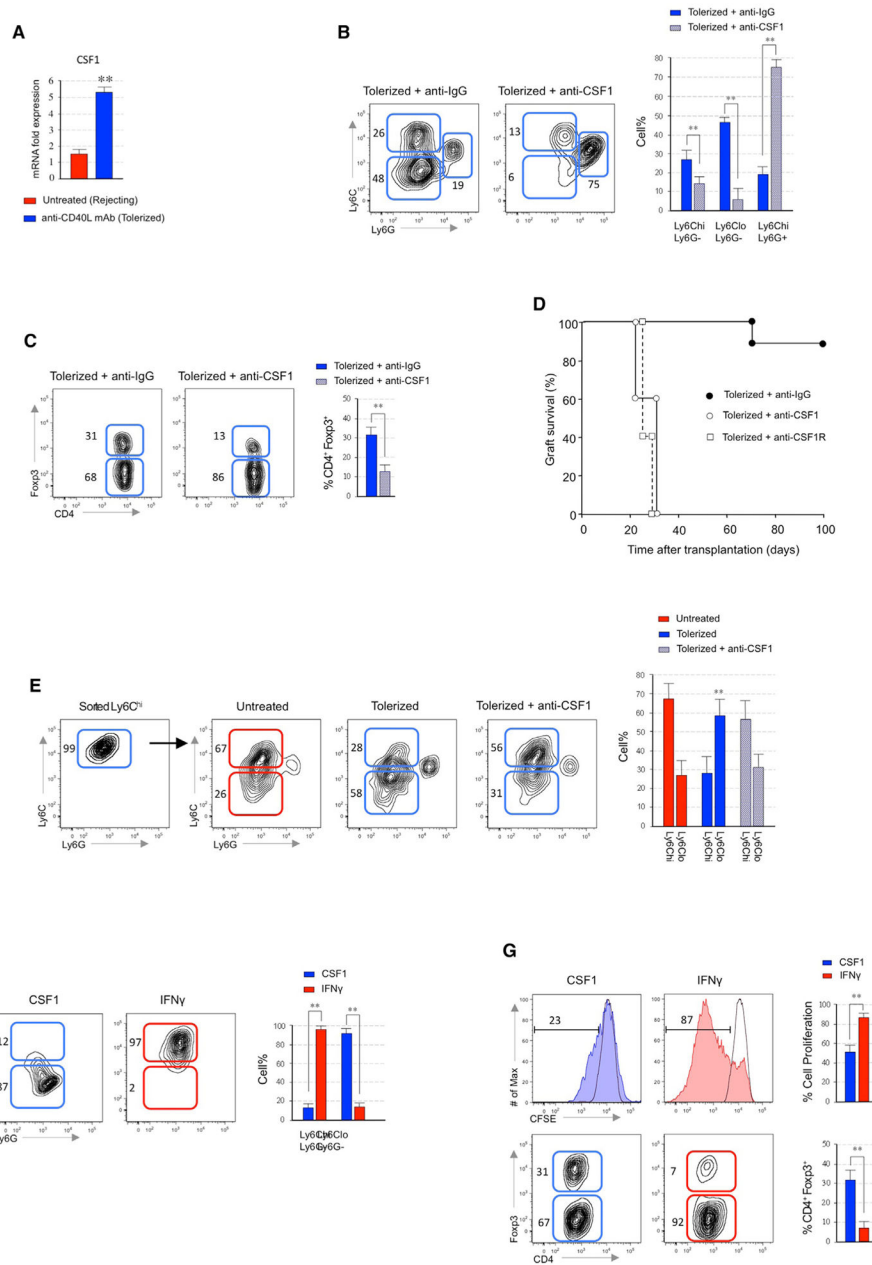


Figure 4. CSF1 Mediates the Development of Suppressive Macrophages

(A) CSF1 expression in cardiac allografts. Cardiac allografts were harvested 5 days after transplantation from tolerized and rejecting recipients. Total single cell suspensions were analyzed for CSF1 measured by real-time PCR. Bar graphs represent mean ± SEM of three independent experiments (**p < 0.01).

(B) Representative and quantitative flow cytometry results of recipient myeloid cell subsets in the allografts of tolerized WT recipients co-treated with anti-CSF1 mAb. Results represent mean ± SEM (n = 4 mice per group of 3 independent experiments).

(C) Representative and quantitative flow cytometry results of Fcγ3 expression on CD4 T cells in the allografts of tolerized recipients on day 21 post-transplantation following anti-

CSF1 mAb treatment. Results represent mean \pm SEM (n = 4 mice per group of 3 independent experiments).

(D) Effects of CSF1 and CSF1R blockade on graft survival in tolerized WT recipients (n = 12 mice/group).

(E) Representative and quantitative flow cytometry results of adoptively transferred CSF1R⁺Ly6C^{hi} bone marrow cells into recipient mice treated with anti-CD40L mAb \pm anti-CSF1 mAb 5 days after transplantation. Results represent mean \pm SEM (n = 3 mice per group of 3 independent experiments).

(F) Representative and quantitative flow cytometry results of in vitro cultured Ly6C^{hi} bone marrow cells with either recombinant CSF1 or IFN γ for 96 hr. Results represent mean \pm SEM of three independent experiments.

(G) Suppressive function of Ly6C^{hi} bone marrow cells after CSF1 or IFN- γ in vitro treatment. Results represent mean \pm SEM of three independent experiments.

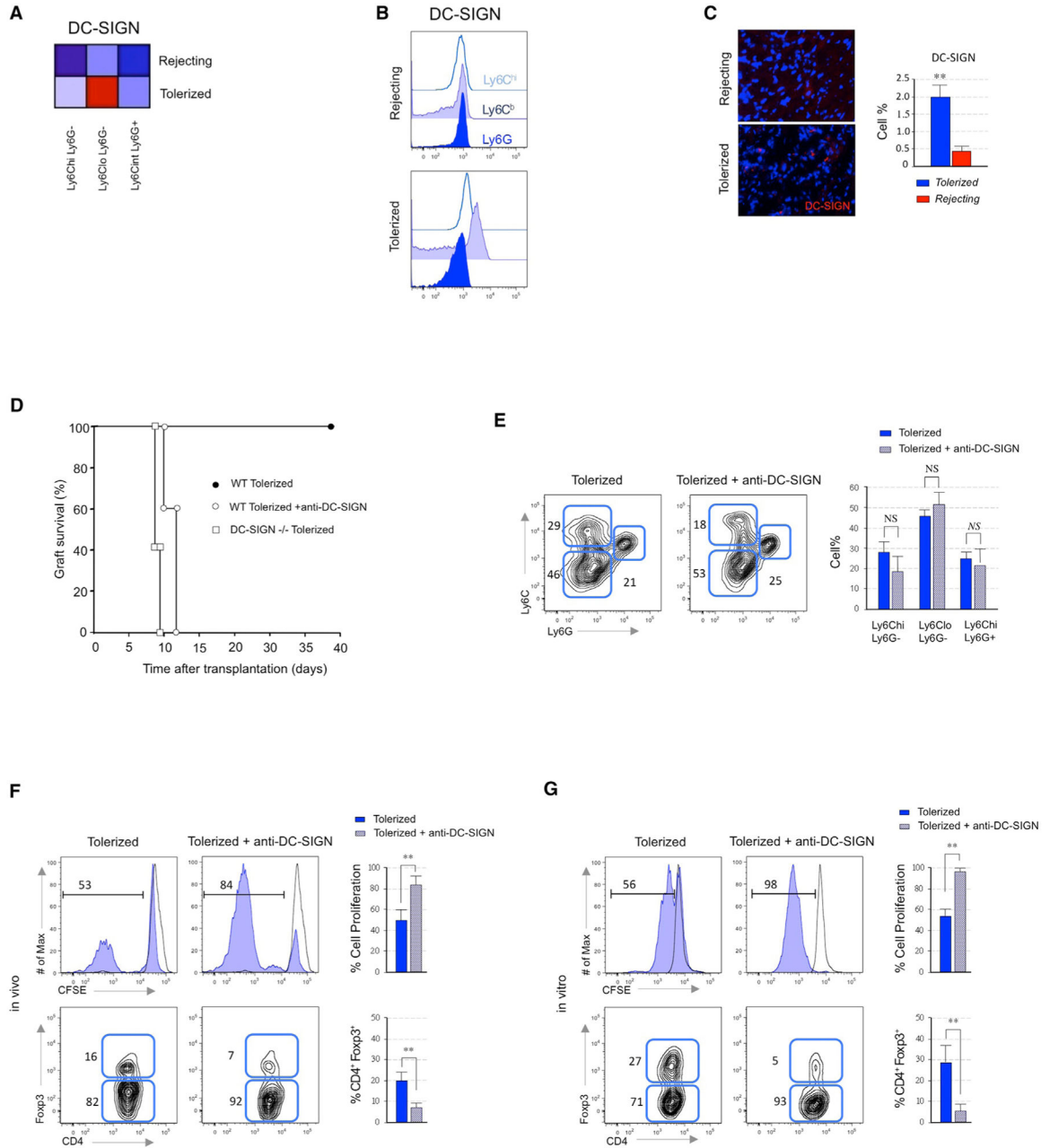


Figure 5. DC-SIGN Controls the Function of Suppressive Macrophages

(A and B) Heatmap derived from microarray data (A) and flow cytometry expression (B) of DC-SIGN in myeloid subsets from the allografts of tolerized and rejecting recipients at day 5 post-transplantation (means of n = 3 per group). Flow cytometry plots are representative of three independent experiments.

(C) Quantitative immunofluorescent analysis of tolerized and rejecting allografts at day 5 post-transplantation. Bar graphs represent frequency of DCSIGN⁺ cells expressed as percentage of a total of 1,000 DAPI nucleated cells from the allografts of tolerized and

rejecting mice. Results represent mean \pm SEM of 10 tissue sections from 4 cardiac allografts per group (**p < 0.01).

(D) Effects of DC-SIGN blockade and DC-SIGN deficiency on graft survival in tolerized WT recipients (n = 12 mice/group).

(E) Representative and quantitative flow cytometry results of recipient myeloid cell subsets in the allografts of tolerized WT recipients co-treated with anti-DC-SIGN mAb. Results represent mean \pm SEM (n = 4 mice per group of 3 independent experiments).

(F) Representative and quantitative flow cytometry results of in vitro suppressive capacity and Treg expansion of Ly6C^{lo} macrophages from tolerized recipients co-treated with anti-DC-SIGN. Results represent mean \pm SEM (n = 4 mice per group of 3 independent experiments).

(G) Suppressive function of Ly6C^{lo} macrophages from tolerized recipients after in vitro treatment with anti-DC-SIGN mAb. Results represent mean \pm SEM of three independent experiments.

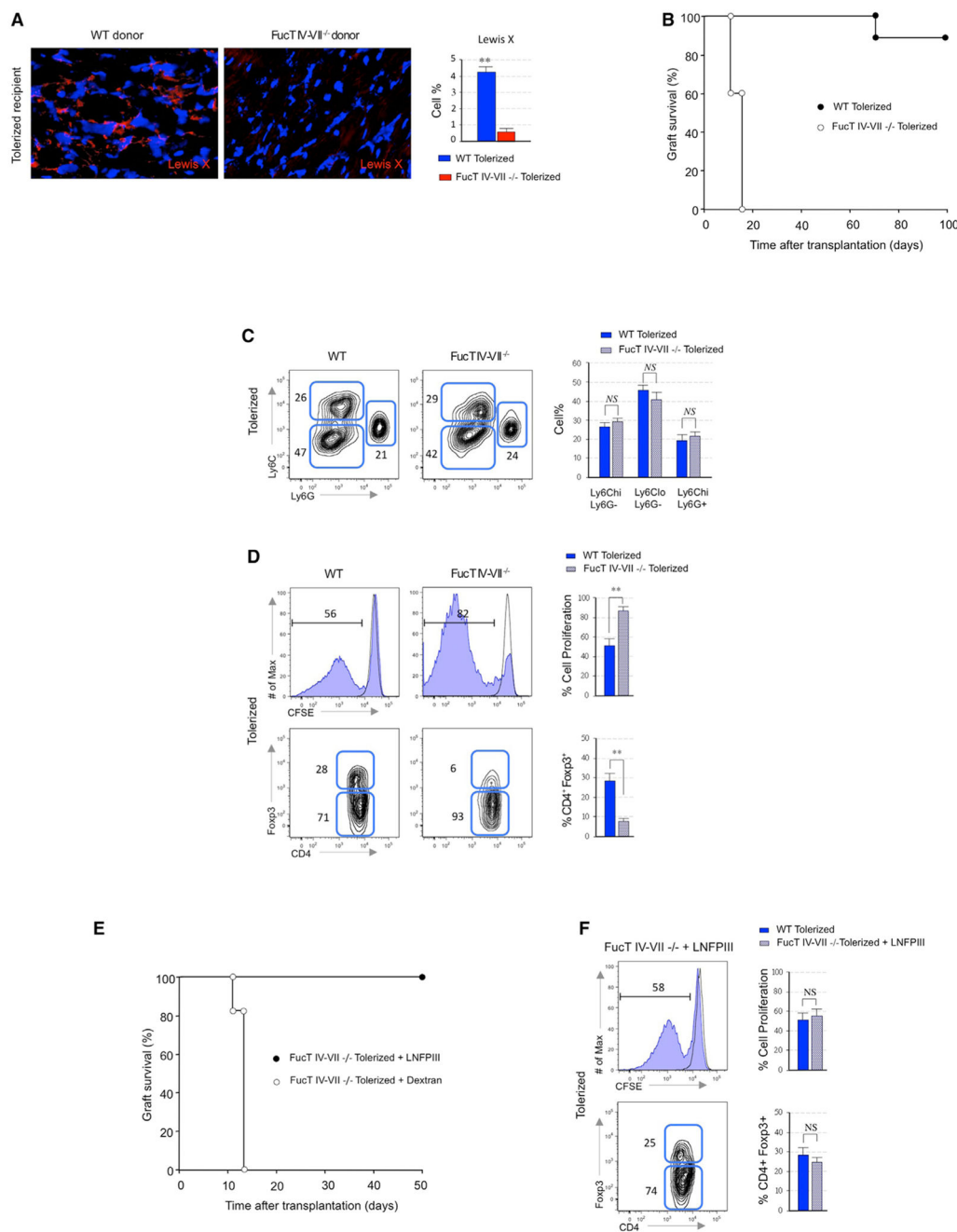


Figure 6. Fucosylated DC-SIGN Ligands Are Required for Macrophage Mediated Suppression
 (A) Quantitative immunofluorescent analysis of tolerized and rejecting allografts at day 5 post-transplantation. Bar graphs represent frequency of Lewis X⁺ cells expressed as percentage of a total of 1,000 DAPI nucleated cells from tolerized mice receiving WT and fucosyltransferase (Fut) IV and VII double-deficient donor allografts. Results represent mean ± SEM of ten tissue sections from four cardiac allografts per group (**p < 0.01).
 (B) Effects of Lewis^X deficiency on graft survival in tolerized WT recipients (n = 12 mice/group).

(C) Representative and quantitative flow cytometry results of recipient myeloid cell subsets in tolerized mice type receiving WT and FucT IV-VII dKO donor allografts. Results represent mean \pm SEM (n = 4 mice per group of 3 independent experiments).

(D) Representative and quantitative flow cytometry results of in vitro suppressive capacity and Treg expansion of Ly6C^{lo} macrophages from the allografts of tolerized mice receiving WT and FucT IV-VII dKO donor allografts. Results represent mean \pm SEM (n = 4 mice per group of 3 independent experiments). (E) Effects of Lacto-N-fucopentaose III (LNFPIII) on graft survival in tolerized wild-type recipients (n = 8 mice/group).

(F) Representative and quantitative flow cytometry results of in vitro suppressive capacity and Treg expansion of Ly6C^{lo} macrophages from the allografts of tolerized mice receiving WT and FucT IV-VII dKO donor allografts following administration of LNFPIII. Results represent mean \pm SEM (n = 4 mice per group of 3 independent experiments).

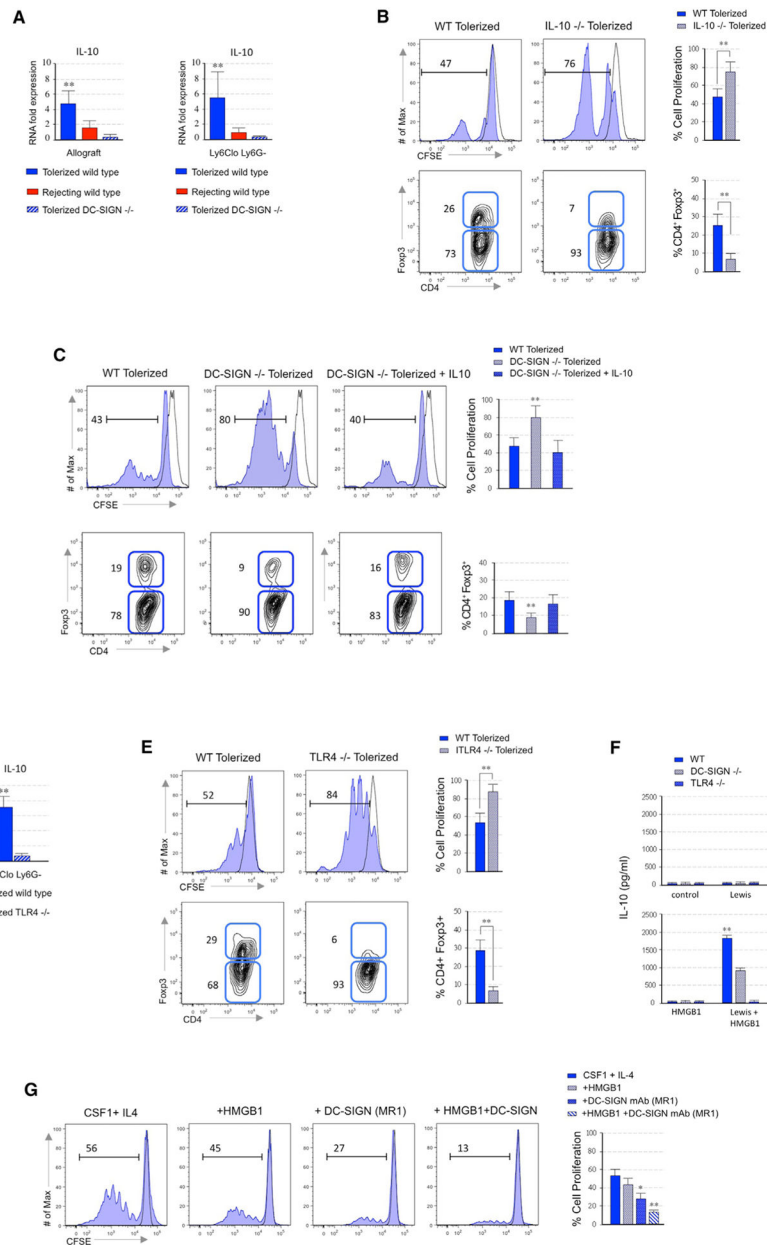


Figure 7. IL-10 Is Essential for DC-SIGN Mediated Suppression

(A) IL-10 expression in cardiac allografts and Ly6C^{lo} macrophages. Cardiac allografts from WT untreated, WT tolerized, and DC-SIGN KO tolerized recipients were harvested 5 days after transplantation. Total single cell suspensions and Ly6C^{lo} cells were analyzed for IL-10 measured by real-time PCR. Bar graphs represent mean ± SEM of three independent experiments (**p < 0.01).

(B) Representative and quantitative flow cytometry results of in vitro suppressive capacity and Treg expansion of Ly6C^{lo} macrophages from the allografts of tolerized IL-10 deficient recipient mice. Results represent mean ± SEM (n = 4 mice per group of 3 independent experiments).

(C) Representative and quantitative flow cytometry results of in vitro suppressive capacity and Treg expansion of Ly6C^{lo} macrophages from the allografts of tolerized DC-SIGN deficient recipient mice receiving in vitro IL-10 stimulation for 72 hr at 10 ng/ml. Results represent mean \pm SEM (n = 4 mice per group of 3 independent experiments).

(D) IL-10 expression in cardiac allografts. Cardiac allografts from tolerized WT and TLR4 KO recipients were harvested 5 days after transplantation. Total single cell suspensions were analyzed for IL-10 measured by real-time PCR. Bar graphs represent mean \pm SEM of three independent experiments (**p < 0.01). (E) Representative and quantitative flow cytometry results of in vitro suppressive capacity and Treg expansion of Ly6C^{lo} macrophages from the allografts of tolerized TLR4 deficient recipient mice. Results represent mean \pm SEM (n = 4 mice per group of 3 independent experiments).

(F) IL-10 expression in stimulated bone marrow cells from WT, DC-SIGN-deficient, and TLR4-deficient mice. Bone marrow cells were stimulated with Lewis^X (10 μ g/ml) and recombinant HMGB1 (10 μ g/ml) for 72 hr in vitro stimulation (control group non-stimulated). Supernatants of single cell suspensions were analyzed for IL-10 measured by ELISA. Results represent mean \pm SEM of three independent experiments (**p < 0.01).

(G) Representative and quantitative flow cytometry results for in vitro suppressive capacity of human monocytes cultured with CSF1 plus IL-4. Results represent mean \pm SEM of three independent experiments (*p < 0.05, **p < 0.01).