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Memory T Cells Mediate Cardiac Allograft Vasculopathy and are Inactivated by Anti-OX40L Monoclonal Antibody

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Abstract

Purpose—Cardiac allograft vasculopathy (CAV) is a major complication limiting the long-term survival of cardiac transplants. The role of memory T cells (T_{mem}) in the pathogenesis of CAV remains elusive. This study investigated the role of T_{mem} cells in the development of CAV and the therapeutic potential of targeting the OX40/OX40L pathway for heart transplant survival.

Methods—T_{mem} cells were generated in Rag-$1^{-/-}$ C57BL/6 (B6) mice by homeostatic proliferation (HP) of CD40L null $CD3^+$ T cells from B6 mice. Rag-$1^{-/-}$ B6 mice (H-2^b) harboring

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Conflict of Interest None of the authors have any conflicts of interest.

T_{mem} cells received cardiac allografts from BALB/c mice (H-2^d), and were either untreated or treated with anti-OX40L monoclonal antibody (mAb) (0.5 mg/mouse/day) for 10 days.

Results—Six weeks after HP, the majority of transferred CD40L$^{-/-}$ T cells in Rag-1$^{-/-}$ B6 mice were differentiated to CD44^{high} and CD62L^{low} T_{mem} cells. BALB/c heart allografts in Rag-1$^{-/-}$ B6 recipient mice in the presence of these T_{mem} cells developed a typical pathological feature of CAV; intimal thickening, 100 days after transplantation. However, functionally blocking the OX40/OX40L pathway with anti-OX40L mAb significantly prevented CAV development and reduced the T_{mem} cell population in recipient mice. Anti-OX40L mAb therapy also significantly decreased cellular infiltration and cytokine (IFN-γ, TNF-α and TGF-β) expression in heart allografts.

Conclusions—T_{mem} cells mediate CAV in heart transplants. Functionally blocking the OX40/OX40L pathway using anti-OX40L mAb therapy prevents T_{mem} cell-mediated CAV, suggesting therapeutic potential for disrupting OX40-OX40L signaling in order to prevent CAV in heart transplant patients.

Keywords

Heart transplantation; Cardiac allograft vasculopathy; Memory Tcell; OX40 pathway; Anti-OX40L antibody therapy

Introduction

Heart transplantation is the most effective therapy for end-stage heart failure or severe coronary artery disease [1]; however, the survival of heart transplants still remains a significant clinical challenge. It has been suggested that chronic rejection or cardiac allograft vasculopathy (CAV) of heart transplants contributes to 30 % of deaths at 5 years following cardiac transplantation [2]. CAV is defined as a type of arteriosclerosis, characterized by concentric intimal hyperplasia with luminal narrowing from distal small vessels to all of the intramyocardial and epicardial arteries of the allograft [3–5]. To date, there is no effective treatment available for the prevention or treatment of CAV [6].

Memory T (T_{mem}) cells are a subpopulation of activated T cells that persist after the initial T cell response. Typically, after such a response and in the absence of antigen stimulation, the majority of T cells die off, either to prevent further damage to the tissue or to make space for future T cells, but T_{mem} cells survive and provide continual immune protection to the host [7]. Early studies have shown that in coronary arteries from patients with CAV, most infiltrating T cells express the phenotype of T_{mem} cells [8, 9], suggesting that this subtype of T cells may play an important role in the development of CAV [10]. However, this hypothesis has not yet been tested.

OX40 (CD134), initially viewed as a T-cell activation marker [11], is a member of the tumor necrosis factor receptor (TNFR) superfamily [12]. OX40 expression has also been observed in other types of leukocytes, such as regulatory T (Treg) cells [13], natural killer T (NKT) cells [14], natural killer (NK) cells [15], and neutrophils [16]. The ligand of OX40 (OX40L, CD252) is a member of the TNF superfamily [12] and can be found on dendritic cells (DCs), B cells, and inflamed vascular endothelial cells [17]. It has been suggested that OX40-

OX40L interaction is one of key pathways for activation of multiple cellular components in the immune response and is becoming an emerging target for immunosuppression [18]. In addition to its stimulatory function in effector T cells, the OX40-OX40L signaling pathway has been found to play a key role in the survival and homeostasis of T_{mem} cells [19–21], which has been supported by the fact that treatments with anti-OX40 agonists result in higher production of T_{mem} cells [19, 22, 23], whereas blocking OX40 signaling during the secondary exposure completely suppresses T_{mem} cell expansion [23]. The objective of this study is to investigate the role of T_{mem} cells in CAV development, and to test the therapeutic potential of disrupting OX40-OX40L interaction in the prevention of CAV in a murine model.

To investigate the activities of T_{mem} cells in the pathogenesis of CAV, we used a murine model of CAV that is mediated by CD40L deficient T_{mem} cells. We generated CD40L deficient T_{mem} cells by the homeostatic proliferation (HP) of naïve CD40L$^{-/-}$ T cells in Rag-1$^{-/-}$ mice that do not have mature lymphocytes (T and B cells) [24]. The rationale for this experimental model being; firstly, induction therapy using a lymphocyte-depleting agent, such as alemtuzumab or rabbit anti-thymocyte globulin, expands CD4 T_{mem} cells that predominate during acute rejection episodes in patients [25], which may be due to the fact that compared with naïve T cells, T_{mem} cells are more difficult to deplete, presumably because of their survival advantages over naïve T cells [26], and are likely to have faster kinetics of HP after lymphoablation [27]. The HP of naïve T cells in an “empty” host also results in the generation of T_{mem} cells [28, 29]. Secondly, cardiac allografts in mice can survive for 100 days in CD40L deficient recipients, but develop CAV [30], and in sensitized recipients, CD40-CD40L interaction is absent or not required for the activation of T_{mem} cells that mediate allograft rejection [31, 32].

Materials and Methods

Animals

Male adult BALB/c (10–12 week old, H-2^{d}) or Rag-1$^{-/-}$C57BL/6 (B6) mice (10–12 weeks old, H-2^{b}) (Jackson Laboratories, Bar Harbor, MA, USA) were used as donors or recipients respectively. CD40L$^{-/-}$ B6 mice (10–12 weeks old) (Jackson Laboratories) were used as a donor of naïve CD3^{+} T cells for generating T_{mem} cells in Rag-1$^{-/-}$ B6 mice by HP. Animals were housed in the Animal Care Facility of the University of Western Ontario (London, ON) and handled following the protocol approved by the animal research ethical review board of the University of Western Ontario according to the Canadian Council on Animal Care guidelines [33].

Adoptive Transfer of Purified T Cells and T_{mem} Phenotype Analysis

To establish a recipient model harboring CD40L$^{-/-}$ T_{mem} by HP in Rag-1$^{-/-}$ B6 mice, CD3^{+} T cells were isolated using CD3-positive selection—MACS® Technology (Miltenyi Biotec Inc., Auburn, CA, USA) from the spleens and lymph nodes of CD40L$^{-/-}$ B6 mice. These CD3^{+}T cells (2×10^{6} cells/mouse; >97 % purity) were adoptively transferred into syngenic Rag-1$^{-/-}$ B6 mice. After undergoing HP for 6 weeks, T_{mem} cells were phenotypically analyzed using flow cytometry with flurochrome-conjugated antibody staining; anti-CD44-

fluorescein isothiocyanate (FITC) and anti-CD62L-phycoerythrin (PE) (eBiosciences, San Diego, CA, USA).

Heterotopic Cardiac Transplantation

Intra-abdominal heterotopic cardiac transplantation was performed as previously described [34]. In this study, surgery involved the transplantation of fully MHC-mismatched hearts from BALB/c donors (H-2^d) to naïve Rag-$1^{-/-}$ B6 recipients (H-2^b) or those harboring T_{mem} cells. The heartbeat of the grafts was monitored and evaluated daily by direct abdominal palpation in double-blind fashion to detect the state of cardiac health/rejection [34].

Experimental Groups

Three groups were included in this study: (1) Controls, naïve Rag-$1^{-/-}$ B6 mice receiving cardiac allografts from BALB/c mice (n =3); (2) Rag-$1^{-/-}$ B6 mice harboring T_{mem} cells were transplanted with BALB/c cardiac allografts without any treatment (n =8); and (3) Rag-$1^{-/-}$ B6 mice harboring T_{mem} cells were transplanted with BALB/c cardiac allografts, and were treated with rat anti-OX40L monoclonal antibody (mAb) (clone RM134L, rat IgG2b; BioXcell, West Lebanon, NH, USA) (0.5 mg/mouse/day, intraperitoneal injection) for 10 days (day 0–10) (n =8). Heart graft samples were collected and analyzed on postoperative day (POD) 100.

Graft Histology

Formaldehyde-fixed, paraffin-embedded tissue samples were sectioned at 4 μm, and stained with hematoxylin and eosin [35]. The sections were examined for severity of rejection, particularly CAV, by a pathologist in a blinded fashion [36]. Criteria for graft rejection included evidence of intimal thickening with luminal narrowing, fibrosis and cellular infiltration.

Immunohistochemistry

Cryosections embedded in Tissue-Tek O.C.T (Skura Finetek, Torrance, CA, USA), mounted on gelatin-coated slides were stained using an avidin-biotin immunoperoxidase method (Vector Laboratories, Burlingame, CA, USA) [34]. Intragraft T cell infiltration was detected using primary antibody anti-mouse CD4 (clone YTS 191.1.2; Cedarlane Laboratories Canada, Burlington, ON), and anti-mouse CD8 mAbs (clone 53–6.7: BD Biosciences—Canada, Mississauga, ON), while intragraft monocyte/macrophage infiltration was identified with an anti-Mac-1 mAb (clone M1/70; Cedarlane Laboratories Canada). Negative stain controls were those sections stained omitting the primary antibodies. Antibody reactivity was evaluated on five randomly selected high-powered bright-phase microscope fields of each tissue section obtained from eight animals per group.

Determination of Cellular Phenotypic Expression

Cell phenotypes were analyzed using a FACS Calibur flow cytometer (Becton Dickinson Canada Inc., Mississauga, ON). All FITC-, PE- and CyChrome (Cy)-conjugated goat or rat anti-mouse antibodies were purchased from BD Biosciences—Canada, Cedarlane Laboratories Canada, or *e*Bioscience. All of flow cytometric analyses were compared to

their appropriate control goat or rat Ig isotypes [34]. Phenotypic analysis of T_{mem} cells involved triple flurochrome-conjugated antibody staining with anti-CD4 or -CD8-FITC, -CD44-PE-Cy5 and -OX40-PE.

Real-Time RT-PCR

Quantitative analyses of transcript (mRNA) levels of tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β and Interferon (IFN)-γ were performed by real-time RT-PCR. Briefly, total RNA was extracted from heart grafts with a spin column, according to the manufacturer's protocol (Qiagen Inc.—Canada, Toronto, ON). cDNA pools were synthesized with the First-Strand Synthesis System according to the manufacturer's protocol (Stratagene/Agilent Technologies, Santa Clara, CA, USA). Primers were designed using Primer Express, primer designing software from Applied Biosystems (Life Technologies—Invitrogen, Burlington, ON): TNF-α: 5′-GGCTGCCCTGTCCTTCCTAGATA and 5′-CGAGCTAG TGACACTCTGACACTCC; TGF-β: 5′-TTGCTTCAGCTC CACAGAGA and 5′-TGGTTGTAGAGGGCAAGGAC; IFN-γ: 5′-CGGCACAGTCATTGAAAGCCTA and 5′-GTTGCTGATGGCCTGATTGTC; β-actin: 5′-GATGACGATATCGCTGCGCTG and 5′-GTACGACCAGAGGCAT ACAGG. The gene sequences were obtained from the www.ncbi.nlm.nih.gov database. Real-time quantitative PCR was performed on standardized quantities of cDNA using the Brilliant SYBR Green QPCR Master Mix kit, and amplified DNA products were generated and detected using the Mx4000 system (Stratagene/Agilent Technologies). Each PCR amplification condition was set up in triplicate. β-actin amplification was used as an endogenous control. The normalized delta threshold cycle value and relative expression levels ($2^{-\Delta\Delta Ct}$) were calculated according to the manufacturer's protocol (Stratagene/Agilent Technologies).

Statistical Analyses

Histological and immunohistological findings were analyzed non-parametrically using ANOVA-on-rank Statistical comparisons of cytokine levels and cell phenotype differences were performed using Student's t-test. All these statistical analyses were performed using SigmaPlot computer software (Systat Software, Inc., San Jose, CA, USA). Differences with p values≤0.05 were considered significant.

Results

HP Generates CD40L Deficient T_{mem} Cells in Transplant Recipients

It has been demonstrated that T_{mem} cells can be generated from syngeneic naïve T cells in immunodeficient mice via HP [28, 37]. To generate CD40L deficient T_{mem} cells in transplant recipients, $CD3^+$ T cells were isolated from the spleens and lymph nodes of $CD40L^{-/-}$ B6 mice, and adoptively transferred into syngenic Rag-$1^{-/-}$B6 mice. After 6 weeks of HP, the transferred T cells acquired high levels of CD44 ($CD44^{high}$) and low expression of CD62L ($CD62^{low}$) (Fig. 1), a typical phenotype of T_{mem} cells [38] that was 86.13±5.22 % of total splenocytes in these recipient Rag-$1^{-/-}$ B6 mice (n =3). This result confirmed that transferred T cells lost their naïvety, and acquired features of T_{mem} cells in the Rag-1 deficient B6 mice.

CD40L Deficient T_{mem} Cells Induce CAV that is Prevented by Anti-OX40L mAb Treatment

In order to verify if T_{mem} cells could induce CAV development, and OX40 pathway blockade would be effective at preventing graft CAV, fully MHC mismatched BALB/c heart allografts were transplanted into Rag-$1^{-/-}$ B6 recipient mice harboring CD40L deficient T_{mem} cells (*Group* 2) compared to those without T cell transfer (*Group* 1). In addition, one half of recipient mice in *Group* 2 were randomly selected for anti-OX40L mAb treatment (*Group* 3) to determine the role of OX40 pathway blockade in the transplant outcomes. On POD 100 the cardiac allografts in naïve recipient Rag-$1^{-/-}$ B6 mice (*Group* 1) showed normal histology—without CAV but the presence of mild cellular infiltration in perivascular area (Fig. 2a), whereas in recipients harboring T_{mem} cells (*Group* 2), six out of eight graft samples developed severe changes, one showing moderate intimal thickening—a typical pathological feature of CAV (Fig. 2b). Furthermore, treatment with anti-OX40L mAb (*Group* 3) resulted in complete prevention of CAV development in cardiac allografts, indicated by the lack of any pathological changes of CAV—intimal thickening in eight allografts from antibody-treated recipients on POD 100 (Fig. 2c), however, mild myocardial interstitial fibrosis was noticed in these grafts.

Treatment with Anti-OX40L Decreases T_{mem} Cell Population in Transplant Recipients

To confirm if anti-OX40L mAb therapy or OX40 pathway interference specifically impaired T_{mem} cell development in transplant recipients, the population of T_{mem} cells with OX40 expression in the splenocytes was measured in transplant recipients on POD 100. As expected, in untreated recipients (*Group* 2) there was a high proportion of $CD4^+OX40^+CD44^{high}$ or $CD8^+OX40^+CD44^{high}$ T_{mem} cells (Fig. 3), while in those receiving anti-OX40L mAb treatment (Group 3), the number of both CD4 and CD8 T_{mem} cells was significantly reduced (Fig. 3, P <0.001, *Group* 2 vs. 3, n =8). These results suggest that the OX40 pathway plays a significant role in the generation of T_{mem} cells that mediate CAV development in cardiac allografts.

Treatment with Anti-OX40L is Associated with a Reduction of Cellular Infiltration and Cytokine Expression in Cardiac Allografts on POD 100

To further understand the mechanism by which treatment with anti-OX40L mAbs prevents CAV, the impact of anti-OX40L mAb therapy on cellular infiltration and cytokine expression in transplants was examined on POD 100. Immunohistochemistry data showed massive cellular infiltration of CD4, CD8, and Mac-1 positive cells in cardiac allografts from untreated recipient animals on POD 100 (Fig. 4a, c, e), whereas analysis of grafts from recipient animals treated with anti-OX40L mAb showed almost no cellular infiltration on the same day (Fig. 4b, d, f) in this limited number of samples.

The expression of the intragraft cytokine profile (TNF-α, IFN-γ and TGF-β) was also examined in these transplants. In untreated control grafts, there were high levels of TNF-α, IFN-γ and TGF-β on POD 100 (Fig. 5), however in grafts treated with anti-OX40L mAb therapy these cytokines were significantly reduced (Fig. 5, P <0.01, *Group* 2 vs. 3, n =5). Taken together, these results suggest that anti-OX40L mAb decreases not only the T_{mem} cell population in spleens but also cellular infiltration ($CD4^+$ and $CD8^+$ T cells, and macrophages) and cytokine (TNF-α, IFN-γ and TGF-β) expression in transplants.

Discussion

We believe that this experimental study demonstrates for the first time that CD40L deficient T_{mem} cells induce CAV in cardiac allografts, and blockage of the OX40 signaling pathway using anti-OX40L mAb reduces T_{mem} cell development and prevents CAV in a mouse cardiac transplantation model. Our findings are consistent with another similar study by Nakano et al. in a different animal model, where they reported that genetic deletion of OX40L suppresses the formation of atherosclerosis in mice [39]. Thus, we believe that the OX40 pathway can be used not only for immunosuppression as suggested in literature [18], but also for the prevention of CAV in cardiac transplant recipients.

To better understanding the role of T_{mem} cells in CAV, we developed a novel T_{mem} cell-mediated CAV model, in which two criteria are met: 1) T_{mem} cells are a major Tcell population in recipient mice and, 2) CAV develops in cardiac allografts. Rag-$1^{-/-}$ B6 mice were chosen as recipients in this model because these mice do not produce mature T and B lymphocytes on their own [24], and T_{mem} cells can be generated in these mice by adoptive transfer of naïve T cells as reported previously [37, 40]. We have also found that although heart transplants from BALB/c mice in fully MHC class II-mismatched recipient $CD40L^{-/-}$ B6 mice can survive for 100 days, they ultimately develop atherosclerosis or CAV[30], suggesting that $CD40L^{-/-}$ alloreactive T cells do not induce acute allograft rejection, but can cause subacute immune-mediated vascular injury, resulting in CAV development in long-term survived grafts. Therefore, we felt that a T_{mem} cell-mediated CAV model could be established where $CD3^+$ T cells from $CD40L^{-/-}$ B6 mice are adoptively transferred into Rag-$1^{-/-}$ B6 mice to generate T_{mem} cells in these recipient mice. After 6 weeks of HP, heart grafts from BALB/c mice were transplanted into the Rag-$1^{-/-}$ B6 recipients to develop CAV. Indeed, after 6 weeks of adoptive transfer of naïve T cells into Rag-$1^{-/-}$ B6 mice, more than 86 % of splenocytes became $CD44^{high}CD62^{low}$ T_{mem} cells (Fig. 1), and induced CAV in survived cardiac allografts in these recipients (Fig. 2). However, one has to acknowledge the limitations of this model: 1) although the majority of splenocytes are ‘memory’ type T cells, other immune effector cells, such as macrophages, NK cells and NKT cells, may contribute to the pathogenesis of CAV as well; 2) it is still unknown if T_{mem} cells generated by HP from naïve T cells are the same as seen in coronary arteries from patients with CAV.

It has been shown that in patients with CAV, the activated T_{mem} cells in their coronary arteries are a major population of infiltrating mononuclear cells [8]. In addition, in vitro endothelial cells, but not vascular smooth muscle cells (VSMC), up-regulate OX40L expression in response to proinflammatory cytokines, which enhances T_{mem} cell responses to VSMC [41], and in vivo treatment with anti-OX40L mAb in animals receiving allotransplants inhibits T_{mem} cell expansion via apoptosis [37, 42] and IFN-γ production of $CD4^+$ T_{mem} cells [37]. The present study further confirms these observations in our unique model; $CD40L^{-/-}$ T_{mem} cells, generated by HP, have the capacity to induce CAV in cardiac allografts (Fig. 2) and in vivo treatment with anti-OX40L mAb reduces the number of T_{mem} cells in the spleens (Fig. 3) and infiltrating $CD4^+$ and $CD8^+$ T cells in grafts (Fig. 4), which correlates with the absence of CAVin cardiac allografts. All these studies may suggest that T_{mem} cells that depend on OX40L-OX40 signaling are the pathogenic effector T cells for

CAV in heart transplantation. We have to acknowledge that CAV in our model is mainly induced by the activation of $CD40L^{-/-}$ T_{mem} cells, and the efficacy of anti-OX40L mAb treatment against CAV is only demonstrated in the absence of other important leukocytes, such as T regulatory cells, which is also a limitation of this study.

It has been documented in literature that effector T_{mem} cells produce both IFN-γ and TNF-α [43–46]. In the coronary arteries from heart transplant patients, IFN-γ-producing T_{mem} cells are only detected in CAV vessels, and these cells also express TGF-β [9, 47]. The present study demonstrates similar results; higher levels of intragraft IFN-γ, TNF-γ and TGF-β are associated with infiltrating T_{mem} cells in grafts with CAV in untreated controls as compared to grafts without CAV in the anti-OX40L mAb treated group. To understand how these cytokines contribute to T_{mem} cell development and CAV, it is reported that IFN-γ can directly stimulate VSMC proliferation via mTORC1 signaling [48–50]. TNF-α is a nonspecific inflammatory factor that can cause microvascular injury [51], and stimulates both T_{mem} cell development [52] and proliferation and migration of VSMC [53], while TGF-β blocks activated-induced cell death in both primary and memory Th1 effectors, promoting T_{mem} cell survival [54].

In conclusion, the data from this study for the first time suggest that effector T_{mem} cells have the capacity of inducing CAV in cardiac allografts in an experimental model, and blocking the activation of OX40 receptors through neutralization with anti-OX40L mAb significantly reduces the recipient's ability to generate T_{mem} cells, either through initial generation and/or re-activation of pre-existing T_{mem} cells. The absence of T_{mem} cells in the recipient results in the protection of the heart transplants from CAV development. Our study provides proof-of-concept that targeting OX40-OX40L signaling in T_{mem} cells has potential for preventing CAV and prolonging heart transplant survival in patients.

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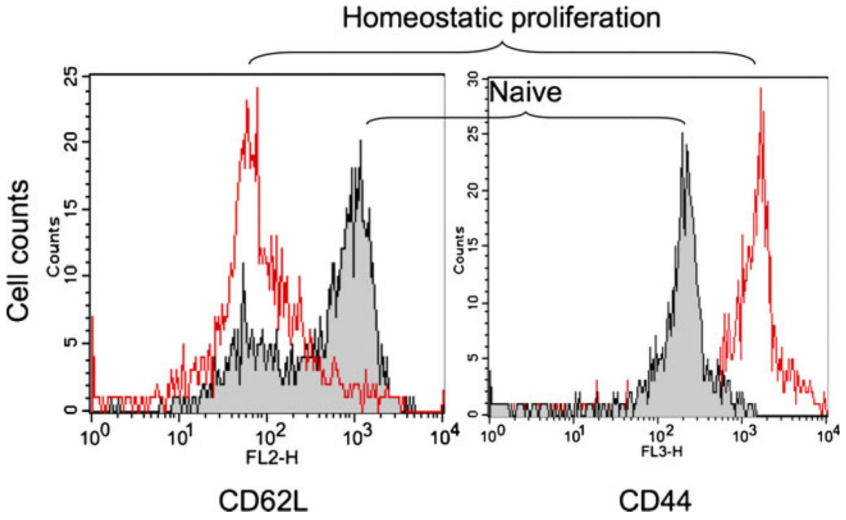

Fig. 1.

Phenotypic analysis of CD40L$^{-/-}$ B6 mouse naive and T_{mem} cells. Naive CD3^{+} T cells from CD40L$^{-/-}$ B6 mice were adoptively transferred into Rag-1 deficient B6 mice, and allowed to undergo homeostatic proliferation for 6 weeks. The phenotypes of both cell types were determined using a flow cytometry with flurochrome-conjugate antibody staining with anti-CD44-FITC and anti-CD62L-PE. Data are a representative of three mice

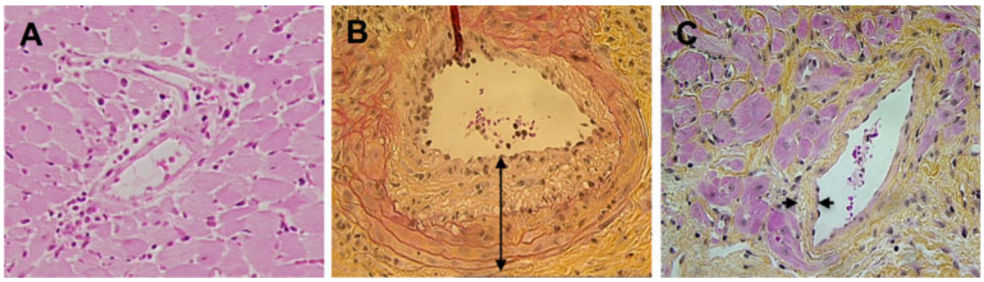

Fig. 2.

CAV development in cardiac allografts in Rag-1 deficient recipients. BALB/c mouse heart allografts in Rag-$1^{-/-}$ B6 mouse recipients were harvested on POD 100. CAV was evaluated in H&E stained paraffin sections. Data are presented as a typical image of vascular lesion in the arteries. **a** Heart grafts from naïve Rag-$1^{-/-}$ recipients—lack of T_{mem} cells (*Group* 1, *n* =3). **b** Heart grafts from Rag-$1^{-/-}$ recipients harboring T_{mem} cells (*Group* 2, *n* =8), showing severe CAV. **c** Heart grafts from Rag-$1^{-/-}$ recipients harboring T_{mem} cells and receiving anti-OX40L mAb treatment (Group 3, *n* =8)

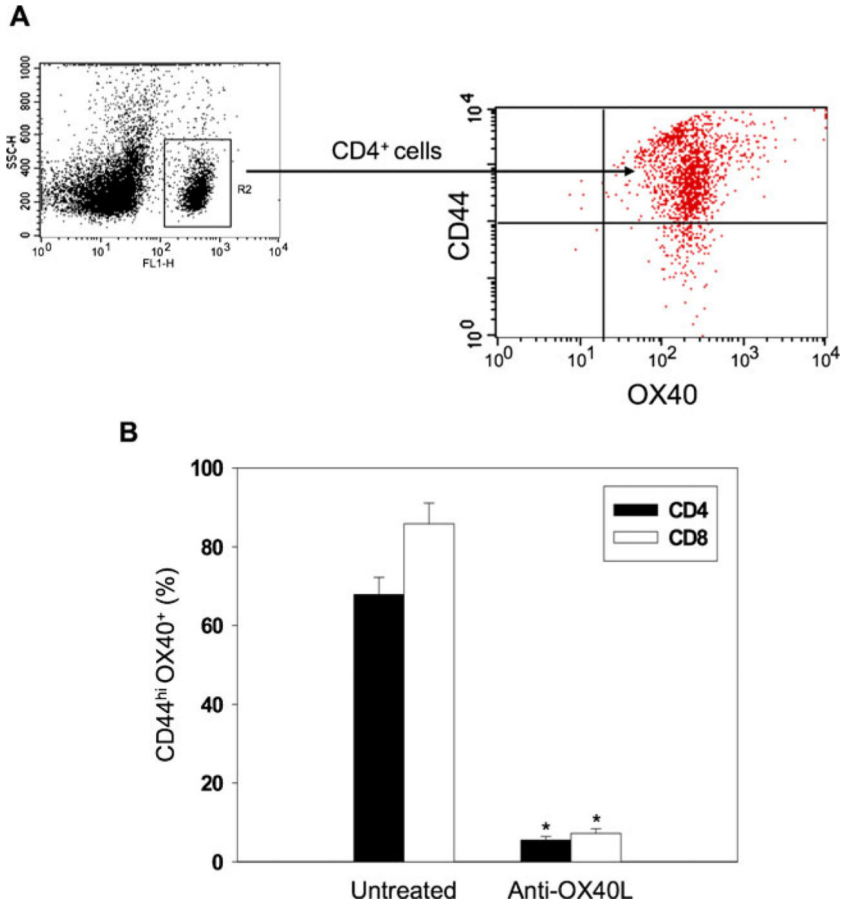

Fig. 3.

Regulation of $CD4^+OX40^+CD44^{hi}$ and $CD8^+OX40^+CD44^{hi}$ T cells by anti-OX40L mAb treatment in allograft recipients. The splenocytes were harvested from the recipient mice on POD 100. **a** Representative dot plots of OX40 and CD44 staining of $CD4^+$ cells. **b** Frequency of $CD4^+OX40^+CD44^{hi}$ and $CD8^+OX40^+CD44^{hi}$ T cells were analyzed by flow cytometric analysis, and expressed graphically in terms of mean percentages (±SEM) that were statistically compared using Student's *t*-test test (**P* <0.001, vs. untreated, *n* =8)

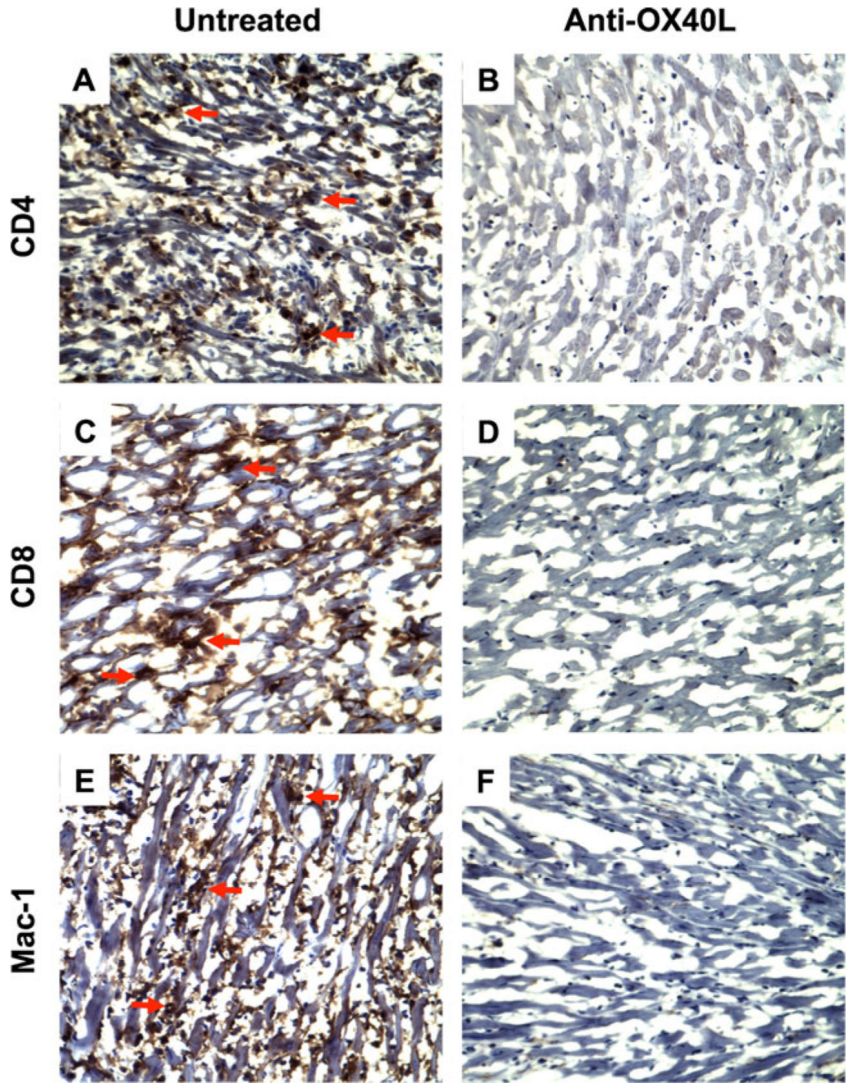

Fig. 4.

Regulation of cellular infiltration (CD4 and CD8 Tcells, and macrophages) in heart allogarfts by anti-OX40L mAb treatment. Cardiac allografts in Rag-$1^{-/-}$ B6 recipients were harvested on POD 100. The CD4, CD8 and Mac-1 expressing cells in the sections of the grafts were localized by immunohistochemistry. Data are presented as a representative immunoperoxidase staining for each of $CD4^+$, $CD8^+$, and Mac-1^+ cells. **a, c** and **e** A typical microscopic view of $CD4^+$ T cells, $CD8^+$ T cells, or Mac-1^+/macrophages in the sections of the grafts from Rag-$1^{-/-}$ recipients harboring T_{mem} cells (*Group* 2, untreated, *n* =8). The *red arrows* indicated positively stained (*brown*) cells. *b, d* and *f* A typical microscopic view of CD4, CD8, or Mac-1 staining in the sections of the grafts from Rag-$1^{-/-}$ recipients harboring T_{mem} cells and receiving anti-OX40L mAb treatment (*Group* 3, *n* =8). Cytoplasm: *blue*. Nucleus: *dark blue*

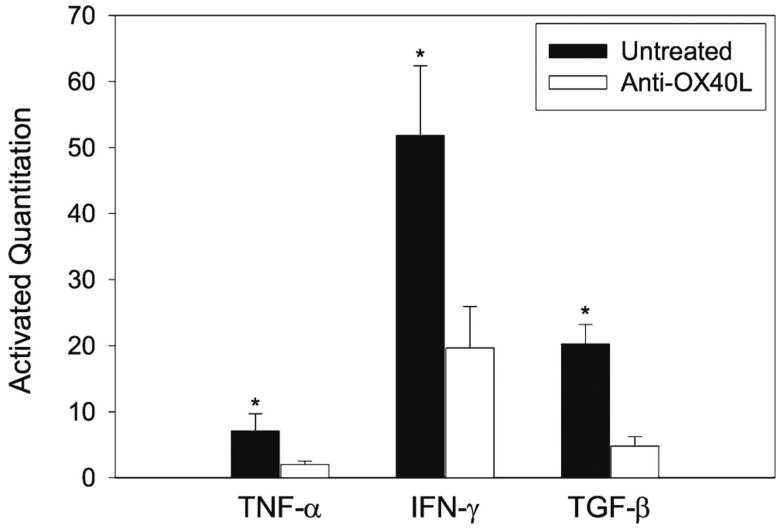

Fig. 5.

Attenuation of cytokine expression in cardiac allografts by anti-OX40L mAb treatment. Cardiac allografts in Rag-$1^{-/-}$ B6 recipients were harvested on POD 100. Five tissues from each group were randomly selected for total RNA extraction. The intragraft expression of TNF-α, IFN-γ, TGF-β in the grafts in untreated versus anti-OX40L mAb-treated groups was analyzed by real-time RT-PCR. Data are presented as mean percentages (±SEM) that were statistically compared using Student's *t*-test test (**P* <0.05 vs. untreated controls, *n* =5)