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Review

Evidence for an Immunoregulatory Role of OX2 with Its Counter Ligand (OX2L) in the Regulation of Transplant Rejection, Fetal Loss, Autoimmunity and Tumor Growth

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Abstract. Transplantation has emerged as an effective treatment for patients with end-stage organ failure. Current regimens of non-specific immunosuppressive drug treatment, which are needed life-long to prevent graft rejection, have numerous adverse side effects and increase the risk of opportunistic infections and malignancy. A major goal is to develop immunotherapeutic protocols that achieve specific tolerance. Such protocols would decrease and eventually eliminate the reliance on non-specific drug therapy. We showed that portal vein delivery of donor antigen prolongs the survival of vascularized and non-vascularized allo- and xeno-grafts, and that increased graft survival is associated with altered cytokine production and augmented expression of the molecule OX2. This review documents further evidence for a more general immunoregulatory role for the interactions of OX2 and its ligand, OX2L.

Key words: OX2; immunoregulation; tolerance; transplantation; autoimmunity; fetal-loss syndrome.

Introduction

Immunological tolerance has been described in terms of three models: **deletion**, in which TCR^+ cells are eliminated from the T cell repertoire[37, 55]; T cell **anergy**; and **suppression**[35, 39]. "Anergy" models hypothesize that activation of TCR^+ cells requires antigen--TCR triggering (signal 1) and "costimulation" by other ligand: counter-ligand interactions (signal 2). Cells encountering antigen without costimulation, when later rechallenged with antigen in the context of costimulatory molecules, no longer respond. They are considered anergized, but not deleted, as determined by the persistence of clonotypic TCR^+ cells[11]. "Suppression" or "infectious tolerance"[8] accounts for the observation that in some instances unresponsiveness can be adoptively transferred with cells from tolerant animals. We pioneered analysis of the prolongation of allograft survival in rodents following antigen-specific portal vein (pv) immunization. Splenic T cells are at least temporarily anergized by hepatic mononuclear cells[15, 29, 30], though unresponsiveness is later transferrable by $\gamma\delta TCR^+$

Abbreviations used: OX2L – ligand for OX2, MLC – mixed leukocyte culture, DC – dendritic cells, Mph – macrophages, mAb – monoclonal antibody.

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cells[30]. This potential role for γδT cells in transplantation tolerance was a novel and unexpected finding, though such cells had been implicated in immunoregulation in other models, including pregnancy[53], oral tolerance induction[46], and suppression of graft-versus-host-disease (GvHD) after allogeneic bone marrow transplantation[47].

Graft unresponsiveness following pv immunization occurred in association with a functionally important preferential activation of $CD4^+$ cells producing type-2 cytokines (IL-4, IL-10, IL-13 and TGF-β) rather than type-1 cytokines (IL-2, IL-12 and IFN-γ)[15, 16, 27, 30]. rIL-12, along with anti-IL-10, reversed graft prolongation and the type-2 cytokine predominance which followed pv immunization[28], while rIL-13, along with anti-IL-12, extended allograft survival[25]. Injection of adenovirus vector constructs expressing IL-10/TGF-β led to more prolonged mouse renal graft survival and further polarization in cytokine production following pv immunization[17, 40].

Antigen-presenting cells (APC) are thought to regulate T cell activation for graft rejection vs tolerance. The function of APC is controlled by their expression of cytokines[2, 9, 43, 52] and costimulatory molecules, including CD80, CD86, CD40 and ICAM-1. Many costimulatory molecules on APC are expressed during maturation and/or exposure of APC to local cytokines[33, 58], and synergistic inhibition of graft rejection has been achieved by the combination of specific monoclonal antibody (mAb) to CD40 and CTLA4Ig, which blocks CD80/CD86 costimulation[39]. However, tolerance is not reproducibly induced by blocking costimulation alone. Paradoxically, we found that dendritic cells (DC), the most potent provider of immunogenic signals, were a powerful tolerogen when infused via the pv[22], implying that there was something fundamentally missing in the conventional models used to explain tolerance regulated by T cell: APC interaction.

Evidence that a Novel Molecule, OX2, Is Implicated in the Regulation of Graft Rejection

We found that tolerance in pv-immunized mice was an correlated with increased expression of a number of mRNA species[20], one of which encodes OX2. This member of the immunoglobulin supergene family is expressed on the surface of dendritic cells[60] and has 92% (77%) homology at the amino acid level with the equivalent rat (human) molecules[6]. Despite a sequence homology between OX2 and CD80/CD86, it signals T cells differently from other costimulator molecules[4], and we hypothesized, instead, that OX2 delivers a "tolerizing" signal[6]. Interestingly, a recently developed OX2 KO mouse shows dysregulation of macrophage activation along with spontaneous development of autoimmune encephalomyelitis (reference added in proof).

Evidence that increased expression of OX2 regulates tolerance to renal and skin allografts

Our first evidence confirming that increased expression of OX2 following pv immunization was important for increased graft survival was that infusion of anti-OX2 monoclonal antibodies blocked graft prolongation, both in mice and rats[20, 23]. Anti-OX2 also blocked the polarization to type-2 cytokine production seen in these animals. Blocking "costimulation" mediated by interaction of CD80/CD86 with their ligands (CD28, CTLA4)[48], using a mixture of anti-CD80/-CD86, acted synergistically with the blockade of OX2.

We next asked whether continued expression of OX2 following pv immunization was necessary for prolongation of allograft survival. FACS and PCR analysis indicated that increased hepatic/splenic DC expression of OX2 was seen throughout the first 15–20 days post pv immunization, corresponding to the improved graft survival over this time. By infusing anti-OX2 into mice at various times following pv immunization and renal allografting, we showed that abrupt termination of graft survival followed anti-OX2 treatment, regardless of the time post-transplant at which infusion of mAb began[21]. Termination of this functional increase in OX2 expression by mAb was followed by a reversal in the altered cytokine production which followed pv immunization. Finally, we constructed a soluble fusion protein for OX2 (OX2:Fc), in which the extracellular domain of murine OX2 was linked to a mutated murine IgG2a Fc region lacking complement and complement-receptor binding domains[18]. OX2:Fc is immunosuppressive and mimicks the effects of pv immunization on allo- and xenograft rejection[18].

Since successful pregnancy can be seen, immunologically, as prolonged allograft survival, we speculated that the immunomodulation mediated by OX2 which we saw in our allograft models might be similarly reflected in normal pregnancy. There are a number of murine models in which inbreeding in certain strain combinations leads to a high rate of spontaneous fetal-loss, and in other combinations leads to low rates of fetal loss. Interestingly, in the former, we found that infusion of OX2:Fc provided protection (from fetal-loss syndrome), while in the latter infusion of anti-OX2

mAbs resulted in spontaneous abortion of pregnancy[7]. Thus we speculated that OX2 was indeed implicated in the regulation of the alloimmune response occurring during normal pregnancy.

The intracytoplasmic region of OX2 has no sequence identity with known signaling kinases, nor does it have a consensus sequence for either the "immunoreceptor tyrosine activation motif" (ITAM) or inhibitor motif (ITIM)[51, 57]. It also lacks typical SH2 or SH3 domains to serve as "docking sites" for adapter molecules which might co-opt other protein kinases/phosphatases in a signaling cascade[44, 49]. Thus we suggested that the ligand-binding activity of the extracellular domains was the biologically important region of the molecule. This, in turn focused our attention on identification of the ligand for OX2 (hereafter OX2L), using OX2:Fc as a tool.

Characterization of cells expressing OX2L and some analyses of their functional properties

Using FITC-coupled OX2:Fc, we showed that LPS--stimulated macrophages, but not DC, bound OX2, as indeed did a subpopulation of ConA-activated $\alpha\beta TCR^+$ cells and most (>80%) ConA-activated $\gamma\delta TCR^+$ cells[31]. This was in agreement with an older study from Barclay's group suggesting the existence of a ligand for OX2 on macrophages[50]. Barclay's group, in association with DNAX, recently succeeded in cloning an OX2L from macrophages, finding it to be a novel molecule of the Ig supergene family and, simultaneously, confirming the signaling properties of the cytoplasmic tail of this molecule following engagement of OX2 which we had predicted (BARCLAY and SEDGEWICK, personal communication). We are currently in the process of preparing rat mAbs to OX2L following cloning of OX2L using[125] I-OX2:Fc as a probe, and an expression library prepared by Stratagene (USA), with mRNA derived from a 1:1 pool of LPS or ConA-stimulated mouse spleen cells. We have already found that stimulation of $OX2L^+$ macrophages by OX2:Fc leads to even more pronounced inhibition of allogeneic stimulation in culture, as assessed by CTL and cytokine induction, and of graft rejection *in vivo*[31]. The DNAX group has described dysregulation of macrophage activation as a prominent phenotype of the OX2 KO mouse. However, the potential role of OX2L expression on $\alpha\beta$ or $\gamma\delta TCR^+$ cells is unexplored. To date it is not even known if the ligand expressed in these different cell populations is identical. It is thus possible that *in vivo/vitro* effects of anti-OX2L, and/or of OX2L:Fc (also under construction), will be complicated by differential effects mediated at the cell surface of these independent cell types. This may be particularly the case if binding affinities (of OX2:OX2L) differ at the cell surface in these cell subpopulations.

The role of OX2 in immunoregulation by $OX2L^+$ $\alpha\beta TCR^+$ or $\gamma\delta TCR^+$ cells

Increased production of IL-10 and TGF-β is implicated in increased graft survival following pv immunization[20, 25]. The initial source of the cytokines contributing to the polarization of T cells[54], and the mechanism(s) controlling their production, is unknown. We showed that $\gamma\delta TCR^+$ cells are involved in immunoregulation after pv immunization[19, 25], and that persistent expression of OX2 was essential for detection of $\gamma\delta TCR^+$ cells capable of adoptive transfer of increased graft survival[21]. As noted above, a subpopulation of $\alpha\beta TCR^+$ cells, and most $\gamma\delta TCR^+$ cells, were found to express $OX2L$[31]. Possible explanations which incorporate these data include the hypothesis that $\gamma\delta TCR^+$ cells are relatively insensitive to OX2:OX2L interactions, or the alternative hypothesis that they are "hypersensitive" to costimulation by the CD80/CD86:CD28/CTLA4 interaction. Another explanation suggests that OX2: :OX2L paradoxically stimulates $\gamma\delta TCR^+$ cells. We are planning to investigate this by stimulating $OX2L^+\alpha\beta$ and γδT cell hybridoma cells[24] (selected using anti-OX2L mAbs) *in vitro* with purified allogeneic DC or OX2:Fc or with combinations of DC and OX2:Fc.

Regulation of transcription of OX2

T cell activation is regulated by the integration of signals resulting from TCR and costimulator molecule engagement. This ignores (temporarily) the potential role for exogenous cytokines in cell activation, beyond their role in regulating expression of costimulator molecules. For simplification, we can consider, from the perspective of the ligands expressed on APC, the "key" players in costimulation to be members of the CD80/CD86 family, CD40 and now, OX2. Understanding the manner in which expression of these gene products is regulated is important to understanding how T cell activation is regulated. The endogenous levels of expression of these molecules is controled by cytokines in the milieu[61, 63], and culturing DC in the presence of different cytokines further alters their costimulatory function[42]. IL-12 and IFN-γ increases T cell activation (for IL-2 production), while DCs cultured with IL-10 and IL-4 have decreased stimulatory activity (for IL-2 production)[41, 42]. Characterizing the regulation of gene

transcription, and the promoter, enhancer and silencer regions in genes which are responsive to cis/trans-activating factors, can often suggest novel mechanisms for regulating biological function and would provide valuable information on mechanisms involved in the endogenous and cytokine-activated differential expression of costimulator molecules. As an example, studies examining the chromatin configuration of the human CD80 gene in intact nuclei from various cell types identified a tissue-specific deoxyribonuclease I hypersensitive site 3 kb upstream of the transcription start site as a cell type-specific enhancer region[62]. This 183 bp region was responsive to both lipopolysaccharide and dibutyryl cAMP, which regulate CD80 expression. Deletional and site-directed mutagenesis revealed the presence of multiple, functionally critical cis elements within this region, one of which was a nuclear factor NF-κB consensus sequence. This is consistent with data implicating members of the NF-κB family in signal-transduction pathways relevant to CD80 expression.

We have screened a human P1 phage library with a probe for a fragment in the V-region (exon 3) of human OX2 to isolate clones for the human OX2 gene. A sublibrary (Sau3A1 endonuclease digestion) was generated in pBluescript SK II (Stratagene) and screened with probes representing various regions of the human OX2 cDNA. Several clones were identified with the probe for exon 1, and 5' sequencing has thus far provided some 1700 bp of the human OX2 promoter region (CHEN et al., submitted). Using both primer extension and 5'RACE, we have identified a region 58 bp from the translational start site as the likely transcriptional initiation site. Current studies using luciferase reporter constructs with the OX2 promoter region are planned to investigate in detail the transcriptional regulation of basal OX2 expression in different cell types (e.g. DC versus non-expressing endothelial cells), as well as the cis elements responsible for inducible expression of OX2.

The role of OX2:OX2L interactions in tumor immunity

Interest in the field of tumor immunology has increased in the last several years with the growing evidence that it is possible to identify, and immunize animals for protection with, discrete tumor antigens[1, 12, 56]. Considerable energy has been invested in trying to understand how natural tumor is regulated *in vivo*, and how this could be manipulated to the benefit of the tumor-bearing host. In this regard, a number of groups have reported on the use of DC coated with tumor antigen, or expressing tumor antigen (following transfection with cDNAs encoding the antigen), as immunogens to induce tumor rejection[10, 14, 32, 38, 41]. An alternative approach has used tumor cells themselves, transfected with gene(s) such as those controlling cytokines or expression of costimulatory molecules (CD80/CD86) on the cell surface, as an immunogen[5, 14, 34, 36, 45]. In an interesting model using as recipients animals receiving allogeneic bone marrow transplantation, which mimicks a therapy used for treatment of leukemia in man, BLAZAR et al.[3] reported that it was possible to promote a graft-versus-leukemia (GvL) effect, without unwanted GvHD, by pre-immunizing tumor-transplanted mice with tumor cells transfected to express CD80, but not CD86.

Given our evidence that interactions between OX2 and OX2L were important in the physiological regulation of immunity per se (as confirmed, for instance, by the effect of infusion of OX2:Fc on antibody responses to nominal antigen[7]) we have recently begun investigating whether these same interactions might contribute to regulation of tumor immunity. Growth of EL4 tumor was studied in C57BL/6 mice immunized to the syngeneic tumor cells using either a bone marrow transplant model (following reconstitution with T-depleted C3H bone marrow cells), or by immunization with CD80-transfected tumor cells in Freund's Adjuvant. In each case, using either manipulation with OX2:Fc or anti-OX2 mAbs, we could show that tumor growth is subject to control by the same OX2:OX2L interactions as we documented in an allogeneic transplant model (GORCZYNSKI et al., Clin. Exp. Immunol., in press).

Conclusions

T cell activation is regulated by the surface expression of both costimulatory (CD40, CD80, CD86) and regulatory (OX2) molecules on APC as well as the cytokine milieu in which antigen is encountered. We have shown that there exists a synergy in immunosuppression following blockade of costimulation and triggering of OX2L on macrophages by infusion of soluble OX2:Fc. Under physiological conditions, increased OX2 expression on DC follows pv donor-specific immunization, and results in increased allograft survival and altered cytokine production in cells from transplanted animals, with polarization to type-2 cytokines (IL-4, IL-10, TGF-β). OX2L itself is also expressed at the surface of activated αβ and $\gamma\delta TCR^+$ cells. While the functional role for OX2L expression on activated T cells is unknown, $\gamma\delta TCR^+$ cells are capable of adoptive transfer of increased graft survival. One prediction

of our work on immunoregulation following OX2:OX2L interactions is that organs expressing OX2 as a transgene should be accepted as allografts with minimal immunosuppression. This is under investigation. More recent data from our own group, and DNAX (see above), suggest immunoregulation by OX2:OX2L interaction can be extended to autoimmune disorders, fetal-loss syndrome, and tumor immunity.

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