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Anti-CD28 Antibodies Modify Regulatory Mechanisms and Reinforce Tolerance in CD40Ig-Treated Heart Allograft Recipients[1]

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Blockade of CD40-CD40 ligand (CD40L) costimulation has been shown to synergize with that of CTLA4/CD28-B7 to promote transplant tolerance. To date, however, CD28-B7 interactions have been prevented using B7-blocking reagents like CTLA4-Ig that inhibit CD28-B7 together with CTLA4-B7 interactions. In this study, we have tested anti-CD28 Abs to prevent selectively CD28-B7 interactions while preserving CTLA4-B7 in addition to CD40-CD40L blockade. In the LEW.1W to LEW.1A rat combination, interfering with CD40-CD40L interactions by CD40Ig administration through gene transfer resulted in indefinite heart allograft survival due to the appearance of clonotypic $CD8^+CD45RC^{low}$ regulatory T cells that were capable of transferring the tolerant state to naive animals. However, cardiac transplants in these recipients systematically developed chronic rejection lesions. Whereas anti-CD28 Ab monotherapy only delayed acute rejection and failed to induce tolerance, coadministration of anti-CD28 Abs and CD40Ig resulted in the long-term acceptation of allografts without chronic rejection lesions in 60% of the recipients, reduced the level of intragraft mRNA transcripts for cytokines and immune factors, and fully abrogated alloantibody production. In addition, the nature of regulatory cells was modified: the $CD8^+CD45RC^{low}$ clonotypic T cells described in the CD40Ig-treated animals could not be found in cotreated animals, and the other $CD8^+CD45RC^{low}$ cells had no regulatory activity and a different cytokine expression profile. Instead, in cotreated recipients we found IDO-dependent non-T cells with regulatory activity in vitro. Thus, the addition of a short-term anti-CD28 treatment with CD40Ig resulted in decreased heart allograft chronic rejection lesions, complete inhibition of Ab production, and modified regulatory mechanisms. ***The Journal of Immunology,*** **2007, 179: 8164–8171.**

T cell costimulatory pathways have drawn considerable interest in transplantation as targets for the prevention of allograft rejection. CD40-CD40 ligand (CD40L)[5] and CD28-B7 are the most extensively studied T cell costimulatory pathways, and their role in supporting T cell activation and acute allograft rejection has been well established (1). Interaction between CD28 on T cells and B7-1/B7-2 on APCs plays an important role in T cell-mediated immune responses, including activation and proliferation of alloreactive T cells (2). The CD40-CD40L costimulatory pathway cooperates with the CD28-B7 pathway to activate T cells (3). Nevertheless, blockade of the CD40-CD40L pathway (using anti-CD40/CD40L mAb, the recombinant molecule CD40Ig, or CD40 knockout mice) and the CTLA4/CD28-B7 pathway with CTLA4Ig, alone or in combination, circumvents acute allograft rejection and yet cannot prevent the development of chronic rejection lesions (4–9), demonstrating that these treatments do not induce complete tolerance. We recently reported that CD40Ig treatment of rats resulted in heart allograft acceptance mediated by $CD8^+CD45RC^{low}$ T cells acting through IFN-γ production that, in turn, induces IDO expression by graft endothelial cells (10). These regulatory cells were present in the spleen and were able to transfer graft acceptance to secondary recipients, yet with a chronic rejection lesion developing within 4 months.

CD28-B7 interactions can also be selectively inhibited using anti-CD28 Abs (11). Because these Abs target CD28 and not B7, they do not inhibit the CTLA4-B7 interactions that are important for the development of tolerance (12, 13) and for the function of regulatory T cells (Treg) (14). Therefore, as compared with

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[5] Abbreviations used in this paper: CD40L, CD40 ligand; AdCD40Ig, adenoviral vector coding extracellular portion of mouse CD40 fused to human IgG1 constant domain coding sequences; Addl324, adenovirus dl324 (noncoding adenovirus); AU, arbitrary unit; BN, Brown Norway; CR, CD40Ig plus anti-CD28 Ab-treated allograft recipient developing histological lesions of chronic rejection; C_t, threshold cycle; FoxP3, Forkhead box P3; HO-1, heme oxygenase-1; GITR, glucocorticoid-induced TNFR-related protein; HPRT, hypoxanthine phosphoribosyltransferase; IP, infectious particle; MCF, mean channel fluorescence; 1-MT, D-1-methyl-tryptophan; Tol, CD40Ig plus anti-CD28 Ab-treated allograft recipient displaying no histological lesions of chronic rejection; Treg, regulatory T cell.

CTLA4Ig, targeting CD28 might promote the development of regulatory mechanisms (15). Several studies have indeed demonstrated that the selective blockade of CD28 reduced T cell reactivity in autoimmunity (16, 17) and transplantation (18). In the rat, the JJ319 modulating CD28-specific mAb (19, 20) induced tolerance to kidney (21, 22) but not to heart allograft (11, 23). However, in a rat model of chronic rejection after heart allograft it promoted long-term survival (23).

The purpose of the current study was to investigate the effect of selectively targeting CD28 in a model of chronic vascularized cardiac allograft rejection following CD40L blockade (10). We hypothesized that the modulation of CD28 expression, by allowing for conserved CTLA4-B7-1/B7-2 interactions, would lead to (or reinforce) regulatory mechanisms and consequently result in full allograft tolerance. Our results identify a critical role for the CD40-CD40L and CD28-B7 costimulatory pathways in the development of different regulatory mechanisms in rat. Coblockade of both pathways resulted in a form of tolerance not supported by Treg cells but associated with IDO-dependant suppressive cells in the non-T cell compartment.

Materials and Methods

Animals and cardiac allograft models

Eight- to 12-wk-old male Lewis.1W (LEW.1W, haplotype RT1^u) and Lewis.1A (LEW. 1A, haplotype RT1^a) congenic rats (Centre d'Elevage Janvier) differ in their entire MHC regions. Heterotopic LEW.1W heart transplantation was performed as previously described (24). Graft survival was evaluated by palpation through the abdominal wall. Studies described here have been performed in accordance with the institutional guidelines of the Institut National de la Santé et de la Recherche Médicale (INSERM).

Recombinant adenovirus coding for CD40Ig and gene transfer

The adenoviral vector coding for the extracellular portion of mouse CD40 fused to the coding sequences of the constant domains of human IgG1 (AdCD40Ig) and the noncoding adenoviral vector Addl324 have been described previously (7, 25). For gene transfer, recombinant adenoviruses (5×10^{10} infectious particles (IP) in 150 μl) were slowly injected into the portal vein. Serum CD40Ig was detected using a sandwich ELISA as previously described (7).

Anti-CD28 administration

The modulating JJ319 (IgG1 anti-rat CD28) mouse hybridoma was a gift from Dr. Thomas Hünig (Institute of Virology and Immunobiology, University of Würzburg, Würzburg, Germany). JJ319 was given at a dose of 4 mg/kg/day i.p. from days 0 to 7 posttransplantation. Control IgG1 was the 3G8 mAb (IgG1, anti-human CD16, with no cross-reaction with rat tissues).

Histological and morphometric analysis of cardiac grafts

The upper third of the graft was fixed in paraformaldehyde and embedded in paraffin. Five-micrometer coronal sections were stained with hematoxylin-eosin-saffron. Tissues were analyzed by a pathologist (K.R.) blinded to the groups and chronic rejection was evaluated as previously described (26). The percentage of vessel occlusion by intimal thickening was determined using the following scoring system: 0, no occlusion; 1, <20%; 2, 20–50%; 3, 50–80%; and 4, >80%. Vasculitis was quantified using the following scoring system: 0, no leukocyte adhesion to the endothelium; 1, leukocyte adhesion to the endothelium; 2, leukocyte infiltration of the intima; 3, fibrosis of the intima; and 4, leukocyte infiltration of the medium. The percentage of pathological vessels was scored taking into account the presence of vessel occlusion and/or vasculitis. Three sections of at least three different biopsy levels were analyzed for each graft. Only vessels that displayed a clear internal elastica interna were scored.

Immunohistological analysis of myocardial infiltration by mononuclear or polynuclear cells was evaluated blindly using a mixture of two anti-leukocyte CD45 mAbs (OX1 and OX30) by two investigators according to an arbitrary scale and scored as follows: −, negative; +, weakly infiltrated (1 to 5% of the surface area); ++, moderately infiltrated (5 to 15% of the surface area); and +++, strongly infiltrated (15 to 30% of the surface area) (7).

Ab detection

Alloantibodies were analyzed by cytofluorometry following the incubation of Con A-activated allergenic spleen cells with diluted (1/10) heat-inactivated rat serum and then with biotin-conjugated F(ab′)$_2$ goat anti-rat IgG Abs (γ-chain specific) (Jackson ImmunoResearch Laboratories) or with a mouse anti-rat IgM mAb (MARM-7; Technopharm). Ab binding was revealed using FITC-coupled streptavidin or FITC-coupled F(ab′)$_2$ goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). Levels of anti-SRBC Abs were assessed similarly using SRBC as targets. Cells were analyzed using a FACScalibur cytofluorometer (BD Biosciences) and the results were expressed as mean channel fluorescence (MCF) for each serum.

Cell purification

Erythrocytes were removed from spleen cell suspensions by hypotonic lyses. T cells were purified from total splenocytes after nylon wool adherence and depletion of 3.2.3 (CD161), OX42 (CD11b/c), and OX12 (Ig κ-chain) mAb-reactive cells using magnetic beads (Dynal). CD8$^+$CD45RC^{low} T cells were purified from the spleen using a FACSAria flow cytometer (BD Biosciences) as previously described (10).

Mixed lymphocyte reactions

Splenocytes or pure T cells were seeded in triplicate (10^5 cells/well) into round-bottom 96-well plates (Nunc) and evaluated for their proliferative response against irradiated APCs (5×10^4 cells/well). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, 1% nonessential amino acids, 1% HEPES, and 5×10^{-5} 2-ME (all from Sigma-Aldrich). APCs were myeloid dendritic cells enriched from LEW.1W or third party Brown Norway (BN) spleen fragments digested with collagenase D (2 mg/ml), purified as low density cells from Nycodez density gradients, and cultured overnight in complete medium as previously described (7). MLRs were performed in the absence or presence of D-1-methyl-tryptophan (1-MT) (Sigma-Aldrich) at 200 μM.

Cells were cultured for 5 days at 37°C, and 1 μCi of [^{3}H]thymidine deoxyribose was added to each well for the final 8 h of culture. [^{3}H]thymidine incorporation was quantified using a scintillation counter.

For coculture experiments, graft recipient or control LEW.1A splenocytes or T cells (10^5 cells) were added to responding splenocytes from naive LEW.1A rats (10^5 cells) and irradiated LEW.1W or BN stimulator APCs (5.10^4 cells/well) in round-bottom 96-well plates.

Results were expressed as the percentage (Δ mean cpm $\pm$ SD after the subtraction of proliferation in the presence of culture medium) of proliferation of cells from treated recipients as compared with that of cells from control-treated recipients. Alternatively, specific cpm (cpm of the assay from which is subtracted the cpm of stimulator plus APCs alone) are shown.

Quantitative RT-PCR

Messenger RNA transcript analysis was performed by real-time quantitative PCR. Total RNA was isolated using TRIzol (Invitrogen Life Technologies) and amplified using the SuperScript RNA amplification system (Invitrogen Life Technologies) according to the manufacturer's instructions. Ten micrograms of RNA were reverse transcribed using an Moloney murine leukemia virus reverse-transcriptase kit (Invitrogen Life Technologies). Real-time quantitative PCR was performed with a GenAmp 7700 sequence detection system (Applied Biosystems) using SYBR Green PCR core reagents (Applied Biosystems). The following primer pairs were used: hypoxanthine phosphoribosyltransferase (HPRT; see Ref. 6); IFN-γ (see Ref. 6); IL-13 (see Ref. 6); IL-2, 5′-CCTTGTCAACAGCGCACCC-3′ and 5′-GCTTTGACAGATGGCTATCC-3′; IL-6, 5′-CAAAGCCAGAGTCA TTCAGAGC-3′ and 5′-GGTCCTTAGCCACTCCTTCTGT-3′; IL-10 (see Ref. 6); heme oxygense-1 (HO-1; see 27); IDO, 5′-GCTGCCTCCCATTC TGTCTT-3′ and 5′-TGCGATTTCCACCATTAGAGAG-3′; TGFβ1 (see 6); CTLA4, 5′-GGCAGACAAATGACCAAGTGAC-3′ and 5′-TCTGAA TCTGGGCATGGTTCT-3′; perforin, 5′-AGCCTCCACTCCACCCTG ACT-3′ and 5′-GTTGTTTCTTCTTCTCCTCGC-3′; Forkhead box P3 (FoxP3), 5′-CCCAGGAAAGACAGCAACCTT-3′ and 5′-CTGCTTGG CAGTGCTTGAGAA-3′; and glucocorticoid-induced TNFR-related protein (GITR), 5′-GCAGACTTTGGACCAACTGTTC-3′ and 5′-AGCGGC TGGGTATTGACCT-3′. The PCR method and the $2^{-\Delta C_t}$ quantification method (where C_t is threshold cycle), after normalization to HPRT values, have been described previously (28). The arbitrary units (AU) are defined as the fold change in mRNA levels in a given sample (Q) relative to levels in a calibrator (CB), in this case a syngeneic graft. The calibrator is the 1× the expression of each gene. The AU are calculated as follows: AU = $2^{-\Delta C_t}$, where $\Delta\Delta C_t = (C_{tTarge} - C_{tHPRT})_Q - (C_{tTarget} - C_{tHPRT})_{CB}$. To use

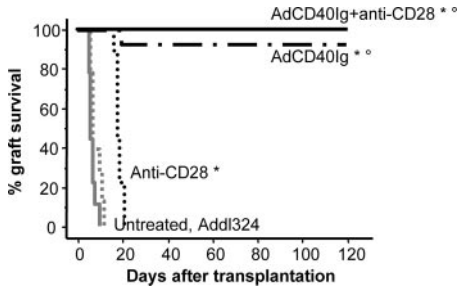

FIGURE 1. Cotreatment with anti-CD28 and CD40Ig leads to indefinite allograft survival. LEW.1A rats were transplanted with LEW.1W hearts. Recipients were either untreated ($n = 9$), or received on day 0 an i.v. injection of 10^{10} IP of the noncoding adenovirus Addl324 ($n = 9$) or the AdCD40Ig adenovirus ($n = 27$). One group was treated with 4 mg/kg/day anti-CD28 mAb from day 0 to day 7 after transplantation ($n = 11$). One other group received a combined treatment with AdCD40Ig (10^{10} IP on day 0) and anti-CD28 (4 mg/kg/day from day 0 to day 7, $n = 16$). Graft survival was evaluated by daily palpation of heartbeat through the abdomen. *, $p < 0.05$ vs untreated or Addl324-treated grafts; ○, $p < 0.05$ vs anti-CD28.

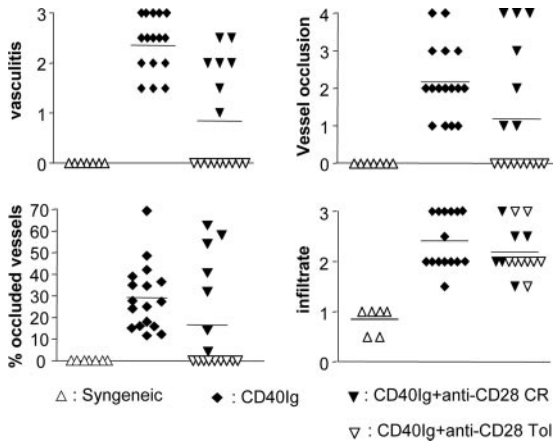

FIGURE 2. CD28 blockade protects against chronic allograft vasculopathy. Long-surviving grafts were collected on day 120 after transplantation from syngeneic nontreated recipients and allogenic recipients transduced with AdCD40Ig and treated or not treated with anti-CD28 Abs. A morphometric analysis of chronic rejection after staining with hematoxylin-eosin-saffron was performed by a pathologist in a blinded fashion. The severity of each criterion was graded using a scoring system described in *Materials and Methods*. Syngeneic, $n = 7$; AdCD40Ig, $n = 17$; AdCD40Ig plus anti-CD28, $n = 16$. In that group, animals considered as Tol are represented with inverted open triangles and those considered as CR with inverted filled triangles. Individual observations (units as described in *Materials and Methods*) are reported with the horizontal bar representing the mean.

the $2^{-\Delta C_t}$ method, the efficiency of the PCR for each gene must be >96%, as determined by the gradient of the $C_t = f(\log(\text{target DNA}))$ curve. Specific amplification products were checked by amplicon melting curves.

TCR clonotype analysis

Qualitative and quantitative analyses of the TCR repertoire have been performed at TcLand. Briefly, CDR3 length distribution (CDR3-LD) alteration was analyzed using Immunoscope software (29). CDR3 length distribution alterations were measured according to Gorochov et al. (30). Vβ/HPRT transcript ratios were measured by real-time PCR (31).

Statistical analysis

Statistical significance was evaluated using the Mann-Whitney U test (analysis of two groups) or Kruskal-Wallis tests followed by a Dunn's post hoc test (analysis of more than two groups) and a Kaplan-Meier analysis of graft survival (log-rank test); $p \leq 0.05$ was considered significant. The Grubb's test was used to determine outliers and $p < 0.05$ was considered significant.

Results

Induction treatment with anti-CD28 Abs induces tolerance in a fraction of CD40Ig-treated allograft recipients

In the rat strain combination used here, monotherapy with an anti-CD28 mAb (JJ319) from day 0 to day 7 after heart transplantation resulted in a significant prolongation of allograft survival (18.7 ± 1.6, $n = 11$, $p < 0.0001$) as compared with untreated controls that uniformly rejected their graft (6.6 ± 0.5 days, $n = 9$) (Fig. 1). As previously shown (7), administration of CD40Ig alone resulted in long-term allograft survival (>120 days, $n = 27$) in 93% of the recipients compared with recipients treated with the noncoding adenovirus (8 ± 0.8 days, $n = 9$). Treatment with anti-CD28 mAb in addition to the administration of CD40Ig resulted in indefinite allograft survival in all recipients (Fig. 1).

A morphometric analysis of chronic rejection lesions performed 120 days after transplantation indicated that the grafts of CD40Ig-transduced recipients, unlike syngenic grafts, displayed vascular lesions as well as mononuclear cell infiltration (Fig. 2). In the group treated with both CD40Ig and anti-CD28 mAb, nine of the 16 grafts analyzed exhibited no vasculopathy (the pathognomic lesions of chronic rejection), and the other seven displayed vascular chronic rejection lesions undistinguishable from those treated with CD40Ig only. In all allograft recipients, however, a substantial level of fibrosis was noted (data not shown). The cotreated recipients displaying no chronic rejection lesions are thus operationally tolerant and are referred to as "Tol," and those with chronic rejection lesions are called "CR" recipients.

Graft infiltration and cytokine expression

An immunohistological analysis showed that infiltrating cells in the allografts 120 days after transplantation represented up to 30% of the analyzed surface area in the CD40Ig-treated recipients (Table I). The initial administration of anti-CD28 mAb to CD40Ig-treated recipients decreased this cell infiltration. Infiltration in cotreated recipients usually represented only 1–5% of the analyzed surface area in these grafts whether or not the recipients presented chronic rejection lesions. One cotreated recipient displaying chronic rejection lesions, however, presented a slightly stronger infiltration that qualified as moderate (Table I). To analyze cytokine expression associated with tolerance and chronic rejection, total RNA was extracted from the allografts of CD40Ig-treated and

Table I. *Immunohistological analysis of graft-infiltrating leukocytes at a late time point after transplantation*[a]

Group	OX1/OX30
Naive	−, 0/+
Syngeneic	−, 0/−
AdCD40Ig	++, +/++, ++, +, ++, +, +++, +++
AdCD40Ig plus anti-CD28 Tol	+, +, +
AdCD40Ig plus anti-CD28 CR	+, +, ++, +

[a] Graft biopsies were analyzed by immunostaining, and the intensity of the staining was evaluated according to an arbitrary scale and scored as follows: −, negative; +, weakly infiltrated (1 to 5% of the surface area); ++, moderately infiltrated (5 to 15% of the surface area); and +++, strongly infiltrated (15 to 30% of the surface area). The recipients cotreated without chronic rejection lesions were thus operationally tolerant and are referred as "Tol" and those with chronic rejection lesions as "CR". Each symbol represents one graft.

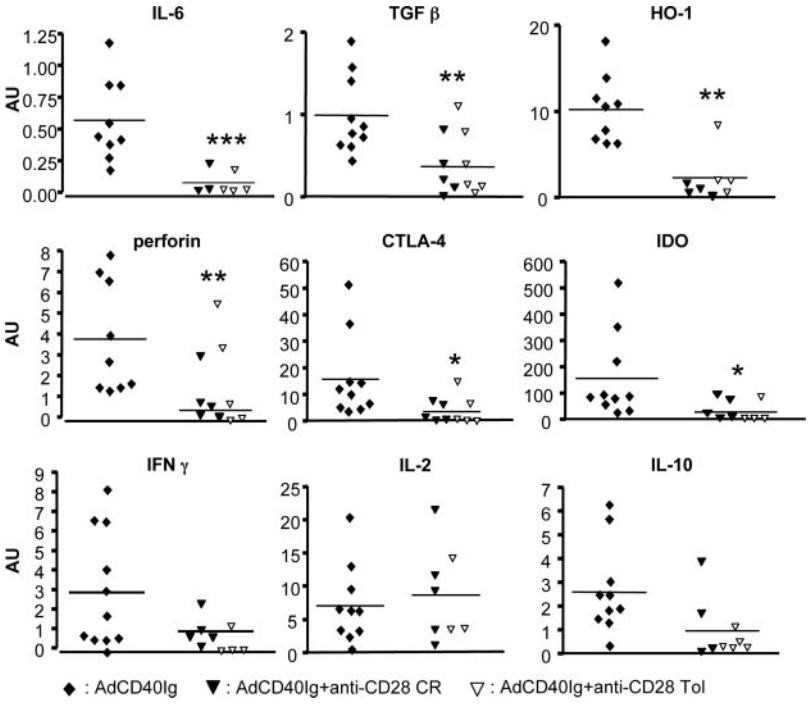

FIGURE 3. Inhibition of cytokine expression in heart grafts of long-surviving recipients following CD28 and CD40L blockade. Total RNA was extracted from cardiac grafts 120 days after gene transfer for the groups receiving AdCD40Ig alone (diamond, $n = 10$) or in combination with anti-CD28 Abs (triangles, $n = 11$). Animal that were ascribed as tolerant (Tol) by the histopathological analysis are represented by open triangles, whereas those that had lesions of vasculitis and occluded vessels are represented by black triangles. Total RNA was then analyzed by quantitative RT-PCR for IL-6, TGFβ1, HO-1, perforin, CTLA4, IDO, IFN-γ, IL-2, and IL-10 transcription. Messenger RNA levels were normalized to the level of HPRT and expressed as the fold change relative to corresponding mRNA levels in syngeneic grafts. Individual observations (in AU) are reported with the horizontal bar representing the mean. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

CD40Ig plus anti-CD28 Abs-treated animals on day 120 posttransplantation. Transcripts for IL-6, perforin, and also for CTLA4, HO-1, TGFβ, and IDO were significantly reduced in CD40Ig plus anti-CD28-treated recipients, whereas those for IL-2 and IL-10 were not. Transcripts for IFN-γ presented also a trend for a decrease (Fig. 3). No difference was detected in the expression of any of these cytokines within the CD40Ig plus anti-CD28 group whether or not the recipients had developed chronic rejection lesions (Fig. 3).

Tolerant recipients treated with CD40Ig and anti-CD28 mAb displayed inhibited alloantibody responses

To assess the mechanisms by which the selective blockade of CD40-CD40L and CD28-B7 pathways resulted in the development of tolerance or chronic rejection lesions, serum levels of alloantibodies were measured in long-surviving recipients 120 days after transplantation. As previously shown (7), IgG alloantibody levels were reduced in the sera of CD40Ig-treated recipients with long-surviving grafts compared with those of control (Addl324)-treated recipients (Fig. 4). The CR recipients in the CD40Ig plus anti-CD28-treated group showed IgG levels not significantly different from those observed in CD40Ig alone-treated recipients. In contrast, this Ab response was considerably reduced and not different from the background in sera of Tol recipients. This reduction was statistically significant for total IgG (MCF of 341.9 $\pm$ 64.7 vs 23.3 $\pm$ 12.8, $p < 0.05$), IgG1 (127.6 $\pm$ 35 vs 11.6 $\pm$ 7.4, $p < 0.05$) and IgG2b (71.8 $\pm$ 16.6 vs 8.4 $\pm$ 0.9, $p < 0.05$).

Anti-CD28 mAb prevents immunization with SRBC in CD40Ig-treated animals

To analyze whether the absence of alloimmunization in Tol recipients was specific for alloantigens, we injected SRBC i.v. on the day of transplantation. Within 3 wk, CD40Ig plus 3G8 irrelevant Ab-treated recipients developed IgG and IgM anti-SRBC Abs, whereas CD40Ig plus anti-CD28 mAb-treated recipients did not develop any responses (Fig. 5).

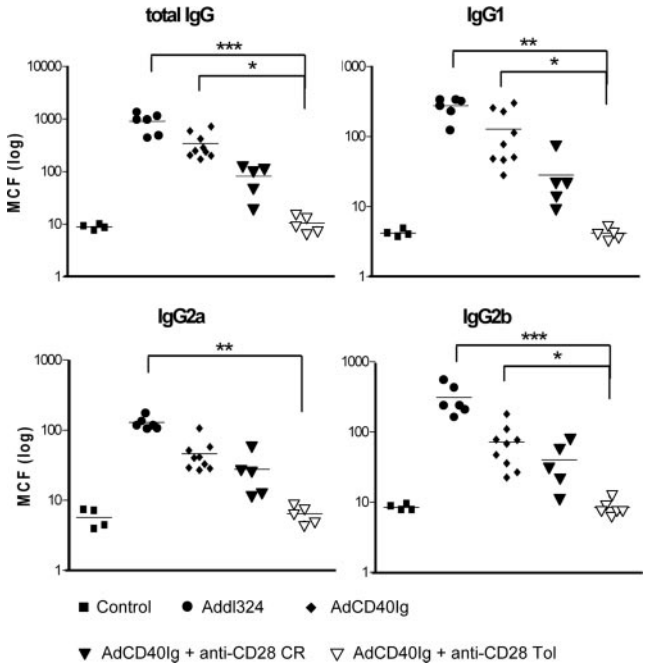

FIGURE 4. Inhibition of alloantibodies in chronic rejection-free anti-CD28 plus CD40Ig-treated recipients. LEW.1A rats were transplanted with LEW.1W hearts. Recipients received on day 0 an i.v. injection of either 10^{10} IP of the noncoding adenovirus Addl324 (black dots; $n = 6$) or 10^{10} IP of the AdCD40Ig adenovirus (diamonds; $n = 9$), or they received a cotreatment combining AdCD40Ig and anti-CD28 Abs (4 mg/kg/day from day 0 to day 7; triangles; $n = 11$). These cotreated recipients are represented separately according to whether they were ascribed as tolerant (open symbols; $n = 5$) or as having chronic rejection lesions (black symbols; $n = 5$) by the histopathological analysis. Naive animals were used as negative control (squares; $n = 4$). Sera diluted 1/10 were analyzed 120 days after transplantation by flow cytometry after reaction with donor-type spleen cells for the presence of total IgG alloantibodies and the IgG1, IgG2a, or IgG2b subclasses. Note that the *y*-axis is in logarithmic scale. Individual observations (MCF) are reported with the horizontal bar representing the mean. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.005$.

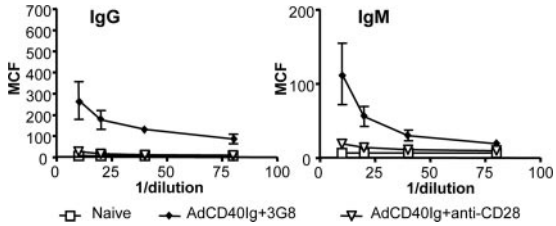

FIGURE 5. Humoral responses against cognate Ags. LEW.1A rats were transplanted with LEW.1W hearts. Recipients were cotreated with CD40Ig plus 3G8 irrelevant Abs (diamonds; $n = 2$) or with CD40Ig plus anti-CD28 Abs (4 mg/kg/day from day 0 to day 7; open triangles $n = 3$). Recipients were also immunized i.v. on day 0 with 10^7 SRBC. Sera collected on days 17 were analyzed for IgG and IgM reactivity against SRBC by flow cytometry. Serum from naive animals was used as negative control (open squares). Results are expressed as MCF for the indicated dilution of serum (means ± SD).

Adoptive transfer of tolerance is modified by anti-CD28 mAb

We demonstrated previously that in CD40Ig-treated recipients of heart allografts, Treg cells from the spleen with a $CD8^+$ $CD45RC^{low}$ phenotype are able to transfer tolerance to secondary grafted recipients (10). To determine whether anti-CD28 treatment influences the nature of these regulatory cells, we performed additional adoptive transfer experiments. LEW.1A rat recipients of LEW.1W heart transplants were sublethally irradiated (4.5 gray) and injected i.v. on day 0 with 2×10^8 spleen cells from Addl324, CD40Ig, or CD40Ig + antiCD28 mAb-treated LEW.1A recipients of LEW.1W grafts. These spleen cells were collected >120 days after transplantation. Transfer of spleen cells from CD40Ig-treated recipients resulted in the indefinite survival of heart grafts in secondary recipients, demonstrating that regulatory cells in the spleen are sufficient for inhibiting rejection. In contrast, spleen cells from CD40Ig plus anti-CD28 Ab-treated recipients failed to induce any prolongation of survival after transfer (Fig. 6). This indicated that spleen cells from cotreated animals did not contain similar transfer-competent regulatory cells.

Cytokine expression by $CD8^+CD45RC^{low}$ T cells is modified in CD40Ig plus anti-CD28 mAb-treated animals

To further understand the functional differences in $CD8^+CD45RC^{low}$ T cell populations, we sorted these cells from the spleen of CD40Ig-treated and CD40Ig plus anti-CD28 mAb-treated animals on day 120 posttransplantation. An average of 5×10^6 $CD8^+CD45RC^{low}$ cells could be extracted from the spleens and the treatments did not modify this number. Total RNA was extracted from these cells, and transcripts for cytokines and regulatory proteins were analyzed by quantitative PCR. Transcripts for IFN-γ and TGFβ were significantly reduced in CD40Ig plus anti-CD28 mAb-treated recipients, as compared with CD40Ig treated alone recipients. The strongest difference appeared to be the expression of IFN-γ, because no expression could be measured in $CD8^+CD45RC^{low}$ T cells from cotreated animals (Fig. 7*A*). In contrast, transcripts for IL-10 and IL-2 were more abundant in $CD8^+CD45RC^{low}$ T cells from cotreated animals. We found no correlation between the Tol (Fig. 7*A*, open symbols) or CR (Fig. 7*A*, filled symbols) status of the cotreated animals and the cytokine expression levels. The expression of transcripts for FoxP3 and GITR was not different between the two groups (Fig. 7*A*) as well as the expression of IL-13 and IL-4 (data not shown).

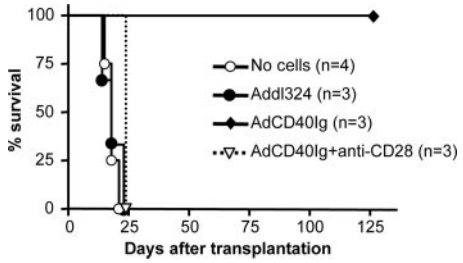

FIGURE 6. Adoptive transfer of tolerance. Graft survival was evaluated in irradiated (4.5 gray) LEW.1A recipients transplanted with LEW.1W hearts (day 0) receiving 2×10^8 splenocytes from recipients transduced with the noncoding adenovirus Addl324 ($n = 3$) or CD40Ig alone ($n = 3$) or combined with anti-CD28 Abs ($n = 3$). Transferred splenocytes were extracted 120 days posttransplantation. Control irradiated heart graft recipients receiving no cells were also analyzed ($n = 4$).

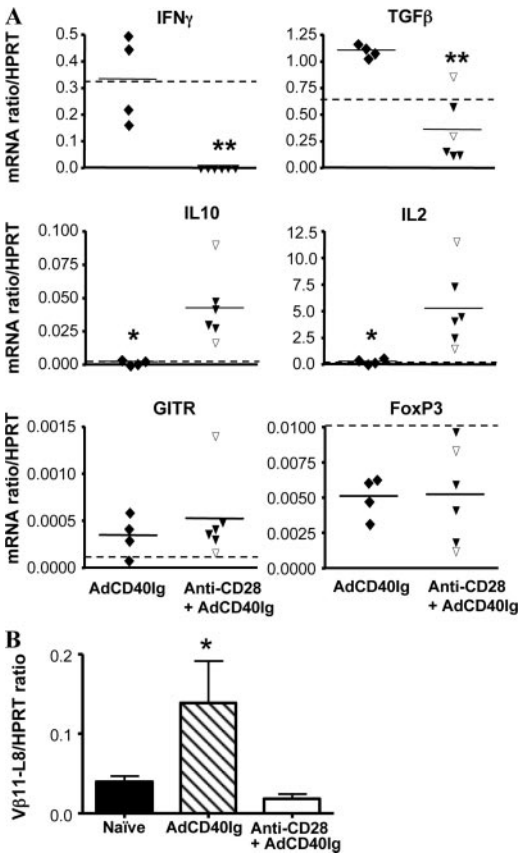

FIGURE 7. Cytokines and clonotype analysis in $CD8^+CD45RC^{low}$ T cells from CD40Ig and anti-CD28 plus CD40Ig-treated recipients. *A*, Total RNA was extracted from spleen $CD8^+CD45RC^{low}$ T cells 120 days after transplantation from rats receiving CD40Ig alone (diamonds, $n = 4$) or CD40Ig plus anti-CD28 mAb (triangles, $n = 6$). Animal were ascribed as tolerant (open symbols) or CR (filled symbols) by the histopathological analysis. Messenger RNA transcripts were then analyzed by quantitative RT-PCR for IFN-γ, TGFβ1, IL-10, IL-2, GITR, and FoxP3. Messenger RNA levels were expressed as the abundance relative to HPRT mRNA levels. Individual observations are reported with the horizontal bar representing the mean. *, $p < 0.05$; and **, $p < 0.01$. Dotted line represents the previously published (see Ref. 10) messenger RNA level of the indicated cytokine in $CD8^+CD45RC^{low}$ T cells from naive animals. *B*, Quantitative analysis of TCR Vβ11 transcripts with an 8-aa-long CDR3 region (L8). Messenger RNA was extracted from $CD8^+CD45RC^{low}$ T cells purified from the spleen of naive animals ($n = 3$) or from heart graft recipients treated with CD40Ig alone ($n = 4$) or combined with anti-CD28 Abs ($n = 4$). Transcripts were analyzed with the TcLandscape technology, as described in *Materials and Methods*. Results are mean ratio of Vβ11-L8 transcripts ± SD as compared with HPRT. *, $p < 0.05$.

Anti-CD28 Abs prevent amplification of clonotypic CD8$^+$CD45RC^{low} Treg cells

We had observed that heart transplant recipients treated with CD40Ig developed an accumulation of the Vβ11 family transcripts as well as an alteration of the Gaussian profile of the CDR3 length distributions in this Vβ family within the CD8$^+$CD45RC^{low} T cell population, with an increased representation of transcripts with an 8-aa-long CDR3 (10). In this study, we determined that this accumulation did not occur in the CD8$^+$CD45RC^{low} T cell population sorted from CD40Ig-treated animals that also received anti-CD28 Abs (Fig. 7*B*), demonstrating that the public TCR response to common donor Ag(s) in the CD8$^+$CD45RC^{low} regulatory T cells was absent.

IDO-dependant non-T cell-based regulatory mechanisms in tolerant recipients treated with CD40Ig plus anti-CD28 mAb

To investigate regulatory mechanisms that might contribute to the tolerant state in the absence of transfer-competent regulatory T cells, the proliferative response of splenocytes from controls or CD40Ig-treated recipients with or without anti-CD28 Abs was analyzed on day 120 posttransplantation. The splenocytes from long-surviving graft recipients consistently displayed decreased proliferative responses to donor-type APCs (27.4 $\pm$ 5, 52.2 $\pm$ 9.8, and 28.7 $\pm$ 14.2% for CD40Ig, CR, and Tol cotreated recipients, respectively) as compared with Addl324-treated controls (considered as 100%, $n = 6$) (Fig. 8*A*). The unresponsiveness of spleen cells was not donor specific because it was also observed when third party BN APCs were used as stimulators (30.1 $\pm$ 6.3, 51.3 $\pm$ 8, and 42 $\pm$ 14.3% for CD40Ig, CR, and Tol recipients, respectively) (Fig. 8*A*). In contrast with unfractionated splenocytes, the proliferation of purified T cells stimulated by donor and third party APCs showed no significant difference between the groups (Fig. 8*B*). These observations suggested that alloreactivity was controlled by non-T cells in the spleen and that the CD8$^+$CD45RC^{low} T cells identified by transfer experiments have no suppressive activity in vitro under the conditions used here. To further characterize this regulatory mechanism dependent on non-T cells, we added 1-MT, an inhibitor of IDO, to the culture medium in mixed lymphocyte reactions. With 1-MT, spleen cells from anti-CD28 plus CD40Ig-treated recipients recovered their proliferation capacities against donor and third party APCs, whereas spleen cells from CD40Ig alone-treated recipients were not sensitive (Fig. 8*C*). The same was true for the reactivity against third party APCs, indicating that the IDO-dependent suppression was not donor specific.

To evaluate whether these non-T cell-based regulatory mechanisms could also inhibit the proliferation of naive T cells, suppression experiments were undertaken where responding naive recipient-type T cells were stimulated by donor APCs in the presence of graft recipient spleen cells. We observed that only splenocytes from the Tol recipients were able to induce a moderate although significant inhibition of the proliferative response of naive T cells in a MLR (Fig. 8*D*, 40% $\pm$ 11% reduction of the response obtained when spleen cells from Addl324-treated recipients were added, $p < 0.05$). In that case, the inhibition appeared donor specific because no reduction in the proliferation of BN APC-stimulated naive LEW.1W T cells was observed (Fig. 8*D*). Finally, to confirm that regulation observed in vitro from Tol recipient splenocytes was the result of the action of non-T cells, we tested whether purified T cells could exhibit a regulatory activity in suppression assays. The data indicated that the inhibitory activity was not displayed by T cells (Fig. 8*E*).

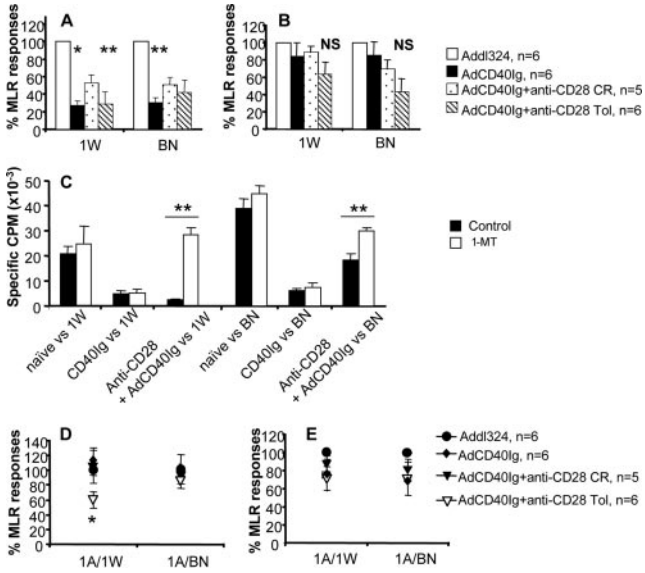

FIGURE 8. Proliferative activity of splenocytes and T cells from cardiac recipients. *A* and *B*, Splenocytes (*A*) and purified spleen T cells (*B*) were harvested on day 120 posttransplantation from cardiac recipients treated with either Addl324 or CD40Ig with or without anti-CD28 Abs. MLR responses were assessed against irradiated first party LEW.1W (1W) or third party BN APCs as indicated. *C*, Proliferation in MLR of splenocytes from naive animals and from CD40Ig with or without anti-CD28 Ab-treated graft recipients was measured with or without 1-MT, an inhibitor of IDO. *D* and *E*, The suppressive activity of splenocytes (*D*) and T cells from the spleen (*E*) was assessed by adding a 1:2 cell ratio to naive MLR reactions consisting of LEW.1A splenocytes responding to irradiated LEW.1W or BN APCs. Proliferation was measured after 5 days by incorporation of [^{3}H]thymidine during the last 8 h of the culture. In all experiments, cell viability was confirmed by the ability of the cells to proliferate upon ConA stimulation. Results in *A*, *B*, *D*, and *E* are the percentage (mean $\pm$ SD of three experiments) of cell proliferation relative to the proliferation of cells from Addl324-treated recipients. In these experiments, specific counts ranged between 30,000 and 100,000 cpm. Results in *C* are means of triplicates $\pm$ SD from one representative experiment of four and are expressed as specific cpm. Animals assigned as CR or Tol are represented separately in *A*, *B*, *D*, and *E*). *, $p < 0.05$; **, $p < 0.01$.

Discussion

In this study, we demonstrated that the perioperative addition of anti-CD28 Abs to CD40Ig treatment reinforces the tolerance to a cardiac allograft and modifies the regulatory mechanisms. We previously demonstrated that the administration of CD40Ig to heart transplant rat recipients induced CD8$^+$CD45RC^{low} regulatory T cells in the spleen that were able to transfer tolerance to secondary recipients (10). In the present study we also observed in this model that despite their suppressive activity in vivo after transfer (Fig. 6), T cells from the spleen responded normally in a MLR (Fig. 8*B*) and had no regulatory activity in vitro (Fig. 8*E*). Because unfractionated spleen cells presented reduced proliferation capacities against APCs, we hypothesized that a control of alloreactivity should occur through the action of suppressive non-T cells. In coculture experiments, however, spleen cells from CD40Ig-treated recipients had no suppressive activity on naive T cells (Fig. 8*D*). Thus, in vitro neither T cells nor non-T cells from CD40Ig-treated recipients could block a naive MLR whereas the alloreactivity of recipient splenocytes was inhibited. This suggested a form of suppression active only on primed alloreactive T cells. In Tol recipients treated with anti-CD28 Abs in addition to CD40Ig, the regulatory mechanism appeared different. First, the adoptive transfer of spleen cells failed to transfer tolerance to secondary recipients (Fig. 6). Second, the transfer-competent CD8$^+$CD45RC^{low} Treg

cells identified in CD40Ig-treated recipients (10) presented a modified cytokine profile; they contained no messenger for IFN-γ and expressed low levels of TGFβ (Fig. 7*A*), two cytokines implicated in the suppressive activity of $CD8^+$ T cells (32, 33). In contrast, they presented more transcripts for IL-10 and IL-2, possibly indicating an activated rather than a regulatory profile. Messenger RNA for TGFβ and IFN-γ were also found reduced in graft biopsies from anti-CD28 Abs plus CD40Ig-treated recipients as compared with CD40Ig treatment alone (Fig. 3), pertaining to the correlation between the expression of these cytokines, the presence of $CD8^+CD45RC^{low}$ Treg cells, and their regulatory properties. In addition, the expansion of $CD8^+CD45RC^{low}$ T cells with a Vβ11 CDR3-L8 clonotype was completely abrogated in CD40Ig-treated recipients that also received anti-CD28 Abs (Fig. 7*B*). These data strongly support the idea that in CD40Ig-treated graft recipients, clonotypic $CD8^+CD45RC^{low}$ T cells are the major regulatory mechanism and that these cells do not develop if anti-CD28 Abs have been initially administered. A third observation pointing to differences between CD40Ig and cotreated recipients was that the proliferation of alloreactive T cells from the spleen was repressed by non-T cells (Fig. 8, *A* and *B*) and that this was under the control of IDO in cotreated animals (Fig. 8*C*). Together, our data indicate that anti-CD28 Abs have induced a shift from a system where transfer-competent regulatory $CD8^+CD45RC^{low}$ T cells presumably interacting with still undefined APCs in a tolerogenic manner control effector T cells to a situation where these cells have not been selected and where a suppression is supported only by IDO-dependent non-T cells.

It was previously demonstrated that CD28 is required for thymic central $CD4^+CD25^+$ Treg development (34) and homeostasis (35) but not for its suppressive function (36). For $CD8^+$ Treg cells, a role of CD28 has not been clear so far; whereas CD8 cells can respond efficiently to Ag in the absence of CD28 costimulation, peripheral tolerance of $CD8^+$ T cells does not occur in vivo in the absence of CD28 (37). In contrast, the expression of CD28 molecules is not necessary for $CD8^+$ Treg generation after contact with $B7^+$ iris pigment epithelial cells (38). Also, after induction therapy in transplantation, suppressive T cells of the $CD8^+CD28^-$ phenotype have been described (39), but whether CD28 is initially required for their development was not elucidated. In addition, regulatory $CD8^+CD45RC^{low}$ cells do not express detectable levels of CD28 (10). In this study we have demonstrated that $CD8^+CD45RC^{low}$ T cells were present in similar numbers in the spleen but modified their cytokine expression profile and lost their regulatory activity in vivo when heart graft recipients were treated with anti-CD28 Abs in addition to CD40Ig. The TCR repertoire analysis also demonstrated that anti-CD28 Abs prevented the expansion of regulatory $CD8^+CD45RC^{low}$ T cells in cotreated animals. Therefore, although the majority of $CD8^+CD45RC^{low}$ cells from CD40Ig-treated animals are $CD28^-$ (10), it is possible that they originate from $CD8^+CD28^+$ cells and that the anti-CD28 mAb used here directly interfered with the acquisition of the regulatory properties of $CD8^+CD45RC^{low}$ cells.

Heart allografts in recipients treated with CD40Ig were still strongly beating 4 mo posttransplantation but systematically presented vasculitis and cell infiltration, the pathognomic characteristics of CR. This indicated that treatment with CD40Ig inhibited acute graft rejection but did not induce full transplant tolerance. In comparison, 50–60% of heart allografts cotreated with anti-CD28 mAb had no lesions of CR and were thus fully tolerated, excepting that they still presented substantial levels of fibrosis. The remaining 40% of heart allografts, presenting CR lesions despite the cotreatment, could not be distinguished from the tolerant recipients by virtue of their anti-mouse Ab response that could otherwise blunt the immunoregulatory effect of anti-CD28 Abs (data not shown). Also, the circulating levels of CD40Ig were similar in Tol and CR cotreated recipients (in all cases >100 μg/ml in the serum on day 10; data not shown). Cellular infiltration and intragraft cytokine expression were reduced in grafts from recipients treated with CD40Ig plus anti-CD28 Abs as compared with CD40Ig treatment alone, whether or not these grafts were found to be devoid of vascular lesions. Infiltration and cytokine expression levels therefore do not seem to be critical parameters correlated with vasculopathy. Rather, alloantibodies were found absent in Tol recipients while they were low but detectable in CR recipients (Fig. 4). It has been previously suggested that Ab induction (40) and chronic allograft vasculopathy (4, 7) are driven primarily by costimulation-dependent adaptative immunity that, in the context of CD40L blockade, might be driven by CD28. Blocking CD28 should therefore be effective in controlling pathogenic costimulation pathway activation and in preventing alloantibody production and chronic allograft vasculopathy. This effect on Ab responses was not restricted to alloantigens, because the coadministration of anti-CD28 Abs with CD40Ig also abrogated Ab responses to SRBC (Fig. 5). The data presented here confirm, in the context of transplantation, a synergy between CD40Ig and anti-CD28 Abs in inhibiting Ab responses.

Inhibiting the CD28-B7 pathway by using anti-CD28 Abs might result in a different outcome than with B7 antagonists such as CTLA4Ig. Indeed, B7 molecules also interact with CTLA4, leading to a direct repression of T cell responses (41) and to the synthesis of IDO by dendritic cells (12). We therefore hypothesized that blocking CD28 and not B7 might reinforce immunosuppression and promote tolerance. The in vivo relevance of an unopposed CTLA4-B7 interaction for transplant tolerance has been illustrated by experiments showing that CTLA4 blockade prevents tolerance induction (14, 42). We observed here an impact of CD40Ig plus anti-CD28 Abs on chronic rejection, whereas in heart transplantation in mice anti-CD40L plus CTLA4-Ig treatment did not result in the complete abrogation of CR (3). Therefore, blocking B7 results in a different outcome in transplantation than blocking CD28, although this is probably also model dependent.

In conclusion, following heart transplantation in the rat treated with CD40Ig, anti-CD28 Abs modified the properties of $CD8^+CD45RC^{low}$ cells, blunted Ab responses, and reduced the development of CR lesions. It seems that the loss of $CD8^+CD45RC^{low}$ T cell-based regulation does not account for the reduction of CR lesions, because both parameters were not correlated; all CD40Ig plus anti-CD28 cotreated recipients presented an absence of suppressive $CD8^+CD45RC^{low}$ T cells whereas 40–50% still presented CR lesions. Rather, a reinforced regulatory activity in the non-T cells compartment and the absence of a specific Ab response are the parameters most clearly associated with an absence of CR lesions.

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Disclosures

The authors have no financial conflict of interest.

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