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CD40/CD154 Blockade Inhibits Dendritic Cell Expression of Inflammatory Cytokines but Not Costimulatory Molecules

Ivana R. Ferrer,1 Danya Liu,1 David F. Pinelli, Brent H. Koehn, Linda L. Stempora, and Mandy L. Ford

Blockade of the CD40/CD154 pathway remains one of the most effective means of promoting graft survival following transplantation. However, the effects of CD40/CD154 antagonism on dendritic cell (DC) phenotype and functionality following transplantation remain incompletely understood. To dissect the effects of CD154/CD40 blockade on DC activation in vivo, we generated hematopoietic chimeras in mice that expressed a surrogate minor Ag (OVA). Adoptive transfer of OVA-specific CD4+ and CD8+ T cells led to chimerism rejection, which was inhibited by treatment with CD154 blockade. Surprisingly, CD154 antagonism did not alter the expression of MHC and costimulatory molecules on CD11c+ DCs compared with untreated controls. However, DCs isolated from anti-CD154–treated animals exhibited a significant reduction in inflammatory cytokine secretion. Combined blockade of inflammatory cytokines IL-6 and IL-12 p40 attenuated the expansion of Ag-specific CD4+ and CD8+ T cells and transiently inhibited the rejection of OVA-expressing cells. These results suggest that a major effect of CD154 antagonism in vivo is an impairment in the provision of signal three during donor-reactive T cell programming, as opposed to an impact on the provision of signal two. We conclude that therapies designed to target inflammatory cytokines during donor-reactive T cell activation may be beneficial in attenuating these responses and prolonging graft survival. The Journal of Immunology, 2012, 189: 4387–4395.

In both bone marrow (BM) and solid organ transplantation models, specifically targeting graft-reactive T cell responses to prevent transplant rejection remains an important goal. Many studies in murine and nonhuman primate models have shown that blockade of costimulatory signals promotes survival of BM, skin, kidney, heart, and islet transplants (1–5). Blockade of the CD40/CD154 costimulatory pathway remains one of the most effective means of inhibiting alloreactive T cell responses and inducing long-term graft survival following transplantation. However, mAbs designed to target CD154 resulted in thromboembolic events in early pilot studies in humans (6). Renewed interest in blockade of this pathway for the prevention of graft rejection has been sparked by promising results from several recent studies in nonhuman primate transplant models using mAbs directed against CD40 (7–9), and clinical trials using CD40 blockers in renal transplant recipients are now underway (10). Thus, the therapeutic potential of targeting this pathway is high, and understanding the effects of CD154/CD40 blockade in transplant models may uncover other novel downstream targets for therapeutic intervention.

Despite the incontrovertible efficacy of blockade of this pathway, the mechanisms underlying its effect are incompletely understood.

Although one report indicated that anti-CD154 mAbs may impact the outcome of graft rejection by specifically binding to and depleting Ag-specific CD4+ T cells that express CD154 following activation (11), subsequent studies using anti-CD40 mAbs showed similar efficacy in both BM and solid organ transplant models in mouse and nonhuman primates (7–9, 12–14). Thus, it is likely that blockade of the CD40/CD154 pathway, rather than Ab-mediated depletion of Ag-specific cells, plays a major role in the observed attenuation of graft rejection. In dendritic cells (DCs), ligation of CD40 by CD154 expressed on activated CD4+ T cells leads to the activation of downstream signaling pathways, resulting in several key events that promote the generation of effective T cell responses. These include increasing MHC expression that would enhance the strength of TCR signals (15, 16); inducing costimulatory molecule expression (e.g., CD80, CD86, OX40L), thus enhancing the strength of “second signals” (17, 18); increased production of proinflammatory cytokines (IL-12, IL-6, IL-1), sometimes referred to as “signal three” (19); and increasing DC longevity (20, 21), thereby enhancing T cell priming via all of the above mechanisms. However, the impact of CD154/CD40 blockade on these aspects of DC biology remains incompletely characterized.

In this study, we hypothesized that blockade of the CD40/CD154 pathway in vivo would result in altered DC phenotype and/or function, which, in turn, could result in suboptimal T cell priming and, thus, lead to protection of hematopoietic chimerism following BM transplantation. To test this hypothesis, we generated hematopoietic chimeras that expressed membrane-bound chicken OVA on all hematopoietically derived cells. Subsequent adoptive transfer of OVA-specific CD8+ OT-I and CD4+ OT-II T cells led to a graft-versus-host disease (GVHD)-like response, as measured by a loss of OVA-expressing cells, a process that was attenuated by treatment with anti-CD154 mAb (MR-1). To dissect the effects of CD154/CD40 blockade on DC activation in vivo, Ag-bearing splenic DCs were isolated from recipients that had received host-reactive CD4+ and CD8+ T cells in the presence or absence of anti-CD154 mAb. Results demonstrated that DCs derived from...
anti-CD154–treated recipients did not differ with regard to their expression levels of MHC or costimulatory molecules but instead exhibited impaired secretion of inflammatory cytokines.

Materials and Methods

**Mice**

BALB/c (CD45.2, H-2K^b^), C57BL/6 (CD45.2, H-2K^b^), and B6-Ly5.2/Cr (CD45.1, H-2K^b^) mice were obtained from the National Cancer Institute (Charles River, Frederick, MD). OT-I and OT-II TCR transgenic mice (C57BL/6 background) were bred to C57BL/6 Thy1.1 congenic animals at Emory University. OT-II × RAG^−/−^ TCR transgenic mice were purchased from Taconic and bred to Thy1.1 congenic animals at Emory University. mOVA mice on a C57BL/6 background (22) were a gift from Dr. Marc Jenkins (University of Minnesota, Minneapolis, MN) and were maintained at Emory University. All animals were housed in pathogen-free animal facilities at Emory University, and all studies were approved by the Institutional Animal Care and Use Committee of Emory University.

**BM isolation and establishment and screening of mOVA BM chimera**

Recipient B6-Ly5.2/CR (CD45.1) (NCI) mice were treated 1 d prior to BM adoptive transfer with 500 µg i.p. busulfan (Busulufex, Otsuka America Pharmaceutical). BM was flushed from femurs and tibias of mOVA mice and transferred into mOVA BM chimeric mice. At the time of transfer, mice were treated with 500 µg anti-CD154 mAb (MR-1), with continued treatment on days 0, 2, 4, and 6 in the indicated groups, or were left untreated in control groups.

**DC isolation and flow cytometric analysis**

Spleens were removed from mice. One milliliter of 2 mg/ml collagenase type 3 (Worthington Biochemical, Lakewood, NJ) in HBSS (with Ca^2+^/Mg^2+^) was injected into the spleen, which was then incubated with 2 ml collagenase solution at 37˚C/5% CO2 for 30 min. Single-cell suspensions were prepared after incubation by passing the spleen through a 3-m syringe plunger on a cell strainer (70 µm) and washing cells with PBS. Single-cell suspensions were stained for flow cytometric analysis with anti–CD11c–allophycocyanin, anti–CD86–FITC, anti–IFN-γ–PE (BD Pharmingen), and anti–IL-12/23p40 (clone C17.8, Bio X Cell) and anti–IL-6R (clone 15A7; Bio X Cell) on days 0, 2, 4, and 6, in the indicated groups, or were left untreated in control groups.

**Assessment of ex vivo cytokine production by DCs**

DCs were isolated as described above, and single-cell suspensions were enriched by negative selection using magnetic beads coated with anti-CD19, anti-CD90.1, and anti-CD90.2 mAbs (Miltenyi Biotec), according to the manufacturer’s instructions. Purified DCs (purity > 70%) were resuspended in RPMI 1640 supplemented with 10% heat-inactivated FBS, 50 µM 2-ME, streptomycin (100 µg/ml), and penicillin (100 U/ml). DCs were then distributed in 200-µl aliquots (6 × 10⁵ cells/well) in a 96-well plate and cultured for 24 h at 37˚C in 5% CO2 in duplicate. The cultured cell supernatants were measured for the levels of inflammatory cytokines by a cytometric bead array (CBA; BD Pharmingen), according to the manufacturer’s instructions.

**T cell intracellular cytokine staining**

To measure cytokine production by Ag-specific T cells, surface and intracellular stains were performed with mAbs to CD8-Pacific Orange (Invitrogen), CD4-Pacific Blue (Invitrogen), Thy1.1-PerCP (BD Pharmingen), TNF–PE–Cy (BD Pharmingen), and IFN-γ–FITC (BD Pharmingen). Spleens of chimeric mice were processed into single-cell suspensions and plated onto 96-well flat-bottom plates at 10⁶ cells/well. Cells were stimulated with 10 nM OVA257–264 (GenScript) and 10 µM OVA323–339 (GenScript) in the presence of 10 µg/ml brefeldin A for 5 h. Cells were processed with an intracellular staining kit (BD Biosciences), according to the manufacturer’s instructions. As a positive control, cells were stimulated with...
10 ng/ml PMA and 1 μg/ml ionomycin (Sigma). Data were acquired on a LSR II flow cytometer (Becton Dickinson) and analyzed using FlowJo software (TreeStar).

**DC purification, RNA isolation, and real-time PCR analysis**

For DC isolation and purification, single-cell suspensions were first enriched by negative selection using magnetic beads coated with anti-CD19, anti-CD90.1, and anti-CD90.2 mAbs, as described above (yielding ~70% purity of CD11c+ cells). Following enrichment, CD11c+ Thy1.1+ cells were further purified by FACS sorting on a BD FACS Aria. Following FACS sorting, DC populations were >90% CD11c+ cells.

RNAs from the sorted DCs were isolated using an RNeasy Isolation kit (Qiagen). Reverse transcription of the RNA into cDNA was performed using a TaqMan reverse-transcription kit (Roche). Mouse immune array cards (Applied Biosystems) were used to obtain a real-time PCR analysis of the selected immune-related genes. Arrays were run on a 7900HT Real-Time PCR System from ABI.

**Results**

**CD154/CD40 pathway blockade prevents Ag-specific T cell-mediated host cell destruction**

To evaluate the impact of CD154/CD40 blockade on the induction and/or abortion of donor-reactive T cell responses, we developed a modified GVHD model in which Ag-specific CD4+ and CD8+ T cells recognized and rejected cognate Ag-bearing hematopoietic cells following BM transplantation. Using an OVA-expressing transgenic mouse (mOVA, B6 background), we generated hematopoietic chimeras in which busulfan-treated B6 host mice (CD45.1+) received a CD45.2+ BM transplant expressing a single defined alloantigen (OVA). Adoptive transfer of 5 x 10^6 Ag-specific CD8+ (OT-I) and 10^6 Ag-specific CD4+ (OT-II) T cells were sufficient to induce rejection of the CD45.2+ mOVA-expressing BM cells, such that by day 6, the number of donor-derived (CD45.2+) peripheral B cells was significantly reduced (Fig. 1A). Both CD4+ and CD8+ T cells were required to mediate this effect, because adoptive transfer of either OT-I or OT-II cells alone failed to result in rejection (Supplemental Fig. 1A, 1B). Importantly, treatment of animals with a short course of CD154 blockade (MR-1) on days 0, 2, 4, and 6 posttransfer resulted in protection of the BM from rejection (Fig. 1). These results indicated that blockade of the CD154/CD40 pathway alone was sufficient to prevent T cell-mediated destruction of OVA-expressing peripheral leukocytes and preserve hematopoietic chimerism.

**CD154/CD40 blockade attenuates Ag-specific T cell expansion and effector function**

To address the potential mechanisms by which this protection occurred, we analyzed the host-reactive CD4+ (OT-II) and CD8+ T cell accumulation but not entry into cell division. mOVA chimeric mice were adoptively transferred with 5 x 10^6 CFSE-labeled Thy1.1+ OT-I and 10^6 CFSE-labeled Thy1.1+ OT-II cells and treated with four doses of MR-1 on days 0, 2, 4, and 6, where indicated. (A and D) Representative flow plots of Ag-specific Thy1.1+ T cell responses in the spleens of treated mice. Data displayed are gated on Thy1.1+ CD8+ or CD4+ T cells on day 4 posttransfer. Frequency of accumulated Ag-specific CD8+ (B) and CD4+ (E) T cells at day 4 posttransfer. Absolute numbers of accumulated Ag-specific CD8+ (C) and CD4+ (F) T cells at day 4 posttransfer. (G and H) CFSE analysis of cell division of Ag-specific CD8+ T cells at days 2 and 3 posttransfer in both untreated and anti-CD154–treated recipients. Data shown are gated on Thy1.1+ CD8+ T cells in spleens of mice. Data are representative of two or three experiments with a total of 8–12 mice/group. Statistics shown are mean ± SEM. *p ≤ 0.05, **p < 0.0001.
(OT-I) T cell responses in anti-CD154–treated animals. We observed that although host-reactive CD8+ T cells expanded dramatically in untreated animals by day 4 posttransfer (Fig. 2A–C), treatment with anti-CD154 resulted in a significant diminution in the Ag-specific CD8+ T cell response (p < 0.0001). A similar result was observed for Ag-specific CD4+ T cell responses, which exhibited a >2-fold reduction in the presence of CD154/CD40 blockade (Fig. 2D–F). This was true for both T cell frequencies and absolute numbers (Fig. 2A–F).

To address whether reduced frequencies of host-reactive T cells were due to reduced activation and proliferation or increased cell death, host-specific CD8+ T cells were labeled with CFSE prior to transfer into OVA/B6 chimeras. Results demonstrated similar levels of CFSE dilution in anti-CD154–treated animals compared with PBS-treated animals (Fig. 2E, 2F), suggesting that the reduced accumulation of host-reactive CD8+ T cells was likely due to increased apoptosis, rather than impaired expansion, in the presence of CD40/CD154 pathway blockade.

The functionality of the remaining graft-specific CD8+ T cells was further assessed by intracellular cytokine staining following ex vivo restimulation with cognate Ag peptide over time. Results revealed that anti-CD154 treatment delayed the differentiation of Ag-specific CD8+ T cells into cytokine-producing cells compared with untreated controls. For example, frequencies of IFN-γ–producing cells at day 4 were significantly reduced, as were frequencies of IFN-γ+TNF+ dual-cytokine producers at day 10 (Fig. 3A, 3B). Absolute numbers of IFN-γ–producing cells were significantly reduced in anti-CD154–treated animals at day 4 but not

**FIGURE 3.** CD154/CD40 blockade attenuates Ag-specific T cell expansion and effector function. mOVA chimeric mice were adoptively transferred with 5 x 10^6 OT-I and 10^6 OT-II cells and treated with four doses of MR-1, where indicated. (A) Representative flow plots of longitudinal analysis of intracellular TNF and IFN-γ cytokine staining of Ag-specific Thy1.1+ CD8+ T cells following a 4-h ex vivo peptide stimulation. (B) Summary data of frequencies of IFN-γ–producing cells (day 4) and IFN-γ+TNF+ dual producers (days 10, 17, 24) as a percentage of Ag-specific Thy1.1+ CD8+ T cells. (C) Absolute numbers of total IFN-γ–producing OT-I T cells over time following adoptive transfer. Data are representative of two or three experiments with a total of 8–12 mice/group. Statistics shown are mean ± SEM. *p ≤ 0.05.
CD8+ Ag-reactive T cells are required to precipitate rejection of engraftments, however, in this GVHD model wherein both CD4+ and CD8+ T cells are required to precipitate rejection of Ag-bearing cells (Supplemental Fig. 1A, 1B), we observed that both cell types were required to elicit optimum costimulatory molecule expression (Supplemental Fig. 1C) and cytokine secretion (Supplemental Fig. 1D) from DCs. Because the observed effects of CD40/CD154 blockade on host-reactive T cell responses were likely a direct result of alterations in DC phenotype and functionality, we endeavored to determine which aspects of DC activation were altered in the presence of CD40/CD154 blockade.

We first characterized the frequency and phenotype of splenic DCs in mOVA BM chimeras following the adoptive transfer of host-reactive T cells. mOVA BM chimeras contained 1.75 ± 0.77 × 10^6 total DCs/spleen (Fig. 4A), and >90% of those were CD45.2+ and expressing the OVA Ag (data not shown). Adoptive transfer of host-reactive CD4+ and CD8+ T cells did not result in a change in the total number of splenic DCs at day 3 posttransfer, either in the presence or absence of CD154 blockade (1.59 ± 0.44 × 10^6 and 2.04 ± 1.04 × 10^6, respectively, Fig. 4B). In addition, the relative proportions of CD11c+ CD11b+ CD8α− versus CD11c+ CD11b− CD8α+ DCs were not altered in the presence of CD154/CD40 blockade (Fig. 4C–E). Thus, these data demonstrate that blockade of the CD40/CD154 pathway did not impact the overall quantity or myeloid/lymphoid phenotype of DCs in GVHD recipients.

We next examined the impact of CD154 blockade on the expression of MHC (signal one) and costimulatory (signal two) molecules on DCs. The DC licensing model predicts that CD40 ligation results in upregulation of MHC and costimulatory molecules. This was confirmed in our model with experiments in which an agonistic anti-CD40 mAb (FGK4.5) was injected into mice, and splenic DCs were observed to express high levels of MHC and costimulatory molecules (data not shown). Next, we confirmed that CD4+ T cells in our system could provide the CD40 stimulus necessary for DC licensing. First, as predicted, CD154 was upregulated on Ag-specific CD4+ T cells (data not shown). Second, following adoptive transfer of host-reactive CD4+ and CD8+ T cells into mOVA chimeric recipients, provision of T cell-derived CD154 signals resulted in a statistically significant increase in class I (H2-Kb) expression on the surface of splenic DCs at day 3 posttransfer (Fig. 5). Likewise, expression of CD86 and CD40 were also increased (Fig. 5). Surprisingly, however, treatment with CD40/CD154 blockade failed to attenuate the expression of class I or class II MHC or the expression of costimulatory molecules on total CD11c+ splenic DCs during graft-specific T cell priming (Fig. 5). We also assessed the expression of costimulatory molecules on individual CD11c+ DC subsets (data not shown), as well as on CD11b+ CD11c+ myeloid cells (Supplemental Fig. 2) in animals left untreated or treated with anti-CD154; again, we observed no difference in the expression of class I or class II MHC or CD40, CD80, or CD86. Therefore, these results indicate that diminution in the provision of either signal one or signal two is not the mechanism by which CD154 blockade attenuates graft-specific T cell responses and promotes long-term graft survival.

Next, to assess the impact of CD154 blockade on the provision of signal three in this model, we conducted similar experiments in which host-reactive Thy1.1+ CD4+ and CD8+ T cells were adoptively transferred into mOVA chimeric recipients. On day 3 posttransfer, CD11c+ splenic DCs were FACS sorted and either cultured in vitro for 24 h or cultured in vitro for 24 h and supernatant assessed for cytokine production. Results demonstrated that the adoptive transfer of Ag-specific T cells resulted in a profound increase in the ability of DCs to produce proinflammatory cytokines. Specifically, DCs isolated from T cell adoptive-transfer recipients exhibited an increase in IL-6 and TNF at the protein level, as well as in IL-12 and IL-1β at the mRNA level (Fig. 6). Importantly, DCs isolated from mice that had been treated with CD154 blockade exhibited significantly reduced IL-6, TNF, IL-12p35, and IL-1β production on
Neither IL-12p35 nor IL-1β was detected at the protein level in any of the groups, suggesting that the expression of these cytokines in this in vitro assay was below the limit of detection. These data suggest that a predominant effect of CD154 blockade in this system may be to inhibit the provision of inflammatory cytokines (signal three) to Ag-specific T cells during priming.

Combined IL-6/IL-12p40 blockade attenuates Ag-specific T cell expansion and transiently protects from GVHD

To assess the contribution of reduced provision of signal three on the observed prolongation in graft survival associated with treatment with anti-CD154, we used a similar experimental approach in which adoptive transfer recipients of host-reactive CD4+ and CD8+ T cells were left untreated, were treated with anti-CD154 as a positive control, or were treated with a combination of anti–IL-6R and anti–IL-12p40, two inflammatory cytokines which were diminished in DCs isolated from anti-CD154–treated recipients. We chose to investigate the impact of blockade of IL-6 and IL-12 in these experiments based on previous evidence in the literature indicating that these cytokines can profoundly impact both CD4+- and CD8+-differentiation programs (23–27). Results indicated that although untreated animals exhibited a significant loss in OVA-expressing CD45.2+ B cells by day 4 posttransfer (Fig. 7A, p = 0.045), animals treated with either anti-CD154 or the combination of anti–IL-6R and anti–IL-12p40 did not exhibit a significant loss in chimerism. Importantly, combined anti–IL-6R/anti–IL-12p40 protected the animals from the host-reactive immune response to a similar degree as did anti-CD154 at this time point (Fig. 7A). We further evaluated the host-reactive CD4+ and CD8+ T cell responses in these animals and observed that the reduction in the expansion of host-reactive CD4+ and CD8+ T cells in animals treated with combined IL-6/IL-12p40 blockade was comparable to that observed in anti-CD154–treated animals (Fig. 7B, 7C, p < 0.05, compared with untreated controls). However, analysis of both survival of OVA-expressing CD45.2+ B cells and expansion of host-reactive CD4+ and CD8+ T cells at later time points (days 7, 10, and 14 posttransplant) revealed that the protection afforded by IL-6/IL-12p40 blockade was not durable; OVA-expressing cells were eventually rejected, and host-reactive CD4+ and CD8+ T cells expanded to frequencies comparable to those observed in untreated animals (data not shown).

Discussion

We observed that although blockade of CD40/CD154 did not alter the level of expression of class I or class II MHC or costimulatory
molecules on the surface of DCs, it significantly altered the differentiation of these cells, specifically with regard to their ability to secrete the inflammatory cytokines IL-6, IL-12p40, and TNF. These results suggest that blockade of CD40 ligation on DCs during the course of graft rejection critically impacts the provision of signal three to developing donor-reactive T cell populations, with less of an impact on the provision of costimulation (signal two). These data provide a mechanistic basis for the observed synergy between blockade of the CD40 and CD28 pathways and suggest that therapies designed to target the provision of inflammatory cytokines during the generation of donor-reactive T cell responses may be beneficial in attenuating these responses and prolonging graft survival.

Our results demonstrating that CD154 antagonism (MR-1) does not inhibit CD4+ T cell-induced upregulation of class I or class II MHC on DCs are surprising, given the known role of CD154 in DC licensing. Indeed, our results confirmed reports by many other groups demonstrating that activated CD154+ CD4+ T cells led to the upregulation of MHC and costimulatory molecules on DCs (28–30). There are two potential reasons why anti-CD154 failed to inhibit this activity. First, it is possible that the Ab does not completely block cell-associated CD154 binding to CD40 expressed on the surface of DCs. Altered CD154 binding could result in a partial signal delivered to the APCs, resulting in the upregulation of MHC and costimulatory molecules but not the elaboration of inflammatory cytokines. CD40 signaling in DCs is mediated by binding of individual TNFR-associated factors (TRAFs) to the intracellular domain, and previous studies revealed that the CD40 TRAF2/3 binding site is critical for costimulatory molecule expression, whereas the TRAF6 binding site is required for production of inflammatory cytokines (31). Thus, it is possible that binding of anti-CD154 mAb inhibits the ability of TRAF6 but not TRAF2/3 to be recruited to the CD40 intracellular domain, and experiments to test this hypothesis are ongoing. Our results are also consistent with the alternate possibility that there are CD154-dependent mechanisms by which cognate T cells can upregulate costimulatory molecule expression but not cytokine production on DCs.

Our results also suggest that this diminution in inflammatory cytokines likely contributes to the observed ability of anti-CD154 to mitigate antihost T cell responses, in that treatment of animals with a combination of anti–IL-6 and IL-12p40 Abs partially recapitulated the effects of anti-CD154. Specifically, antagonism of these cytokines transiently attenuated the expansion of both Ag-specific CD4+ and CD8+ T cell populations and impaired their ability to reject host cells. These results are supported by evidence in the literature suggesting that both IL-12– and IL-6–mediated signals can function to enhance the expansion and differentiation of Ag-specific T cells. With regard to IL-12, seminal studies showed that this cytokine was as effective as CFA for inducing clonal expansion, differentiation into competent effectors, and generation of long-lived memory cells in Ag-specific T cell populations (23–25). Importantly, the absence of IL-12 in these studies rendered the T cells tolerant (24), again suggesting that inhibition of inflammatory cytokine signaling may be an important facet of CD154 antagonism. Likewise, IL-6 has been identified as a predominant inflammatory cytokine associated with the enhancement of alloimmune responses, insofar as neutralization of IL-6 was shown to significantly delay graft rejection, diminish differentiation of alloreactive effectors, and impact the Th1/Th2 balance during allograft rejection (32, 33). Furthermore, the inhibition of IL-6 and IL-17 was shown to protect cardiac allografts from rapid rejection (34), and the combined reduction of TNF and IL-6 was shown in an in vitro system to reduce allograft-specific T cell proliferation and differentiation (35).

Another interesting aspect of our study was the finding that adoptive transfer of both CD4+ and CD8+ Ag-specific T cells was required for the optimal upregulation of MHC, costimulatory molecules, and cytokines in this model. Traditional DC-licensing models suggest that binding of activated CD4+ T cells is sufficient to license DCs (28–30). However, our data are consistent with other reports in models of viral infection showing that CD8+ T cells can “license” resting DCs (36), and this effect was later shown to be mediated specifically through the elaboration of GM-CSF (37). Thus, future studies on the effect of CD154 antagonism of CD8+ T cell-derived GM-CSF in this model are warranted.

Taken together, this study demonstrates that, although CD40/CD154-pathway blockade does not significantly alter the costimulatory receptor profile of DCs, it does alter their cytokine secretion profile. Although blockade of two such inflammatory cytokines was insufficient to recapitulate the effects of CD40 blockade, the observed attenuation in host-reactive T cell expansion and delay in GVHD progression suggests that decreased inflammatory cytokine production likely contributes to the observed effects of CD40/CD154 blockade. Thus, targeting DC-derived inflammatory cytokines in clinical transplantation could lead to attenuated alloreactivity and improved outcomes.
Frequency of Ag-specific CD8\(^+\) and CD4\(^+\) T cells at day 4 posttransfer. Data are representative of two experiments with a total of six mice/group. Statistics

FIGURE 7. Combined IL-6/IL-12p40 antagonism attenuates Ag-specific T cell expansion and transiently protects from GVHD. mOVA chimeric mice were adoptively transferred with 5 \times 10^5 OT-I and 10^6 OT-II T cells and treated with anti–IL-6R and anti-IL-12p40 on days 0, 2, 4, and 6, where indicated. (A) Total number of CD45.2\(^+\) mOVA\(^+\) B cells in the blood prior to T cell transfer (day 0) and on day 4 posttransfer. (B) Representative flow plots of Ag-specific Thy1.1\(^+\) T cell responses in the spleens of treated mice. Data displayed are gated on Thy1.1\(^+\) CD8\(^+\) or CD4\(^+\) T cells on day 4 posttransfer. (C) Frequency of Ag-specific CD8\(^+\) and CD4\(^+\) T cells at day 4 posttransfer. Data are representative of two experiments with a total of six mice/group. Statistics shown are mean ± SEM. *p < 0.05.

Disclosures

The authors have no financial conflicts of interest.

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