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Low CD86 Expression in the Nonobese Diabetic Mouse Results in the Impairment of Both T Cell Activation and CTLA-4 Up-Regulation

Eva Dahlén, Gunnar Hedlund, and Kim Dawe

The nonobese diabetic (NOD) mouse spontaneously develops autoimmune insulin-dependent diabetes mellitus and serves as a model for human type I diabetes. NOD spleen cells proliferate to a lesser extent than those from C57BL/6 and BALB/c mice in response to anti-CD3. To investigate the cause of this reduced T cell proliferation, costimulatory molecule expression was investigated. It was found that NOD macrophages, dendritic cells, and T cells, but not B cells, expressed lower basal levels of CD86, but not CD80, CD28, or CD40, compared with C57BL/6 and BALB/c. This low CD86 expression was not dependent on the MHC haplotype or on diabetes development since the NOD-related, diabetes-free mouse strains NON (H-2\textsuperscript{b}d) and NOR (H-2\textsuperscript{d}) exhibited similar low levels of CD86 expression and proliferation. Furthermore, following activation, the relative up-regulation of CTLA-4, as compared with CD28, was more pronounced on C57BL/6 and BALB/c T cells as shown by an increased CTLA-4/CD28 ratio. This activation-induced increase in the CTLA-4/CD28 ratio was markedly reduced on NOD T cells compared with the other two strains. The low CD86 expression in NOD mice may account for the reduced increase in both proliferation and the CTLA-4/CD28 ratio, since reducing CD86 expression in C57BL/6 and BALB/c cultures to NOD levels significantly reduces the proliferation and the CTLA-4/CD28 ratio. Therefore, we propose that a low level of CD86 expression in the NOD mouse contributes to a defective regulation of autoreactive T cells by preventing the full activation of T cells and therefore the up-regulation of autoimmune disease, but that this activation may not be strong enough to induce a tolerogenic or protective response (17). For example, the induction of IL-4, which is a cytokine known to protect from diabetes development in the NOD mouse (20, 21), has been shown to be dependent on a greater number of cell divisions than that required for IFN-γ (22), a cytokine implicated in the pathogenesis of IDDM in the NOD mouse (23–26). Thus, IFN-γ production can be found in T cells that have gone through one or more cell cycles whereas IL-4 production can be detected only in cells that have divided at least four times. In addition, the induction of anergy in, or deletion of, T cells has been shown to occur in response to relatively strong T cell activation signals (27–29).

Modulation of costimulation via the CD28/CTLA-4-CD80/CD86 pathway is known to influence diabetes development in the NOD mouse. Modulating the costimulatory signals delivered by CD28- and/or CD86 were blocked using mAb (32). It was found that the blocking anti-CTLA-4 mAb results in the acceleration of diabetes development (31). Additional evidence for the importance of the CD86 signaling pathway has been obtained from studies in which CD80 mAbs inhibits diabetes development in an IL-4-dependent manner (30), therefore arguing for the presence of a defective T cell activation resulting in a reduced induction of tolerance in the NOD mouse. In addition, protection from diabetes development is highly dependent on the inhibitory signals delivered by CTLA-4, since treatment of BDC2.5 TCR transgenic (tg) NOD mice with blocking anti-CTLA-4 mAb results in the acceleration of diabetes development (31). Additional evidence for the importance of this signaling pathway has been obtained from studies in which CD80 and/or CD86 were blocked using mAb (32). It was found that the blocking of CD86 inhibited disease development, whereas blocking CD80 alone or in combination with CD86 accelerated diabetes.

In addition to the modulation of costimulatory pathways, several cytokines have been shown to influence the development of diabetes in the NOD mouse. For instance, TNF-α has been implicated in the initiation of insulitis (33, 34) whereas Th1 cells producing IFN-γ have been implicated in the effector phase of β cell destruction (23–26). In addition, Th2 cytokines such as IL-4 and IL-10 as

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2 Abbreviations used in this paper: NOD, nonobese diabetic; IDDM, insulin-dependent diabetes mellitus; Idd, insulin-dependent diabetes; M6, macrophage; geo MFI, geometric mean fluorescent intensity; DC, dendritic cell; MACS, magnetic cell separation; CD40L, CD40 ligand; NON, nonobese nondiabetic; NOR, nonobese resistant.

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well as TGF-β have been shown to protect from diabetes development in the NOD mouse (20, 21, 35–37). The observations that IL-4 has a protective effect and that CD86 is required for Th2 development (38, 39) contradict those demonstrating the inhibition of diabetes development upon CD86 blockade. Thus, the role of costimulatory molecules in the regulation of the autoimmune responses occurring in the NOD mouse are not yet fully understood.

In this study, we have been able to demonstrate that NOD leukocytes express low levels of CD86 compared with those from C57BL/6 and BALB/c mice. This low CD86 expression may result in an impaired T cell activation since partial blockade of CD86 in C57BL/6 and BALB/c cultures to those found in the NOD inhibits T cell proliferation. In addition, the low levels of CD86 that are expressed in the NOD may result in an impaired up-regulation of CTLA-4 relative to CD28. Therefore we propose that the activation of mechanisms involved in limiting an ongoing immune response are defective in the NOD mouse.

Materials and Methods

Cell culture medium and reagents
Cells were cultured in R10 medium, i.e., RPMI 1640 medium (catalogue no. 12-702F/U1; BioWhittaker, Verviers, Belgium) supplemented with 10 mM HEPES buffer (BioWhittaker), 100 μg/ml sodium pyruvate (BioWhittaker), 100 μg/ml gentamicin (Biological Industries, Kibbutz Beit Haemek, Israel) at 2% L-glutamine (Darmstadt, Germany), and 10% FCS (HyClone, Logan, UT). Stimulating anti-CD3 (clone: 145-2C11), stimulating anti-CD28 (37.51), blocking anti-CD80 (clone: 1G10), blocking anti-CD86 (clone: GL1), hamster IgG, group 1, κ isotype control (clone: A19-3), hamster IgG group 2, λ isotype control (clone: Ha4/8), and rat IgG2a isotype control mAb (clone: R35-95) were purchased as sodium azide free preparations from Becton Dickinson (Mountain View, CA). Stimulating anti-CD40 mAb was produced from the hybridoma FGGK45 (40) (a gift from Professor J. Andersson (Basel Institute for Immunology, Basel, Switzerland) and purified on a Hi-Trap protein G column (Amersham Pharmacia Biotech, Solna, Sweden). LPS was purchased from Sigma (St. Louis, MO). For flow cytometry, mAb directed to CD16/32 (clone: 2.4G2), CD4 (clone: RM4-5), CD8 (clone: 53-6.7), CD19 (clone: 1D3), CD11c (clone: HL3), CD11b (clone: M170), CD28 (clone: 37.51), CTLA-4 (clone: UC10-4F10-11), CD80 (clone: 16-10A1), CD86 (clone: GL1), CD40 (clone: 3/23), CD40L (clone: MR1), and streptavidin-PerCP were purchased from Becton Dickinson, and F4/80 was purchased from Serotec (Oxford, U.K.).

Mice and tissue preparation

Female NOD, C57BL/6, and BALB/c mice were purchased from Bommice (Bombatte Gerd Breeding and Research Center Limited, Ry, Denmark) and were used at 8 wk of age unless otherwise stated. For the experiments in which CD86 expression and spleen cell proliferation of NON and NOR mice were investigated, female NON, NOR, and NOD mice were purchased from The Jackson Laboratory (Bar Harbor, ME). NOD mice from both vendors exhibited a similar incidence of diabetes, this being 85% in both groups. Results from one representative experiment of four are shown.

Sorting and culture of CD4+ and CD11b+ splenocyte populations

CD4+ and CD11b+ cells were sorted using MACS technology (Miltenyi Biotec, Auburn, CA) according to the manufacturers protocol. Briefly, single cell preparations of spleens from four or eight animals per mouse strain were cultured in triplicate and stimulated with 1.5–500 ng/ml soluble anti-CD3 mAb. The proliferation was determined by [3H]thymidine incorporation following 48 h of culture. Results are shown as mean ± SD of the four individuals in each group. Proliferation in NOD cultures was statistically lower than that in C57BL/6 and BALB/c cultures as determined by a one-way ANOVA followed by a Dunnett test in which NOD was compared with either C57BL/6 or BALB/c. Results from one representative experiment of four are shown.

Flow cytometry

Spleen and lymph node cell phenotypes were determined by flow cytometry on a FACSort flow cytometer (Becton Dickinson). Three-color staining with FITC, PE, or biotin-conjugated mAbs was conducted in PBS supplemented with 2% FCS, at 4°C. To inhibit nonspecific FcR-mediated
binding of mAbs, cells were preincubated with 10 μg/ml anti-CD16/32 for 10 min at room temperature. Cells were then incubated with primary Abs directed toward the cell surface molecules of interest for 30 min, with streptavidin-PerCP being used for the detection of biotin-conjugated mAbs.

Based on their forward and side scatter characteristics, live cells were gated and analyzed for expression of the molecules of interest, and the expression was quantified by measuring the geometrical mean fluorescence intensity (geo MFI) for each sample.

F4/80 was used for the detection of Mø, CD19 for B cells, and CD11c for dendritic cells (DC). For the analysis of DC phenotypes, CD11c1 cells from three pooled spleens per sample were enriched using magnetic cell separation (MACS). Thus, cells were labeled with magnetic bead-conjugated mAb directed to CD11c (clone: N418), and the positive cells were separated on a column according to the manufacturer’s protocol (Miltenyi Biotec). Two samples per mouse strain were prepared for each experiment.

For the detection of total, i.e., both intracellular and surface-bound, CTLA-4 in CD4+ and CD8+ T cells, cultured bulk spleen cells were incubated with anti-CD16/32 as before and then stained for surface-bound CD4 or CD8 in PBS supplemented with 2% FCS as described above. Cells were then fixed in 4% paraformaldehyde (Sigma) diluted in PBS for 20 min at 4°C and washed with PBS + 2% FCS twice. Cells were then incubated with mAb diluted in a permeabilization buffer consisting of 0.1% saponin (Riedel-de Haen, Seelze, Germany), 0.1% sodium azide (BDH Laboratory Supplies Poole, Dorset, U.K.), and 1% FCS in PBS (pH 7.4) for 30 min at 4°C and washed twice in permeabilization buffer.

Activated lymphocytes are slightly larger than unactivated lymphocytes, and therefore the background fluorescence of activated cells is higher than that of unactivated cells. Due to the low geo MFI values of samples stained for CTLA-4, and to the fact that we were interested in detecting the true degree of CTLA-4 up-regulation, the background values obtained from the samples incubated with PE-labeled hamster IgG isotype control mAb were subtracted from those obtained when incubating with PE-labeled anti-CTLA-4 mAb. The results are shown as Δ geo MFI. For consistency, since CTLA-4 expression was compared with that of CD28, the same procedure was applied to the CD28 expression values.

**FIGURE 2.** CD86 expression by freshly isolated or anti-CD3-stimulated spleen cells from NOD, C57BL/6, and BALB/c mice. A, Six individual spleen cell samples per mouse strain from 8-wk-old NOD, C57BL/6, or BALB/c mice were analyzed for the expression of CD86 on F4/80+ Mø, CD19+ B cells, CD4+ T cells, or CD8+ T cells by flow cytometry. For DC analysis, two samples of MACS-enriched CD11c+ DC were analyzed per mouse strain. B, Cells from individual spleens were cultured in the presence of 15 ng/ml soluble anti-CD3 mAb and analyzed for the expression of CD86 on Mø and B cells (n = 3) and CD4+ and CD8+ T cells (n = 2) by flow cytometry following 48 h of culture. Results are shown as mean ± SD of the individual samples in each group. Statistics were performed by a one-way ANOVA, which was followed by a Dunnett test comparing NOD with either C57BL/6 or BALB/c if a p value < 0.05 was obtained in the analysis of variance. NS, Not significant in the ANOVA; *, p < 0.05 in Dunnett. Results from one representative experiment out of three are shown.
Statistical analysis

When comparing two groups, statistical analyses were performed using the t test included in Microsoft Excel 97 (Redmond, WA). When comparing several groups, a one way ANOVA was performed using SigmaStat 2.0 (Jandel Scientific, San Rafael, CA). If a statistically significant difference (p < 0.05) was found, the analysis was followed by a Dunnett test in which the NOD or C57BL/6 group, depending on which was relevant for the particular question being asked, was compared with the others as described in the figure legends. The Dunnett test was performed using SigmaStat 2.0. Note that this program states only whether the p value is below 0.05 or not, and therefore only one level of significance is obtained. Thus, the asterisk means that p < 0.05 but not necessarily that it is $p < 0.01$.

Results

NOD T cells exhibit an impaired proliferation

To compare the proliferative response of T cells from diabetes-prone NOD mice with that of normal diabetes resistant mice, bulk spleen cells from 8-wk-old NOD C57BL/6 and BALB/c mice were cultured with soluble anti-CD3 mAb for 48 h, the time point when maximal T cell proliferation was observed in kinetics experiments. Cultures were then pulsed with [3 H]thymidine for 4 h to determine their proliferative response. As shown in Fig. 1, the anti-CD3-induced T cell proliferation is reduced in NOD as compared with C57BL/6 and BALB/c cultures, thus indicating a defect in the mechanism of T cell activation. Similar results were also obtained using bulk spleen cells from 4-wk-old mice (data not shown). The differences in T cell proliferation were not due to a reduced frequency of T cells in NOD spleens as confirmed by flow cytometry (data not shown).

Low basal levels of CD86 expression in the NOD mouse

Optimal T cell proliferation is dependent on signaling through the TCR complex (in this system provided by anti-CD3 mAb) as well as on a second signal provided by the interaction of costimulatory molecules, e.g., the interaction of CD28 with the B7 molecules (19). To investigate whether the poor proliferative response observed in NOD spleen cell cultures was due to a defective level of expression of costimulatory molecule(s), the expression of CD80, CD86, CD28, CTLA-4, CD40, and CD40 ligand (CD40L) was analyzed by flow cytometry. Freshly isolated spleen and pooled popliteal and inguinal lymph node cells from 2-, 4-, 8-, or 14-wk-old NOD, C57BL/6, and BALB/c mice were analyzed for the expression of these molecules. It was found that CD86 expression was significantly lower on Mo, DC, and CD8+ T cells, but not B cells, nor consistently on CD4+ T cells from NOD as compared with C57BL/6 and BALB/c mice (Fig. 2A). In contrast, the expression of CD80, CD28, and CD40 was not consistently decreased on NOD compared with C57BL/6 and BALB/c cells. CTLA-4 and CD40L were undetectable on freshly isolated T cells. The basal levels of CD86 expressed on unactivated B cells were comparable in the three mouse strains. In contrast, following anti-CD3 activation of bulk spleen cell cultures, the up-regulation of CD86 on B cells from NOD mice was reduced as compared with the levels observed on C57BL/6 and BALB/c B cells. For Mo, CD8+ T cells, and CD4+ T cells, the differences in CD86 expression upon anti-CD3 stimulation were similar to those seen on unactivated cells, although the CD86 levels were higher following stimulation (Fig. 2B). When using anti-CD40 mAb or LPS to stimulate bulk spleen cells, NOD responses were not reduced compared with the other two mouse (Fig. 3).

Low CD86 expression in NON and NOR mice

Diabetes development in the NOD mouse is controlled by several genetic loci, which are termed Idd loci (2). NON and NOR mice are related to NOD but do not develop diabetes due to the lack of
The NOD and NON strains were established from different sublines established during the development of a cataract model (the CTS mouse) from ICR mice (41). The NON strain develops neither insulitis nor diabetes. It has NOD alleles on some of the Idd loci but differs from the NOD on others. Thus, it has a different MHC haplotype (H-2nb1) than NOD (H-2g7) and also lacks some additional Idd loci (42, 43). NOR islets exhibit APC infiltration but lack T cell infiltration (44) and consequently do not develop diabetes. The mice are derived from a C57BL/KsJ contamination of a NOD colony and have NOD alleles on several of the Idd loci, including the MHC (45).

To investigate whether the low CD86 expression found in NOD mice may be related to the autoimmune disease developed by these mice or dependent on the MHC haplotype, the CD86 expression on NON and NOR Mø and DC was compared with NOD and C57BL/6. Since our regular animal supplier, Bommice, did not breed NON and NOR mice, these mice were purchased from The Jackson Laboratory. As a control, NOD mice from The Jackson Laboratory were also included in some of the experiments and found to express CD86 at comparable levels to the NOD mice obtained from Bommice (Fig. 4A). As shown in Fig. 4, both NON and NOR APC express similar low levels of CD86 as NOD mice, and this correlates with a reduced proliferative response to anti-CD3 stimulation. Thus, the reduced CD86 expression in NOD mice is probably not dependent on the MHC haplotype of these mice nor is it a consequence of the diabetes developed by these mice, but rather it may reflect a genetic difference between the diabetes-prone NOD mouse and related strains compared with non-diabetes-prone mouse strains.

Having established that NOD, NON, and NOR mice are similar in terms of CD86 expression and T cell proliferation, NOD mice...
were compared with C57BL/6 and BALB/c in the remaining experiments investigating the potential consequences of the low CD86 expression.

**NOD APC exhibit an impaired T cell stimulatory capacity**

Although we have demonstrated that NOD APC express low levels of CD86 compared with C57BL/6 and BALB/c, anti-CD3-induced T cell proliferation is dependent on several factors, and the impaired proliferation of the bulk spleen cell cultures observed in the NOD could be due to defects in the APC as well as the T cell compartment. We therefore wanted to investigate whether APC from the three mouse strains may differ in their ability to activate T cells and whether the T cells from the three strains may respond differently to stimuli. First, we investigated whether the ability of NOD APC to stimulate T cells from either of the three mouse strains was reduced compared with that of APC from C57BL/6 and BALB/c mice. Thus, MACS-sorted CD4⁺ T cells from NOD, C57BL/6, or BALB/c mice were cultured with MACS-sorted Mφ (CD11b⁺ cells) from either of the three strains or with no additional cells in the presence or absence of soluble anti-CD3 mAb. The MLR was found to be very low at the time point investigated, with cpm values below 1000 in the cultures lacking anti-CD3 mAb (Fig. 5A). Indeed, NOD Mφ were found to be less efficient than Mφ from either C57BL/6 or BALB/c mice at supporting anti-CD3-induced T cell proliferation (Fig. 5A), suggesting that the reduced proliferation of NOD spleen cells may be due to the APC from these mice being less efficient in stimulating T cells and that a low CD86 expression may result in a reduced T cell stimulation.

**FIGURE 5.** Comparison of functional abilities of APC and T cells from NOD, C57BL/6 and BALB/c mice. A, CD4⁺ T cells from four pooled spleens and CD11b⁺ macrophages from eight pooled spleens from either NOD, C57BL/6, or BALB/c mice were purified using MACS technology. The cells were cultured in triplicate at a density of 10⁵ T cells and 12.5 × 10³ Mφ per well in all possible combinations in the presence of the indicated concentrations of soluble anti-CD3 mAb. Proliferation was determined by [³H]thymidine incorporation for 4 h following 48 h of culture. Results from one representative experiment out of four are shown. B, CD4⁺ T cells from individually prepared spleens from 8-wk-old NOD, C57BL/6, and BALB/c mice (n = 3) were purified using MACS technology and cultured at a density of 10⁵ cells together with 10⁵ mAb-coated latex beads per well. Proliferation was determined by [³H]thymidine incorporation for 4 h following 48 h of culture. Results are shown as mean ± SD of the three individual samples in each group. Statistics were performed by one-way ANOVA and Dunnett test, and NOD cells were found not to be significantly different from C57BL/6 and BALB/c cells. Results from one representative experiment of four are shown.
To investigate whether the reduced proliferation of NOD bulk spleen cells could also be due to defects in the T cell response, the proliferation of MACS-purified CD4$^+$ T cells from the three mouse strains in response latex beads coated with stimulating anti-CD3 and anti-CD28 mAb was analyzed. Differences in the T cell responses are easiest to detect at suboptimal levels of activatory signals. Therefore, beads coated with 0.15, 0.5, or 1.5 μg/ml anti-CD3 and 0, 1, or 5 μg/ml anti-CD28 mAb were used. As shown in Fig. 5B, CD4$^+$ T cells from NOD mice responded as well as those from C57BL/6 and BALB/c irrespective of the mAb concentrations being used, thus arguing against gross defects in the TCR-CD3- or CD28-mediated signaling in NOD T cells. Thus, these results suggest that NOD APC are incapable of inducing optimal T cell stimulation, whereas no differences in the T cells’ ability to respond to identical stimuli could be observed.

A low CD86 expression results in an impaired T cell proliferation
Since the interaction of the B7 molecules with CD28 is crucial for an optimal T cell activation and proliferation, the reduced levels of CD86 expression in the NOD mouse may account for the impaired CD3- or CD28-mediated signaling in NOD T cells. Thus, these results suggest that NOD APC are incapable of inducing optimal T cell stimulation, whereas no differences in the T cells’ ability to respond to identical stimuli could be observed.

FIGURE 6. Blockade of CD80, CD86, or a combination of CD80 and CD86 in anti-CD3-stimulated spleen cell cultures. Cells from individually prepared spleens from 8-wk-old NOD, C57BL/6, and BALB/c mice (n = 2) were cultured in triplicate and stimulated with soluble anti-CD3 mAb in the presence of blocking anti-CD80, anti-CD86, anti-CD80, and anti-CD86 or rat IgG2a isotype control mAb. Proliferation was determined by [3H]thymidine incorporation for 4 h following 48 h of culture. Results are shown as mean ± SD of individual samples. Proliferation in cultures with blockade of CD86 alone or CD80 and CD86, but not CD80 alone, was found to be statistically lower than that of rat IgG2a control cultures, as determined by a one-way ANOVA followed by a Dunnett test. Results from one representative experiment of three are shown.

FIGURE 7. Procedure for equilibration of the C57BL/6 and BALB/c CD86 expression levels to NOD levels. A, CD86 on APC was partially blocked by the addition of 3 ng/ml anti-CD86 mAb (clone: GL1). Subsequently the remaining CD86 molecules were stained for flow cytometric analysis using PE-conjugated anti-CD86 (GL1-PE). B, The two upper panels show examples of the difference in CD86 expression on NOD and C57BL/6 Ms on unblocked cells. The lower panel shows that, following partial blockade of CD86 on C57BL/6 cells, the levels of CD86 on Ms are similar to those expressed by NOD cells.
spleen cell proliferation observed in these mice. We therefore in-
vestigated the requirement for B7 in anti-CD3-induced T cell pro-
liferation of bulk spleen cell cultures. This was achieved by the
addition of blocking anti-CD80, anti-CD86, singly or in combina-
tion, or by rat IgG2a isotype control mAb to the cultures. We were
able to show that blocking CD86 alone or in combination with
CD80, markedly inhibited the anti-CD3-induced proliferation,
whereas blocking CD80 alone only had a marginal, if any, effect
on proliferation (Fig. 6).

Although it was clear that the T cell proliferation was highly
CD86 dependent, a comparison of the CD86 expression on NOD,
C57BL/6, and BALB/c cells showed that the basal levels of ex-
pression in the NOD were ~50% of those found in the other two
strains. We therefore thought it important to consider whether this
difference in CD86 expression would make a great difference in
terms of the achievable degree of T cell activation.

We therefore investigated whether the equilibration of the CD86
expression levels in C57BL/6 and BALB/c mice to those of NOD
would affect anti-CD3-induced proliferation in the three mouse
strains. This was achieved by using anti-CD86 mAbs to reduce the
level of available CD86 on C57BL/6 and BALB/c spleen cells
such that the level of free, unoccupied CD86 on the cell surface
was at a similar level to that expressed by NOD cells. Following
CD86 binding, a PE-conjugated anti-CD86 mAb of the same clone
(GL-1) as that used for the blocking was used to stain the remain-
ing available CD86 on the cell surface for subsequent analysis by
flow cytometry. In titration experiments, 3 ng/ml anti-CD86
was found to be the optimal concentration for the equilibration
of the CD86 levels expressed on Mφ and B cells in C57BL/6 and
BALB/c cultures to NOD levels. Thus, after 1, 24, and 48 h of
CD86 blockade using 3 ng/ml anti-CD86 mAb, the levels of free,
unoccupied CD86 expressed on Mφ and B cells in C57BL/6 and
BALB/c cultures were similar to NOD levels. Fig. 7 illustrates the
procedure for this partial block. As shown in Fig. 8, the equili-ration of C57BL/6 and BALB/c CD86 expression on Mφ and B cells
to NOD levels significantly inhibited the anti-CD3-induced T cell
proliferation in these strains. This indicates that relatively small
differences in CD86 expression do indeed influence T cell activa-
tion and that the low CD86 expression in NOD may at least par-
tially account for the impaired T cell proliferation observed.

**Impaired up-regulation of CD28 and CTLA-4 in anti-CD3-stimulated NOD cultures**

Given that there is a defective activation of NOD T cells, as re-
lected by the reduced proliferative response, one must consider
how this contributes to the development of the autoimmune dis-
ease in these animals. One possible outcome of a defective initial
T cell activation may be that the mechanisms responsible for the
down-regulation of ongoing T cell responses may not be induced.
One important negative regulator of T cell activation is CTLA-4,
which inhibits T cell proliferation through the inhibition of CD3ζ
chain tyrosine phosphorylation (46) as well as by inducing TGF-β
production (47).

Although no differences were found in the basal expression of
CD28 on unactivated T cells in the three mouse strains, it was of
interest to investigate whether any differences existed in the acti-
vation-induced up-regulation of CD28 or CTLA-4. Therefore,
spleen cells were cultured in the presence or absence of 150 ng/ml
anti-CD3 mAb for 42 h, when maximal CTLA-4 expression and
profound CD28 up-regulation is observed, and the expression of
CD28 and CTLA-4 were analyzed by flow cytometry. It was found
that, although the basal levels of CD28 expressed by CD4+ and
CD8+ T cells did not differ between the three mouse strains tested,
the anti-CD3-induced up-regulation of CD28 was impaired in
NOD T cells as compared with C57BL/6 and BALB/c. The up-
regulation of CTLA-4 was also impaired and to a greater extent
than that of CD28 (Fig. 9). Since much of the CTLA-4 expressed
by T cells is present intracellularly (48), we investigated the levels
of total, i.e., both surface and intracellular, CTLA-4 by staining the
cells with anti-CTLA-4 mAb diluted in a permeabilization buffer
containing saponin. As shown in Fig. 9A, similar to surface bound
CTLA-4, the total CTLA-4 expression was reduced in NOD T
cells compared with C57BL/6 and BALB/c, suggesting that NOD
T cells have a reduced CTLA-4 production rather than a reduced
transport of the molecule to the cell surface.

*The activation-induced increase in the CTLA-4/CD28 ratio is
CD86 dependent*

Since CD28 delivers a positive signal whereas CTLA-4 delivers a
negative signal to the T cell, the net effect of the activation-induced
up-regulation of these molecules is reflected by the CTLA-4/CD28
ratio. This was calculated by dividing the Δ geo MFI for CTLA-4
with that of CD28. As shown in Fig. 10, the CTLA-4/CD28 ratio
is increased upon anti-CD3 stimulation of C57BL/6 and BALB/c
T cells. However, this increase is not seen in NOD T cells, sug-
gest that the induction of inhibitory signals following an immu-
none activation is impaired in NOD mice. These signals are es-
tential for the inhibition and control of an ongoing immune
response and the ability to maintain lymphocyte homeostasis.

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**FIGURE 8.** Effect on anti-CD3-induced spleen cell proliferation by the equilibration of C57BL/6 and BALB/c CD86 expression to NOD levels. Cells from individually prepared spleens from 8-wk-old NOD C57BL/6 and BALB/c (n = 3) were cultured in triplicate and stimulated with 15 ng/ml soluble anti-CD3 in the presence of partially blocking anti-CD86 or rat IgG2a isotype control mAb. Following 48 h of culture, parallel cultures were either analyzed by flow cytometry for CD86 expression or pulsed with [3H]thymidine for 4 h for determination of proliferation. Results are shown as mean ± SD of individual samples in each group. ***, p < 0.001 by t test.**

**TABLE 1.** Impaired up-regulation of CD28 and CTLA-4 in anti-CD3-stimulated NOD cultures

<table>
<thead>
<tr>
<th>Condition</th>
<th>CD86 expression on Mφ</th>
<th>CD86 expression on B cells</th>
<th>Proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>- NOD</td>
<td>50% of those found in the other two strains.</td>
<td>50% of those found in the other two strains.</td>
<td>50% of those found in the other two strains.</td>
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<tr>
<td>- C57BL/6</td>
<td>50% of those found in the other two strains.</td>
<td>50% of those found in the other two strains.</td>
<td>50% of those found in the other two strains.</td>
</tr>
<tr>
<td>- BALB/c</td>
<td>50% of those found in the other two strains.</td>
<td>50% of those found in the other two strains.</td>
<td>50% of those found in the other two strains.</td>
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*Geo MFI = geometric mean fluorescence intensity.*
Thus, the absence of such inhibitory signals has the potential to lead to the development of autoimmunity.

Given that the up-regulation of CTLA-4 is dependent on B7 molecules (48, 49), we next investigated whether the reduced expression of CD86 in NOD accounted for the reduced increase in the CTLA-4/CD28 ratio. Again, bulk spleen cells were stimulated with 150 ng/ml soluble anti-CD3 in the absence or presence of partially blocking concentrations of anti-CD86 mAb as described above, such that the levels of free CD86 molecules on C57BL/6 and BALB/c Mø and B cells were similar to those on NOD cells. Following 42 h of culture, the expression of CD28 and CTLA-4 on CD4⁺ and CD8⁺ T cells was analyzed by flow cytometry. It was

![Flow cytometry histograms](A) showing surface-bound CD28 and CTLA-4 levels and total CTLA-4 expression by CD4⁺ and CD8⁺ T cells. Results are shown as mean ± SD of the individual samples in each group. Statistics were done by a one-way ANOVA followed by Dunnett test comparing NOD with either C57BL/6 or BALB/c. *p < 0.05. Results from one representative experiment of three are shown.

![Flow cytometry histograms](B) showing CD28 and CTLA-4 expression by CD4⁺ and CD8⁺ T cells in NOD, C57BL/6, and BALB/c mice (n = 4) stimulated with 150 ng/ml soluble anti-CD3. Following 42 h of culture, the cells were stained for the expression of CD28 or CTLA-4 on CD4⁺ and CD8⁺ T cells and analyzed by flow cytometry.
found that the low levels of CD86 expressed in the NOD mouse may indeed account for the lack of increase in the CTLA-4/CD28 ratio, since the equilibration of CD86 levels in the C57BL/6 and BALB/c cultures, significantly inhibited the increase in CTLA-4 relative to CD28 (Fig. 11). CD28 up-regulation was found to be independent of CD86 expression, since a partial blockade of CD86 did not inhibit anti-CD3-induced CD28 up-regulation. In fact, partial CD86 blockade resulted in a slightly higher CD28 expression on BALB/c T cells (data not shown).

**Discussion**

In this study we have shown that the basal level of CD86 expression on freshly isolated and unactivated Mø, DC, and T cells is lower in NOD mice compared with C57BL/6 and BALB/c. Basal expression of CD86 on B cells did not differ significantly in the mouse strains investigated. However, upon anti-CD3 stimulation of bulk spleen cell cultures, CD86 was up-regulated on B cells from all three mouse strains, but the increase was less in the NOD compared with C57BL/6 and BALB/c. This may have been a secondary effect due to an initial low CD86 expression by Mφ and DC resulting in a defective T cell activation and an accompanying low CD40L induction (50, 51). On interaction of these partially activated T cells with B cells, the low CD40L expression will result in a poor CD86 up-regulation (52, 53). When strong stimuli such as LPS and anti-CD40 mAb were used to stimulate spleen cells in vitro, proliferation and CD86 up-regulation were not found to be reduced in NOD compared with the other two strains, suggesting that there are no fundamental defects in the pathway for activation-induced up-regulation of CD86 in the NOD mouse.

Furthermore, we found that similar to NOD, the two related mouse strains NON and NOR expressed low levels of CD86 on APC and that a low CD86 expression correlated with a reduced proliferative response to anti-CD3. NON and NOR mice develop neither diabetes nor massive insulitis, although NOR islets have been shown to have some degree of APC infiltration (44). They both share some of the Idd loci with NOD; NON mice have NOD alleles at Idd 4, 5, 8, and 11 (42, 43) and NOR mice have NOD alleles at Idd 1, 2, 3, 6, 7, 8, 10, 12, and 14 (45). Our results indicate that the low levels of CD86 expression found in NOD mice are not dependent on the NOD MHC haplotype since NON mice that have the H-2\textsubscript{nb1} haplotype also express CD86 levels similar to those seen in NOD. In addition, the low levels of CD86 are not a consequence of the autoimmune diabetes developed by NOD mice since the disease-free strains have similar low levels of CD86 expression. We do not know the reason for this low CD86 expression in NOD, NON, and NOR mice. The only Idd locus in which these three mouse strains have been shown to have the same allele is Idd8. However, this allele is considered to protect from...
diabetes (2), and therefore it seems unlikely that this locus is responsible for the low CD86 expression in the NOD mouse. Thus, the low CD86 expression is probably due to an as yet unidentified genetic locus that differs between normal and diabetes-prone mouse strains, possibly the CD86 gene or a gene(s) coding for a factor(s) regulating the expression of CD86. It could be argued that, if the low CD86 expression seen in NOD mice does play a role in diabetes susceptibility, NON and NOR mice, which exhibit similar low levels of CD86 expression, should also develop diabetes. However, diabetes development in the NOD mouse is known to be under polygenic control (2). Since both NON and NOR mice lack NOD alleles at several Idd loci, they do not develop diabetes. Therefore, we suggest that a low level of CD86 expression may contribute to diabetes susceptibility but that it is certainly not sufficient to induce disease.

Proliferation and CTLA-4 induction are both dependent on TCR-CD3 signaling as well as the interaction of the B7 molecules with CD28 (19, 49). The low levels of CD86 expressed in NOD mice may at least partially account for the impairment of T cell proliferation and CTLA-4 up-regulation, since partial blockade of CD86 in C57BL/6 and BALB/c cultures to NOD levels significantly inhibited the above parameters. Since CD86 is expressed constitutively by APC (54), it is likely that it interacts with CD28 expressed by T cells at an early stage of T cell priming. The B7/CD2 interaction has been shown to be important for the up-regulation and stabilization of CD40L expression on the T cell (50), and therefore it is likely that an initial low CD86 expression could lead to a reduced up-regulation of CD40L and thus maintain the low CD86 expression on APC also after the initial APC-T cell interaction.

In addition to the low CD86 expression in the NOD mouse described here, Delovitch and coworkers (15, 16) have shown that signal transduction by the TCR-CD3 complex is defective in NOD, since the recruitment of Grb2, mSos, and PLC-γ1 to the cell membrane and activation of p21<sup>ras</sup> upon TCR cross-linking are all diminished in NOD thymocytes. Therefore, the defective T cell proliferation and CTLA-4 up-regulation observed in NOD mice might be a consequence of the low CD86 levels in combination with the defective TCR-CD3 signaling previously reported. However, on comparison of the proliferative response of NOD, C57BL/6, and BALB/c CD4<sup>+</sup> T cells to anti-CD3 and anti-CD28 mAb bound to latex beads, we were unable to detect any differences in the T cell response between the three strains. The reason for the discrepancies between our results and those obtained by Delovitch and coworkers is not known. However, it should be noted that they used biotinylated anti-TCR-β mAb cross-linked with protein G or streptavidin to stimulate their cells, and that most of the work was done on thymocytes rather than peripheral T cells; this could possibly explain the observed differences.

In addition to the reduced anti-CD3-induced up-regulation of CTLA-4, we also observed that the up-regulation of CD28 is decreased in NOD, although not to the same extent as CTLA-4. This is in agreement with a previous publication (55). Although the low CD86 expression may account for the reduced up-regulation of CD86, it does not account for the reduced up-regulation of CD28, since blocking of CD86 and/or CD80 was unable to prevent CD28 up-regulation (data not shown). The reduced CD28 up-regulation that was observed may be due to defective TCR-induced signaling, but it has also been suggested that the defective expression of CD28 as well as CTLA-4 in the NOD may be due to mutations in these gene loci (55).

Modulation of costimulation via the CD28/CTLA-4/CD80/CD86 pathway is known to influence diabetes development in the NOD mouse. Treatment with stimulating anti-CD28 mAb was shown to inhibit insulitis and diabetes development in an IL-4-dependent manner (30), suggesting an insufficient T cell activation in the NOD mouse. Furthermore, the role of CTLA-4 in the maintenance of T cell homeostasis and tolerance was also shown by the acceleration of diabetes by treatment of BDC2.5 TCR transgenic NOD mice by blocking anti-CTLA-4 mAb (31), thus supporting our notion that a reduced up-regulation of CTLA-4 may contribute to diabetes development in the NOD mouse.

The role of this costimulatory pathway in diabetes development has been further demonstrated by the treatment of young NOD mice with CTLA-4-Ig, anti-CD80, or anti-CD86. It has been shown that treatment with CTLA-4-Ig or anti-CD86 mAb protects from diabetes development whereas anti-CD80 mAb alone or in combination with anti-CD86 mAb accelerates diabetes development (32). The reasons for these in part contradictory effects are not clear. However, one could speculate that the inhibitory effects of the anti-CD86 treatment could be due to the level of costimulation influencing the T cell response such that optimal costimulation results in tolerance or regulatory T cell responses, whereas lower levels of costimulation (such as those expressed in the NOD mouse) result in the activation of Th1 cells. If that is the case, the latter could then be inhibited by the lowering the T cell stimulus below the activation threshold of a productive Th1 response by treatment with anti-CD86 mAb. In addition, the authors (32) speculated that the exacerbating effects of the anti-CD80 treatment could be due to either a direct signaling through the CD80 molecule or that the mAb blocked the down-regulatory CTLA-4-CD80 interaction.

Our results suggest that a low CD86 expression in the NOD mouse may result in the reduced activation of at least two activation-induced T cell responses, these being proliferation and the up-regulation of CTLA-4, which may both contribute to the autoimmune phenotype of these mice. It has been shown that a reduced T cell proliferation may skew the T cell response toward a pathogenic Th1 profile since the ability of T cells to produce IL-4, which is protective against diabetes in the NOD mouse (20, 21), is acquired after a greater number of cell cycles following activation than the production of IFN-γ (22). Interestingly, results from several studies indicate an important role of CD86 in the induction of IL-4 production and Th2 development (38, 39).

Although our results are based on in vitro experiments, it could be speculated that a similar reduced up-regulation of CTLA-4 may occur in vivo in NOD mice. Thus, a reduced up-regulation of CTLA-4 following T cell activation could result in a defective inactivation of the activated autoreactive T cells. CTLA-4 directly inhibits T cell activation by inhibiting tyrosine phosphorylation of the TCRζ chain (46). In addition, CTLA-4 cross-linking induces the production of TGF-β (47), a cytokine able to suppress the function of several cell types including T cells and APC (56) and to protect from diabetes development in the NOD mouse (35-37).

Therefore, in summary, we suggest that, for some as yet unidentified reason, possibly a mild tissue damage, MΦ and DC migrate into pancreatic islets of Langerhans where they encounter and endocytose β cell Ags. Due to the inflammatory environment in and around the islets, APC become activated and mature. They migrate to the draining lymph nodes where they present islet Ags to β cell Ag-reactive T cells. However, due to the low expression of CD86 in combination with defects in CD3 signaling present in NOD T cells, the T cells are suboptimally activated. This poor T cell activation results in the development of autoreactive T cells capable of producing cytokines such as IL-2 and IFN-γ. However, the normal up-regulation of CTLA-4 following T cell activation is reduced. Therefore, the mechanisms that normally limit ongoing T cell responses, such as CTLA-4 signaling and TGF-β production, are also reduced and therefore unable to control the autoreactive response.
References


