Paradoxical Dampening of Anti-Islet Self-Reactivity but Promotion of Diabetes by OX40 Ligand[1]

Natalia Martin-Orozco,[2]* Zhibin Chen,* Laurent Poirot,* Elzbieta Hyatt,* Andy Chen,† Osami Kanagawa,‡ Arlene Sharpe,† Diane Mathis,[3]* and Christophe Benoist[3]*

Costimulatory signals received by diabetogenic T cells during priming by or upon secondary encounter with autoantigen are decisive in determining the outcome of autoimmune attack. The OX40-OX40 ligand (OX40L) costimulatory pathway is known to influence T cell responses, prompting us to examine its role in autoimmune diabetes. A null allele at OX40L completely prevented diabetes development in nonobese diabetic mice and strongly reduced its incidence in a TCR transgenic model (BDC2.5). However, somewhat paradoxically, the initial activation of T cells responsive to islet β cell Ag was slightly faster and more efficient in the absence of OX40L, with an increased degree of cell proliferation and survival in the deficient hosts. Activated T cell migration into and retention within the islets was also slightly accelerated. When challenged in vitro, splenocytes from BDC2.5.OX40L$^{o/o}$ mice showed no altered reactivity to exogenously added peptide, no bias to the Th1 or Th2 phenotype, and no alteration in T cell survival. Thus, the OX40/OX40L axis has the paradoxical effect of dampening the early activation and migration of autoimmune T cells, but sustains the long-term progression to autoimmune destruction. *The Journal of Immunology,* **2003, 171: 6954–6960.**

Type 1 diabetes mellitus is caused by autoreactive T cell destruction of insulin-producing β cells (1). The costimulation that diabetogenic T cells receive during priming by or secondary encounters with autoantigen is one of the decisive signals regulating the progression of diabetes. For example, costimulation through members of the CD28 family expressed on T cells (CD28, CTLA-4, inducible costimulator) is involved in the control of autoreactivity during initiation of insulitis in mouse models, preventing the complete destruction of β cells (Refs. 2, 3 and A. Herman, A. J. McAdam, A. H. Sharpe, D. Mathis, and C. Benoist, manuscript in preparation). Similarly, signaling through CD40/CD154, members of the TNF/TNFR family of costimulatory molecules, is known to be important for diabetes development (4, 5), although not completely essential.

Another TNFR family member, OX40 (CD134, *tnfrsf4*), a contributor to the costimulatory signal for $CD4^+$ T cells, may also be of interest in the context of type 1 diabetes. OX40 is expressed only, and only transiently, on activated T cells (6). OX40 ligand (OX40L)[4] (CD134-L, *tnfsf4*) is expressed on APCs, B cells, macrophages, and dendritic cells (DCs), also after activating stimuli (7–10) and on vascular endothelial cells (11). OX40-OX40L costimulation promotes priming and survival of $CD4^+$ T cells after an antigenic or tumor challenge (10, 12–21). It requires direct interaction between T cells and DCs (22), and may involve induction of the anti-apoptotic Bcl-x_L and Bcl-2 molecules (13). Blocking OX40-OX40L interaction suppressed autoimmune attack by $CD4^+$ T cells in several experimental systems. Treatment with anti-OX40L reduced inflammation in the joints of mice with collagen-induced arthritis (CIA), and OX40/OX40L blockade dampened clinical signs of experimental autoimmune encephalomyelitis (EAE) (10, 21, 23, 24). The mechanism of such protection has not been fully clarified: in the CIA model, $CD4^+$ T cells seemed to have reduced differentiation to the Th1 phenotype and helper activity; in the EAE model, the priming and activation of pathogenic T cells or their migration to the spinal cord were found to be differently affected in the various studies.

The goal of this study was to evaluate the impact of the OX40/OX40L pair on the development of type 1 diabetes. We introgressed the OX40L knockout (KO) mutation (OX40L^o) (19) onto the nonobese diabetic (NOD) or BDC2.5 TCR transgenic (tg) background. The former is the classical mouse model of autoimmune diabetes; the latter is a derivative carrying the rearranged TCR genes from a diabetogenic T cell clone derived from a NOD mouse, and harboring anti-islet T cells at high frequency, thereby greatly facilitating dissection of the events that modulate the activation and effector function of potentially diabetogenic T cells (25). We find that OX40L does make an important contribution to autoimmune diabetes, but it does so in paradoxical manner, not readily congruent with previous conceptions of its role in immune and autoimmune responses.

*Section on Immunology and Immunogenetics, Joslin Diabetes Center, Department of Medicine, Brigham and Women's Hospital, and Harvard Medical School, †Immunology Research Division, Department of Pathology, Brigham and Women's Hospital, Boston, MA 02215; and ‡Department of Pathology and Immunology and Center for Immunology, Washington University School of Medicine, St. Louis, MO 63110

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[2] Current address: The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8.

[3] Address correspondence and reprint requests to Dr. Christophe Benoist and Dr. Diane Mathis, Joslin Diabetes Center, One Joslin Place, Boston, MA 02215. E-mail address: cbdm@joslin.harvard.edu

[4] Abbreviations used in this paper: OX40L, OX40 ligand; DC, dendritic cell; NOD, nonobese diabetic; CIA, collagen-induced arthritis; tg, transgenic; EAE, experimental autoimmune encephalomyelitis; RAG, recombinant-activating gene; KO, knockout.

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Materials and Methods

Mice

NOD/Lt mice were bred in our animal facility (Institutional Animal Care and Use Committee protocol 99-20). BDC2.5 TCR tg mice (25) were backcrossed for over 25 generations onto the NOD (BDC2.5/N) or the B6.H-2^{g7} congenic background that carries the H-2^{g7} MHC on the C57BL/6J (BDC2.5/B6^{g7}) background (26), or with the recombinant-activating gene (RAG)-1 KO mutation (over nine generations). Previously described OX40L KO mice (19) were backcrossed onto the NOD/Lt background for eight generations (OX40L^{o}/NOD) and onto B6.H-2^{g7} (OX40L^{o}/B6^{g7}) for eight generations, and were intercrossed to obtain animals homozygous for the OX40L mutation. These were crossed with BDC2.5/N and BDC2.5/B6^{g7}, respectively, to generate BDC2.5/N-OX40L$^{o/o}$ and BDC2.5/B6^{g7}-OX40L$^{o/o}$ mice (and OX40L-proficient control littermates). Mice were typed for the BDC2.5 transgenes and for being H-$2^{g7/g7}$ by flow cytometric staining of blood samples using mAbs against CD4 (Caltag Laboratories, Burlingame, CA), VB4 (BD PharMingen, San Diego, CA), A^{b} (Y3P mAb) and A^{g7} (40N). Analysis of the stained samples was done on a Coulter XL cytometer, using EXPO32 software. OX40L genotypes were determined by PCR analysis of tail DNA (with a combination of primers specific for the neomycin and OX40L genes (19)).

Mice were monitored for diabetes weekly starting at day 21, and were followed for up to 30 wk. Animals were considered diabetic after two sequential positive measurements of urine glucose and a blood measure above 300 mg/dl.

Histology

Thin sections from different levels of 4% paraformaldehyde-fixed, paraffin-embedded pancreas were H&E stained and examined for the presence of insulitis. All the islets were counted and analyzed to determine the percentage of infiltrated islets and the degree of infiltration.

Adoptive transfers of CFSE-labeled cells

Depending on the particular experiment, splenocytes from either BDC2.5/N or BDC2.5/N.OX40L$^{o/o}$ were pooled. The cell suspension was depleted of erythrocytes in 0.83% NH_4Cl, washed with PBS, adjusted to 10^7 cells/ml in PBS and labeled with 2.5 mM of CFSE (Molecular Probes, Eugene, OR) for 10 min at 37°C. After the incubation time, the cells were washed twice with DMEM supplemented with 20% FCS and adjusted to 10^8 cells/ml. In each experiment, 100 μl of CFSE-labeled cells were injected i.v. into recipient mice (o/o, +/o, or wild-type for OX40L). After transfer (60 h), or when indicated, lymph nodes (LN) were removed, smashed between glass slides, and the cell suspensions were stained with biotin-conjugated anti-CD44 (BD PharMingen), streptavidin-PE Cy5 (DAKO, Glostrup, Denmark), anti-CD4 PE-Texas Red (Caltag Laboratories), Vβ4-PE (BD PharMingen) and anti-BDC2.5 (27). A total of 1×10^6 cells were analyzed per sample in a Coulter XL cytometer using EXPO32 software.

DC flow cytometry analysis

Lymphocytes from OX40L^{o}/NOD and OX40L^{o}/B6^{g7} were harvested and dissociated with collagenase D (30 min at 37°C). The cell suspensions were blocked with anti-CD32/CD16 (BD PharMingen) in FACS wash buffer (PBS supplemented with 3% heat-inactivated horse serum and 0.05% sodium azide). After 15 min at 4°C the cells were washed and stained with anti-A^{k} (clone 10-3-6, BD PharMingen), anti-mouse IgG H+L Cy5 (The Jackson Laboratory, Bar Harbor, ME), anti-mouse CDllc-PE (BD PharMingen), anti-mouse CD11b-biotin (Caltag Laboratories), anti-mouse B220-FITC, Streptavidin-PE Cy7 (Caltag Laboratories) and 0.1 μg/ml Hoechst 133 (Molecular Probes).

Ag presentation assay

Splenocytes from BDC2.5/N.OX40L^{o} mice (o/o, +/o, or +/+ littermates) were depleted of erythrocytes as previously described, washed and cultured in DMEM supplemented with 10% FCS, 2 mM glutamine, nonessential amino acids, sodium pyruvate, penicillin and streptomycin, in the presence of the agonist mimotope peptide 1040–63 of Judkowski et al. (28): RTRPLWVRME, hereafter referred to as BDCmi peptide. Cultures were harvested at 72 h following 18-h exposure to 0.5 mCi [^{3}H]TdR, and incorporated radioactivity was measured in a Direct Beta Counter MATRIX 9600 (Packard Instrument, Meriden, CT). For experiments involving T cells and APCs from different sources, DCs were purified by positive selection through magnetic cell sorting with streptavidin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) using biotin-conjugated anti-mouse CD11c (BD PharMingen) following the manufacturer's instructions, and T cells were purified by magnetic negative selection using mAb conjugated to biotin anti-B220, anti-Aβ^{k} (10-2-16), anti-CD11c, anti-CD11b, anti-CD8a, and anti-CD69.

Adoptive transfer of CD4^{+} T cells into BDC2.5/N-Rag$^{o/o}$ mice.

Purification and adoptive transfer of CD4^{+} T cells were done largely as described (29). Briefly, splenocytes from 5- to 9-wk-old OX40L^{o}/NOD mice or heterozygous littermates were positively selected with anti-CD4 magnetic beads (Miltenyi Biotec), according to the manufacturer's protocol. A total of 95% of the selected cells were CD4^{+}. These cells were injected i.p. into 10-day-old BDC2.5/N-Rag$^{o/o}$ mice, and the recipients were tested from age day 20 for diabetes every 2–3 days.

Results

Lack of OX40L expression can block diabetes development in NOD mice

To evaluate the impact of OX40L on the development of autoimmune diabetes, the OX40L-null mutation (19) was backcrossed onto the NOD/Lt genetic background. To accelerate the natural attrition of diabetes-resistance loci during the backcross, we selected breeders for homozygosity of the NOD alleles at the main diabetes-resistance loci (*idd1* at the MHC, *idd3*, *idd5*, and *idd10*). After eight generations of backcross, the mice were intercrossed and followed for diabetes until 30 wk of age. As illustrated in Fig. 1*A*, the OX40L deficiency had a dramatic impact: all homozygous KO mice were completely protected from diabetes, whereas disease showed its usual course and penetrance in wild-type littermates. In parallel, we evaluated the incidence of insulitic lesions in the pancreas (Fig. 1*B*). In this evaluation, the OX40L deficiency had a strong influence, almost completely eliminating leukocytic infiltration of islets. Thus, OX40/OX40L costimulation seems to play an essential role in the pathogenesis of type 1 diabetes in the NOD mouse, as it does in murine models of other autoimmune diseases (10, 21, 23, 24).

As an independent verification of this conclusion, and to begin dissecting the mechanisms involved, we crossed the OX40L mutation into the BDC2.5/B6^{g7} line. In these TCR tg mice, the congenic H-2^{g7} locus permits the activation of BDC2.5^{+} T cells, whereas disease-accelerating loci from the B6 genetic background contribute to a faster and more reproducible progression to overt diabetes (25, 26) as also happens for *idd7*, *8*, and *14* in NOD mice (30, 31). The incidence of diabetes in this context is ~50%, with peak onset around 5–6 wk. Diabetes was followed in OX40L-deficient and control littermates in two independent cohorts (Fig. 1*C*). In both sets of animals, the OX40L-deficient mice showed a very significant reduction in disease compared with littermates heterozygous or wild-type for OX40L. Both the overall incidence (14.6% in o/o vs 48.6 and 50.0% in +/o and +/+ control groups) and the maximum age of onset (9.5 vs 6 wk median age of onset) were altered in homozygotes. Thus, in this second model of diabetes, for which diabetes susceptibility loci of the NOD genome play essentially no role, the OX40-OX40L pathway affects the development of diabetes, prompting us to study further the root of this effect.

No heightened tolerance in OX40L-deficient mice

One of the most obvious explanations for the profound effect of the OX40L mutation on diabetes development in NOD mice was that tolerance induction in the T cell compartment was affected, as has been suggested in another context (32). Might pancreas-reactive T cells be tolerized more efficiently in the absence of OX40 costimulation? This hypothesis was tested in the BDC2.5 TCR tg model of diabetes, which allows one to track the fate of islet-reactive, CD4^{+} T cells (25). A mAb specific for the BDC2.5 clonotype has recently been generated; BDC2.5/N TCR tg mice were found to

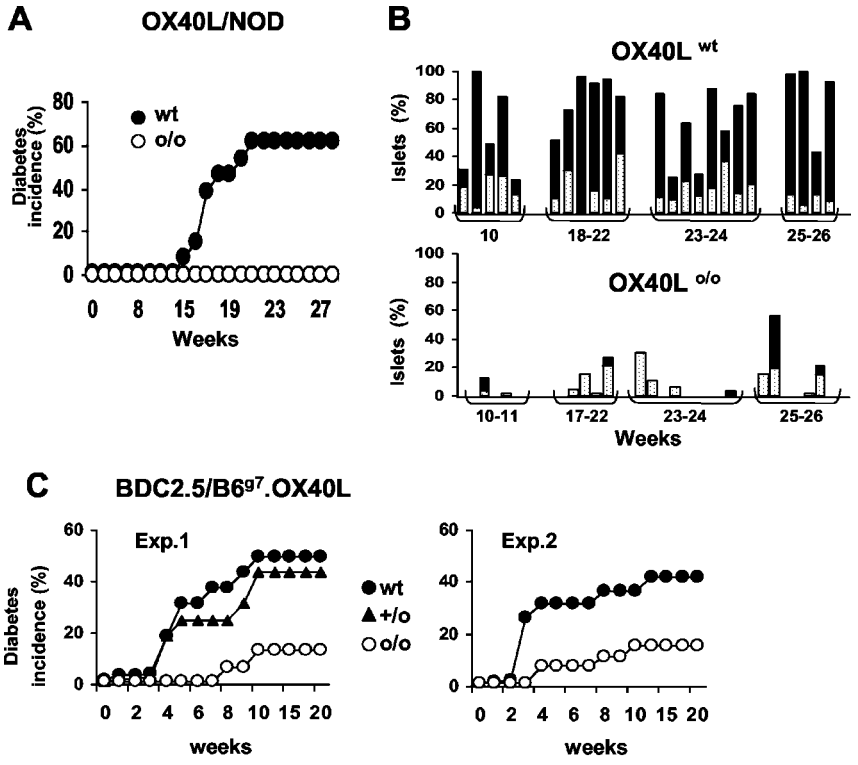

FIGURE 1. Protection from diabetes in NOD or BDC2.5/B6^{g7} mice lacking OX40L. *A*, OX40L$^{o/o}$/NOD mice at the 8th generation backcross to NOD (n = 10), or wild-type littermates (n = 13), were followed for diabetes up to 30 wk of age. *B*, Insulitis was quantitated in pancreata from mice of various ages, with or without the OX40L$^{o/o}$ mutation on the NOD background. Peri-insulitis (□) and true insulitis (■) are shown. Each bar represents an individual mouse. The evaluation of H&E sections was performed in a blind fashion by two independent observers. *C*, Diabetes progression in BDC2.5/B6^{g7} OX40L littermates; two independent cohorts were followed (n in expt. 1: wt = 16, +/o = 16, o/o = 15; n in expt. 2: +/o = 19, o/o = 26).

harbor T cells expressing various levels of clonotypic TCR, anti-islet reactivity varying for cells with different clonotype levels (27). The BDC2.5 transgenes were introduced into the mouse line carrying the OX40L-null mutation on the NOD background (eight generations of backcross) to generate BDC2.5/N.OX40L$^{o/o}$ mice. Flow cytometric analyses of CD4$^+$ T cells in s.c. (inguinal) lymph nodes and pancreatic lymph nodes from BDC2.5/N.OX40L$^{o/o}$ or control BDC2.5/N.OX40L$^{+/+}$ mice are shown in Fig. 2*A*. T cell numbers were normal, and there was no change in the number of cells expressing the islet-reactive clonotype, nor in the clonotype expression profile. Similar observations were made in the thymus, where clonotype-positive cells matured normally (data not shown). These cells appeared reactive in vivo, as their expression of CD69 betrayed cell activation in pancreatic lymph nodes but not in inguinal lymph nodes (Fig. 2*A*).

The lack of diabetes in the absence of OX40 costimulation might also have been explained by induction of anergy in autoreactive T cells. This possibility was tested by stimulating T cells

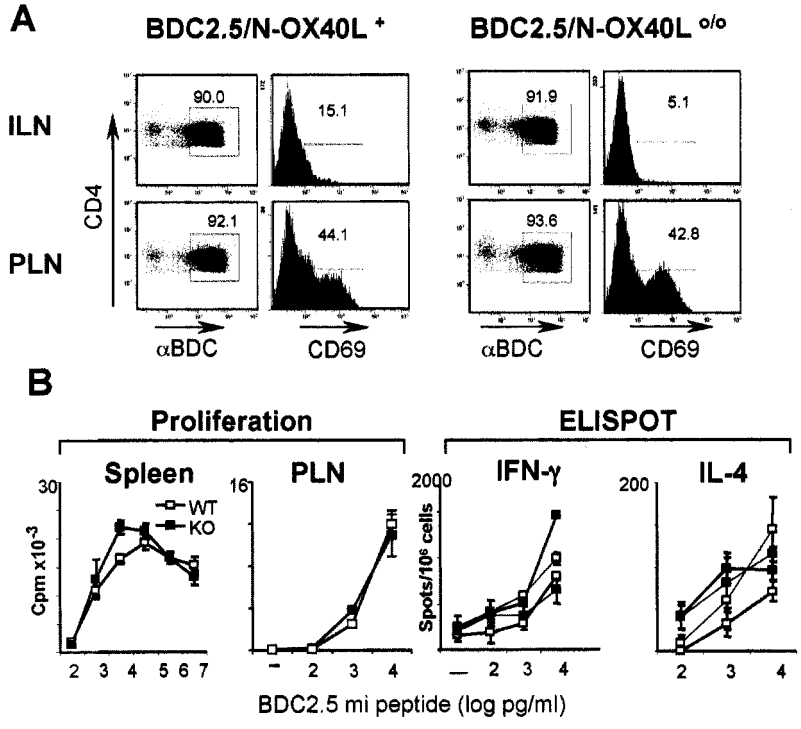

FIGURE 2. Normal repertoire and activation of BDC2.5/N T cells in the absence of OX40L. *A*, Inguinal and pancreatic lymph nodes from BDC2.5/N.OX40L$^{o/o}$ and OX40L^{wt} mice were analyzed by multicolor flow cytometry (stained with mAbs against CD4, the BDC2.5 clonotype, and CD69). Histograms showing CD69 expression are gated on CD4$^+$BDC^{high} cells. *B*, The BDC2.5mi peptide (peptide 1040-63; see Ref. 28) was used to activate splenocytes from BDC2.5/N.OX40L$^{o/o}$ and BDC2.5/N.OX40L$^{+/o}$ littermates. [^{3}H]Thymidine incorporation was measured at 72 h of culture; IFN-γ and IL-4 producing cells were detected at 16 h of culture by ELISPOT. Data are representative of three experiments.

from BDC2.5/N.OX40L$^{o/o}$ mice in vitro with an agonist peptide recognized by the BDC2.5 receptor. The islet β cell Ag recognized by the BDC2.5 clonotype is still unknown, but several agonist mimotope peptides have been described recently (28). As shown in Fig. 2*B*, the BDCmi peptide elicited identical proliferative responses with splenocytes or lymph node cells from KO mice or control littermates. This held true whether cells were harvested after 72 h of culture as in this experiment, or when left in culture for longer periods, up to 120 h (data not shown). In further criss-cross experiments, tg T cells derived from BDC2.5/N.OX40L$^{o/o}$ or OX40L^{wt} mice responded similarly to the BDCmi peptide when presented by OX40L$^{o/o}$ or OX40L^{wt} DCs (data not shown). Comparable numbers of these activated T cells produced comparable amounts of IFN-γ and IL-4, detected in ELISPOT assays (Fig. 2*B*, *right*). Therefore, signals from OX40L do not seem to impact on the numbers and reactivity of islet-specific T cells. Tolerance is not an issue.

More efficient autoimmune activation in the absence of OX40L

We next examined the activation of T cells in vivo, as the deficit in OX40L could conceivably prevent the activation of otherwise normal T cells. In a first set of experiments, we examined the rate of establishment of autoimmune infiltration, to test whether the initial entry of islet-reactive T cells into the target organ might be affected. In BDC2.5 TCR tg mice, the insulitis initiates in a very stereotyped and abrupt fashion, between 14 and 18 days of age (25, 33), a timing found to be similar in other TCR tg systems based on islet-reactive specificities (34–36). As shown in Fig. 3, insulitis was not retarded in BDC2.5/N.OX40L$^{o/o}$ mice compared with OX40L-proficient littermates: it was already detectable at day 15, and actually showed significantly increased leukocyte penetration into the islets compared with that of wild-type littermates (29 vs 9% fully infiltrated islets, $p = 0.032$). By day 21, the wild-type mice had "caught up" somewhat, but there was still a higher proportion of mice with insulitis in the OX40L$^{o/o}$ group (56 vs 36%). Thus, and contrary to expectation, the absence of OX40L did not delay insulitis, but actually accelerated the rate of leukocyte accumulation in target organs.

In a second set of experiments, to test whether OX40L costimulation affected the initial activation of BDC2.5 T cells in vivo, we transferred the same preparations of CFSE-labeled naive splenocytes from BDC2.5/N mice into OX40L$^{o/o}$/NOD and control littermates. We and others have shown that Ag-specific activation of islet-reactive T cells first occurs in the adjacent pancreatic lymph nodes but not in lymph nodes draining other organs (33, 34, 36). As illustrated in the representative experiment of Fig. 4, BDC2.5/N T cells proliferated effectively when transferred into both OX40L-deficient and -proficient mice, with an increased proportion of divided cells in the KO hosts. This trend was analyzed in a number of similar experiments in which we quantitated both the proportion of cells having undergone division ("proliferation index") and the total cell yield, reflecting cell division and survival in the first days of the response. A range of experimental conditions and times (between 48 and 76 h post-transfer) was used, thus spanning the very early stages of the response (very few cells having divided) to more advanced response stages (the majority of cells having undergone several cycles of division). In all cases, the KO recipients showed an increased proportion and absolute number of proliferated cells, even at the earliest times of response (Table I), suggesting that signaling through OX40 actually has a negative effect on T cell proliferation and survival subsequent to stimulation by autoantigens. This observation contrasts with prior suggestions that OX40 costimulation conditions the survival of cells after priming (13, 16, 18), but likely explains the faster onset of insulitis previously shown (Fig. 3).

Given this more active presentation of endogenous Ags in the OX40L-deficient mice, we then analyzed the DC compartment in these mice, as presentation by DCs is the rate-limiting step for T cell activation in the lymph node environment. Might a phenotypic alteration in DCs explain the more effective autoantigen presentation? Lymph node DC populations from OX40L$^{o/o}$/NOD and control littermates were analyzed by flow cytometry, delineating these complex populations with mAbs to CD11c, CD11b, CD8, and DEC205. Overall, the OX40L deficiency did not induce any alteration in overall DC numbers in pancreatic lymph nodes or other lymph nodes (data not shown). The relative proportions of so-called myeloid and lymphoid DCs, ascertained from CD8 and DEC205 stains, were also unaffected. On the contrary, the CD11c^{+}CD11b^{high} subset of

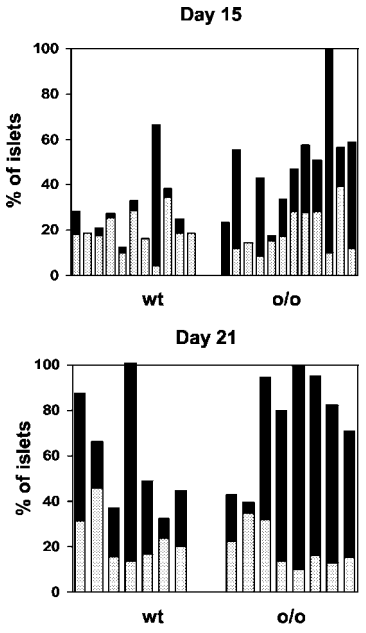

FIGURE 3. Faster insulitis in OX40L deficient BDC2.5/N tg mice. Pancreata from young BDC2.5/N mice, with or without the OX40L$^{o/o}$ mutation (eight generations backcross to NOD/Lt), were tested histologically at 15 and 21 days of age. Pancreatic infiltration represented as in Fig. 1.

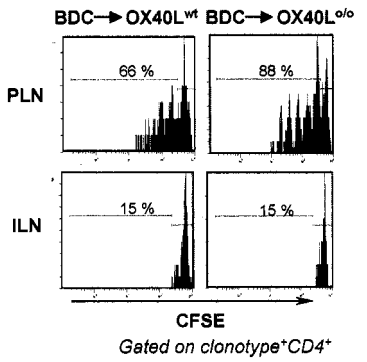

FIGURE 4. BDC2.5 T cells priming is more robust in pancreatic lymph nodes (PLN) of OX40L$^{o/o}$ mice. CFSE-labeled splenocytes from young BDC2.5/N mice were transferred into OX40L$^{o/o}$/NOD and OX40L$^{+/o}$/NOD mice. Pancreatic and inguinal lymph nodes were removed from the recipients 72 h after transfer, and cells were counterstained with mAb against the BDC2.5 clonotype and CD4. The CFSE profiles are gated on CD4^{+}BDC^{high} cells. The results are representative of six experiments (see Table I).

Table I. *Number of BDC2.5$^+$ T cells that undergo priming-derived cell division in PLN of CD134$^{o/o}$/NOD and CD134^{wt}/NOD mice*[a]

	CD134^{wt}		CD134$^{o/o}$	
Experiment	Cell Number[b]	PI[c]	Cell Number	PI
1	3352	3.558386	5800	6.590909
2	2349	0.817328	4940	1.869796
3	294	0.091818	411	0.553163
4	397	0.866812	776	1.558233
5	1089	0.754175	1344	0.913779
6	1129	0.754175	1134	0.913779

[a] Analysis of PLN-derived CFSE-labeled CD4$^+$ BDC2.5$^+$ T cells recovered from CD134L$^{o/o}$/NOD and CD134L^{wt}/NOD mice at 72 h after transfer. Data are from six independent experiments as shown in Fig. 4.

[b] Number of CD4$^+$BDC2.5$^+$ T cells that undergo cell division and decrease in CFSE fluorescence intensity.

[c] PI: Proliferation index = dividing cells/nondividing cells.

myeloid DCs (CD8$^-$DEC205^{low}33D1$^+$) from OX40L$^{o/o}$/NOD mice showed a mild but reproducibly increased expression of MHC class II molecules (A^{g7}) compared with that of the same population in OX40L-positive littermates (Fig. 5, *A* and *B*). This was observed not only in the pancreatic lymph nodes but also in other lymph nodes, and was isolated in that CD86, CD80, and CD40 levels were not affected (data not shown). When cultured in vitro, however, these APCs are not demonstrably more effective at presenting the BDC2.5mi peptide (Fig. 5*C*). Thus, in the absence of OX40L there is induction of MHC class II molecules on DCs as a result of T cell activation or due to enhanced DC maturation, consistent with the increased efficiency of BDC T cell priming and insulitis.

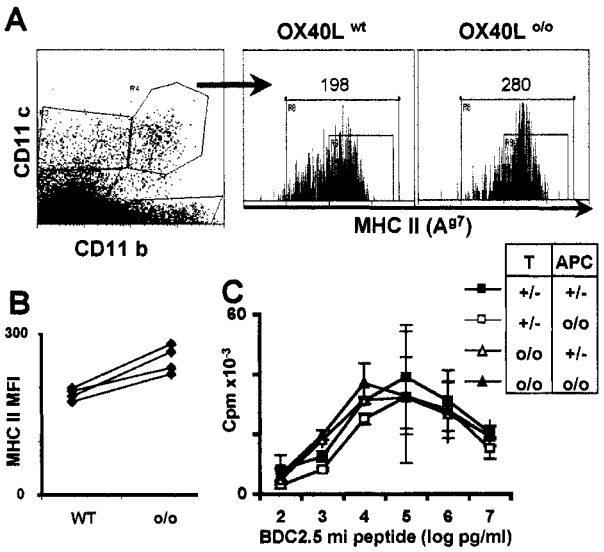

FIGURE 5. CD11c$^+$CD11b$^+$ DCs from OX40L$^{o/o}$ mice display more MHC class II molecules. *A*, DCs were prepared from mesenteric lymph nodes of OX40L$^{o/o}$/NOD and OX40L$^{+/+}$/NOD mice, and stained with mAbs against CD11c, CD11b, Aβ^{g7}, and B220. The histograms showing MHC class II expression are gated on CD11c$^+$CD11b$^+$ cells. *B*, Mean fluorescence intensity (MFI) values for MHC class II expression from CD11c$^+$CD11b$^+$ DC population, from four independent experiments (littermate pairs connected by a line); $p = 0.0086$, two-tailed paired Student's *t* test. *C*, Ag presentation capacity. In reciprocal mixing experiments, CD4$^+$ T cells were purified from BDC2.5/N.OX40L$^{o/o}$ or BDC2.5/N.OX40L$^{+/o}$ littermates, and DCs from OX40L$^{o/o}$/NOD and OX40L$^{+/o}$/NOD mice. They were mixed in wells of 96-well plates (10^5 CD4$^+$ cells, 5×10^4 APCs), and cultured with graded doses of mimotope peptide. Proliferation was measured as incorporation of [^{3}H]thymidine in the last 18 h of a 72 h culture.

Thus, rather paradoxically in light of its requirement for progression to end-stage diabetes, OX40L costimulation actually dampens the early stages of autoimmune T cell activation and islet infiltration.

No detectable regulatory cell defect in the absence of OX40L

It has recently been suggested that OX40L costimulation may be tied to the function of regulatory T cells (37). We performed several experiments to test whether a difference in the regulatory populations might account for the effect of OX40L on development of autoimmune diabetes. The numbers and phenotypic characteristics of CD4$^+$CD25$^+$ cells were very comparable in spleens of OX40L$^{o/o}$/NOD mice and control littermates, as were those of NKT cells (data not shown). We also tested directly the function of regulatory populations in these mice, using a sensitive assay system previously described (29). BDC2.5/N.RAG$^{o/o}$ mice become uniformly diabetic by 4 wk of age, but the normal splenocytes from NOD mice can prevent diabetes when transferred at 10 days of age. As shown in Fig. 6, CD4$^+$ splenocytes from either donor protected BDC2.5/N.RAG$^{o/o}$ mice when 2×10^5 cells were administered (a subsaturating dose repeatedly found to confer protection to only a fraction of the mice; see Ref. 29 and experiments 1 and 2). There was thus no increase in the activity of protective cells in the absence of OX40L, at least by this assay, confirmed when a subeffective dose of cells was injected (5×10^4; experiment 3).

Discussion

In several models of autoimmune diabetes the outcome of autoimmune aggression seems to be determined by the quality or quantity of costimulation received by autoreactive T cells during priming and/or upon tissue invasion (2, 4, 5). We initiated this study to evaluate the role of OX40L in such a context. We used both the NOD/Lt and BDC2.5 TCR tg mouse models of diabetes, crossing the OX40L KO mutation onto the NOD or B6-H2^{g7} backgrounds. The effects on diabetes were quite dramatic: complete protection in NOD mice, strong reduction in BDC2.5/B6^{g7}. We can rule out the influence of a known *idd* locus because the analysis was performed as a segregation study in the experimental cohort after eight backcrosses and selection against *idd3*, *5*, and *10*. As usual, we cannot absolutely rule out the impact of a gene tightly linked to the OX40L locus on *chr1*. Yet this artifact is highly unlikely in this study: first, there are no known *idd* loci in the vicinity of OX40L (and the *idd5* loci upstream on *chr1* were converted early in the backcross); second and most importantly, the effect was observed

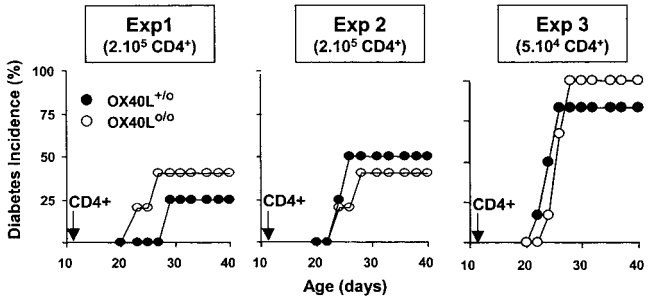

FIGURE 6. Protective T cells are present in normal numbers in OX40L$^{o/o}$ mice. CD4$^+$ T cells from OX40L$^{o/o}$ and OX40L$^{o/+}$ mice were purified using magnetic beads, and transferred to 10-day-old BDC2.5/N.RAG$^{o/o}$ mice, and diabetes was followed in the subsequent days. Colony control BDC2.5/N.RAG$^{o/o}$ mice become uniformly diabetic by 4 wk if not transferred with protective cells. In experiments 1 and 2, 2×10^5 cells were transferred, but only 5×10^4 in experiment 3.

in two different contexts, including BDC2.5/B6^{g7} mice which are inherently insensitive to genetic influences, as the disease develops despite C57BL/6 alleles that normally confer diabetes resistance.

These results are reminiscent of the protection afforded by the OX40L KO mutation against EAE and CIA (10, 21, 23, 24). However, several of our findings are somewhat at odds with previous notions of OX40 function. In particular, deeper investigation of the influence of the OX40L deficiency revealed that this mutation actually enhances anti-islet T cell activation by natural β cell Ag, as well as the initiation of leukocyte infiltration into the islets.

Accelerated T cell priming and insulitis

Our finding of an enhancement of T cell activity in early disease stages contrasts with previous reports claiming that the abrogation of OX40 signaling in vivo in EAE models (by neutralizing mAbs, a chimeric OX40-Ig molecule, or OX40L KO) reduced the expansion of effector CD4$^+$ T cell populations and/or prevented their migration to the target tissue (10, 21, 24). Similarly, other work had shown an impact of OX40/OX40L on early T cell responses in vitro, promoting the expansion and survival of cells in the days just after activation, perhaps through induction of anti-apoptotic molecules (10, 12, 14, 16–18). OX40L-deficient mice exhibit diminished recall responses to exogenously added Ag, suggesting an impairment of DC function (20). In contrast with these reports, we detected no influence of OX40L on T cell survival after activation, either in vivo or in vitro. The key to the difference in effect may lie in the nature of the stimulus: much of the earlier data had been generated in vitro or by priming with exogenous Ag, whereas the autoantigen that stimulates BDC2.5 T cells is presented naturally in the pancreatic lymph nodes, outside of an overtly inflammatory context, and without the adjuvants required for the EAE and CIA models. One might speculate that the impact of OX40 costimulation is modulated by engagement of Toll-like receptors. Whatever the mechanism, the present results establish that the proposed role of OX40 in positive costimulation of T cell priming by enhancing survival does not apply in all situations.

It seems reasonable to suggest that the augmented T cell activation in OX40L-deficient mice is due to the increased expression of MHC class II molecules on DCs, resulting in more efficient Ag processing and presentation. Enhanced class II molecule expression on DCs seemed specific, not reflecting wholesale DC activation, because there was no increased expression of other surface molecules, such as CD40 or the B7 family members. The increased expression of class II molecules is limited to the myeloid subset of DCs, and does not extend to B cells. The change in expression of class II molecules may be due to external influences on the DC population, through retrograde signals from OX40L, which might normally dampen display. In support of this idea is the observation that triggering OX40L activates c-*jun* (22), which has been implicated in MHC class II gene regulation (38). Alternatively, the effect may be intrinsic to the DC, for example by competition between OX40L and class II molecules for chaperone or intracellular transport molecules.

Reduced diabetes

OX40L is clearly essential for full progression to diabetes. Importantly, this was observed both in conventional NOD mice and in BDC2.5/B6^{g7} animals. The latter model, because of the dominant islet-reactive T cell repertoire, is far less sensitive to the complex genetic elements that influence diabetes in NOD mice, and thus provides important confirmation. In keeping with these results, injections of anti-OX40L mAb into very young BDC2.5/N mice did not provoke diabetes (data not shown), as do anti-CTLA-4 or anti-inducible costimulator mAbs when administered at young ages (Ref. 2) and A. Herman et al., manuscript in preparation). Nor did the OK40L KO mutation accelerate disease on the BDC2.5/N background as the CTLA-4 mutation does (39). With this result then, OX40L acts as a positive costimulator. This decreased incidence of diabetes is obviously not due to reduced T cell activation, nor to a reduced frequency of T cells expressing the BDC2.5 clonotype, which can correlate with disease penetrance in other contexts (27, 29). Because the destructiveness of insulitis appears to be linked to Th1 bias (40), and because OX40/OX40L perturbation has been found to affect the Th1/Th2 balance in some contexts (41), the effect of the OX40L mutation on diabetogenesis might have been due to an altered Th1/2 equilibrium. Yet, no such imbalance was observed in this study, nor was an effect on regulatory T cell populations observed, at least in the assays used.

How, then, can one reconcile a faster and more efficient onset of autoimmunity with a less aggressive outcome? The OX40-OX40L pathway may be instrumental in orchestrating the late phases of autoimmune infiltration, once autoreactive T cells have entered the target organ, allowing for the long-term establishment of an insulitic lesion and the subsequent broadening of the autoimmune response. This would explain why the requirement for OX40L is more marked when autoimmune T cells are at a lower frequency (Fig. 1). This interpretation is quite compatible with observations in the EAE system, where OX40L blockade prevented the local spread of inflammation, effective at an early stage of disease when activated T cells are moving into neural tissue (24). Alternatively (or in addition) it is conceivable that T cells, if overly activated at the early stages of the response, may "burn out" faster and thereby not be capable of participating in sustained lesions. Whatever the explanation, it seems that OX40 costimulation can be successively inhibitory or stimulatory.

In general, the analysis of costimulatory genes and molecules has been fraught with surprises and paradoxes, molecules thought to be stimulatory turning out to have inhibitory roles in other contexts (e.g., Ref. 3). These complexities can be ascribed to the number of interacting genes/molecules, and to their joint effects on both positively and negatively acting cell types. The unexpected results on OX40L reported in this study are of the same ilk. Clearly, it is imperative to have a more profound understanding of the activities of costimulatory molecules throughout the progression of a particular autoimmune disease before attempting to prevent or cure it via costimulatory molecule manipulation. Otherwise, the outcome could be disastrous.

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References

1. Tisch, R., and H. McDevitt. 1996. Insulin-dependent diabetes mellitus. *Cell 85:291*.
2. Luhder, F., P. Höglund, J. P. Allison, C. Benoist, and D. Mathis. 1998. Cytotoxic T lymphocyte-associated antigen 4 regulates the unfolding of autoimmune diabetes. *J. Exp. Med. 187:427*.
3. Salomon, B., D. J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J. A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the CD4$^+$CD25$^+$ immunoregulatory T cells that control autoimmune diabetes. *Immunity 12:431*.
4. Balasa, B., T. Krahl, G. Patstone, J. Lee, R. Tisch, H. O. McDevitt, and N. Sarvetnick. 1997. CD40 ligand-CD40 interactions are necessary for the initiation of insulitis and diabetes in nonobese diabetic mice. *J. Immunol. 159:4620*.
5. Amrani, A., P. Serra, J. Yamanouchi, B. Han, S. Thiessen, J. Verdaguer, and P. Santamaria. 2002. CD154-dependent priming of diabetogenic CD4$^+$ T cells dissociated from activation of antigen-presenting cells. *Immunity 16:719*.
6. Calderhead, D. M., J. E. Buhlmann, A. J. van den Eertwegh, E. Claassen, R. J. Noelle, and H. P. Fell. 1993. Cloning of mouse Ox40: a T cell activation marker that may mediate T-B cell interactions. *J. Immunol. 151:5261*.
7. Godfrey, W. R., F. F. Fagnoni, M. A. Harara, D. Buck, and E. G. Engleman. 1994. Identification of a human OX-40 ligand, a costimulator of CD4$^+$ T cells with homology to tumor necrosis factor. *J. Exp. Med. 180:757*.

8. Baum, P. R., R. B. Gayle III, F. Ramsdell, S. Srinivasan, R. A. Sorensen, M. L. Watson, M. F. Seldin, E. Baker, G. R. Sutherland, K. N. Clifford, and et al. 1994. Molecular characterization of murine and human OX40/OX40 ligand systems: identification of a human OX40 ligand as the HTLV-1-regulated protein gp34. *EMBO J. 13:3992*.
9. Ohshima, Y., Y. Tanaka, H. Tozawa, Y. Takahashi, C. Maliszewski, and G. Delespesse. 1997. Expression and function of OX40 ligand on human dendritic cells. *J. Immunol. 159:3838*.
10. Weinberg, A. D., K. W. Wegmann, C. Funatake, and R. H. Whitham. 1999. Blocking OX-40/OX-40 ligand interaction in vitro and in vivo leads to decreased T cell function and amelioration of experimental allergic encephalomyelitis. *J. Immunol. 162:1818*.
11. Imura, A., T. Hori, K. Imada, T. Ishikawa, Y. Tanaka, M. Maeda, S. Imamura, and T. Uchiyama. 1996. The human OX40/gp34 system directly mediates adhesion of activated T cells to vascular endothelial cells. *J. Exp. Med. 183:2185*.
12. Gramaglia, I., A. D. Weinberg, M. Lemon, and M. Croft. 1998. Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. *J. Immunol. 161:6510*.
13. Rogers, P. R., J. Song, I. Gramaglia, N. Killeen, and M. Croft. 2001. OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. *Immunity 15:445*.
14. De Smedt, T., J. Smith, P. Baum, W. Fanslow, E. Butz, and C. Maliszewski. 2002. Ox40 costimulation enhances the development of T cell responses induced by dendritic cells in vivo. *J. Immunol. 168:661*.
15. Weinberg, A. D., M. M. Rivera, R. Prell, A. Morris, T. Ramstad, J. T. Vetto, W. J. Urba, G. Alvord, C. Bunce, and J. Shields. 2000. Engagement of the OX-40 receptor in vivo enhances antitumor immunity. *J. Immunol. 164:2160*.
16. Kopf, M., C. Ruedl, N. Schmitz, A. Gallimore, K. Lefrang, B. Ecabert, B. Odermatt, and M. F. Bachmann. 1999. OX40-deficient mice are defective in Th cell proliferation but are competent in generating B cell and CTL Responses after virus infection. *Immunity 11:699*.
17. Pippig, S. D., C. Pena-Rossi, J. Long, W. R. Godfrey, D. J. Fowell, S. L. Reiner, M. L. Birkeland, R. M. Locksley, A. N. Barclay, and N. Killeen. 1999. Robust B cell immunity but impaired T cell proliferation in the absence of CD134 (OX40). *J. Immunol. 163:6520*.
18. Gramaglia, I., A. Jember, S. D. Pippig, A. D. Weinberg, N. Killeen, and M. Croft. 2000. The OX40 costimulatory receptor determines the development of CD4 memory by regulating primary clonal expansion. *J. Immunol. 165:3043*.
19. Chen, A. I., A. J. McAdam, J. E. Buhlmann, S. Scott, M. L. Lupher, Jr., E. A. Greenfield, P. R. Baum, W. C. Fanslow, D. M. Calderhead, G. J. Freeman, and A. H. Sharpe. 1999. Ox40-ligand has a critical costimulatory role in dendritic cell:T cell interactions. *Immunity 11:689*.
20. Murata, K., N. Ishii, H. Takano, S. Miura, L. C. Ndhlovu, M. Nose, T. Noda, and K. Sugamura. 2000. Impairment of antigen-presenting cell function in mice lacking expression of OX40 ligand. *J. Exp. Med. 191:365*.
21. Ndhlovu, L. C., N. Ishii, K. Murata, T. Sato, and K. Sugamura. 2001. Critical involvement of OX40 ligand signals in the T cell priming events during experimental autoimmune encephalomyelitis. *J. Immunol. 167:2991*.
22. Matsumura, Y., T. Hori, S. Kawamata, A. Imura, and T. Uchiyama. 1999. Intracellular signaling of gp34, the OX40 ligand: induction of c-*jun* and c-*fos* mRNA expression through gp34 upon binding of its receptor, OX40. *J. Immunol. 163:3007*.
23. Yoshioka, T., A. Nakajima, H. Akiba, T. Ishiwata, G. Asano, S. Yoshino, H. Yagita, and K. Okumura. 2000. Contribution of OX40/OX40 ligand interaction to the pathogenesis of rheumatoid arthritis. *Eur. J. Immunol. 30:2815*.
24. Nohara, C., H. Akiba, A. Nakajima, A. Inoue, C. S. Koh, H. Ohshima, H. Yagita, Y. Mizuno, and K. Okumura. 2001. Amelioration of experimental autoimmune encephalomyelitis with anti-OX40 ligand monoclonal antibody: a critical role for OX40 ligand in migration, but not development, of pathogenic T cells. *J. Immunol. 166:2108*.
25. Katz, J. D., B. Wang, K. Haskins, C. Benoist, and D. Mathis. 1993. Following a diabetogenic T cell from genesis through pathogenesis. *Cell 74:1089*.
26. Gonzalez, A., J. D. Katz, M. G. Mattei, H. Kikutani, C. Benoist, and D. Mathis. 1997. Genetic control of diabetes progression. *Immunity 7:873*.
27. Kanagawa, O., A. Militech, and B. A. Vaupel. 2002. Regulation of diabetes development by regulatory T cells in pancreatic islet antigen-specific TCR transgenic nonobese diabetic mice. *J. Immunol. 168:6159*.
28. Judkowski, V., C. Pinilla, K. Schroder, L. Tucker, N. Sarvetnick, and D. B. Wilson. 2001. Identification of MHC class II-restricted peptide ligands, including a glutamic acid decarboxylase 65 sequence, that stimulate diabetogenic T cells from transgenic BDC2.5 nonobese diabetic mice. *J. Immunol. 166:908*.
29. Gonzalez, A., I. Andre-Schmutz, C. Carnaud, D. Mathis, and C. Benoist. 2001. Damage control, rather than unresponsiveness, effected by protective $DX5^+$ T cells in autoimmune diabetes. *Nat. Immun. 2:1117*.
30. Ghosh, P., S. M. Palmer, N. R. Rodrigues, H. J. Cordell, C. M. Hearne, R. J. Cornall, J.-B. Prins, P. McShane, G. M. Lathrop, and L. B. Peterson. 1993. Polygenic control of autoimmune diabetes in nonobese diabetic mice. *Nat. Genet. 4:404*.
31. Brodnicki, T. C., F. Quirk, and G. Morahan. 2003. A susceptibility allele from a non-diabetes-prone mouse strain accelerates diabetes in NOD congenic mice. *Diabetes 52:218*.
32. Bansal-Pakala, P., A. G. Jember, and M. Croft. 2001. Signaling through OX40 (CD134) breaks peripheral T-cell tolerance. *Nat. Med. 7:907*.
33. Hoglund, P., J. Mintern, C. Waltzinger, W. Heath, C. Benoist, and D. Mathis. 1999. Initiation of autoimmune diabetes by developmentally regulated presentation of islet cell antigens in the pancreatic lymph nodes. *J. Exp. Med. 189:331*.
34. Verdaguer, J., J.-W. Yoon, B. Anderson, N. Averill, T. Utsugi, B.-J. Park, and P. Santamaria. 1996. Acceleration of spontaneous diabetes in TCR-β-transgenic nonobese diabetic mice by β-cell cytotoxic $CD8^+$ T cells expressing identical endogenous TCR-α chains. *J. Immunol. 157:4726*.
35. Morgan, D. J., C. Kurts, H. T. C. Kreuwel, K. L. Holst, W. R. Heath, and L. A. Sherman. 1999. Ontogeny of T cell tolerance to peripherally expressed antigens. *Proc. Natl. Acad. Sci. USA 96:3854*.
36. Mintern, J. D., R. M. Sutherland, A. M. Lew, K. Shortman, F. R. Carbone, and W. R. Heath. 2002. Constitutive, but not inflammatory, cross-presentation is disabled in the pancreas of young mice. *Eur. J. Immunol. 32:1044*.
37. Malmstrom, V., D. Shipton, B. Singh, A. Al Shamkhani, M. J. Puklavec, A. N. Barclay, and F. Powrie. 2001. CD134L expression on dendritic cells in the mesenteric lymph nodes drives colitis in T cell-restored SCID mice. *J. Immunol. 166:6972*.
38. Ting, J. P., and J. Trowsdale. 2002. Genetic control of MHC class II expression. *Cell 109(Suppl):S21*.
39. Luhder, F., C. Chambers, J. P. Allison, C. Benoist, and D. Mathis. 2000. Pinpointing when T cell costimulatory receptor CTLA-4 must be engaged to dampen diabetogenic T cells. *Proc. Natl. Acad. Sci. USA 97:12204*.
40. Katz, J. D., C. Benoist, and D. Mathis. 1995. T helper cell subsets in insulin-dependent diabetes. *Science 268:1185*.
41. Ohshima, Y., L. P. Yang, T. Uchiyama, Y. Tanaka, P. Baum, M. Sergerie, P. Hermann, and G. Delespesse. 1998. OX40 costimulation enhances interleukin-4 (IL-4) expression at priming and promotes the differentiation of naive human $CD4^+$ T cells into high IL-4-producing effectors. *Blood 92:3338*.