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Mechanisms of *Mycobacterium avium*-induced resistance against insulin-dependent diabetes mellitus (IDDM) in non-obese diabetic (NOD) mice: role of Fas and Th1 cells

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SUMMARY

NOD mice spontaneously develop autoimmune diabetes. One of the manipulations that prevent diabetes in NOD mice is infection with mycobacteria or immunization of mice with mycobacteria-containing adjuvant. Infection of NOD mice with *Mycobacterium avium*, done before the mice show overt diabetes, results in permanent protection of the animals from diabetes and this protective effect is associated with increased numbers of $CD4^+$ T cells and $B220^+$ B cells. Here, we investigate whether the *M. avium*-induced protection of NOD mice from diabetes was associated with changes in the expression of Fas (CD95) and FasL by immune cells, as well as alterations in cytotoxic activity, interferon-gamma (IFN-γ) and IL-4 production and activation of T cells of infected animals. Our data indicate that protection of NOD mice from diabetes is a Th1-type response that is mediated by up-regulation of the Fas–FasL pathway and involves an increase in the cytotoxicity of T cells. These changes are consistent with induction by the infection of regulatory T cells with the ability of triggering deletion or anergy of peripheral self-reactive lymphocytes that cause the autoimmune disease of NOD mice.

Keywords diabetes tolerance apoptosis cytotoxicity Th1/Th2

INTRODUCTION

The NOD mouse is a classical animal model of spontaneous T cell-mediated autoimmune IDDM. Numerous reports have shown that the autoimmune process in NOD mice can be delayed or prevented by either antigen-specific or broader T cell-specific manipulations if they are performed early in the ontogeny of the immune system of the mice, i.e. before extensive insulitis has occurred [1–6].

Several mechanisms may account for induction of T cell tolerance to self antigens, namely deletion or anergy of autoreactive T cells, suppression/regulation of autoreactive lymphocytes by other T cells, or competitive antigen (or autoantigen) presentation to T lymphocytes [7–10]. The relative contributions of these mechanisms to the maintenance of self tolerance *in vivo* are yet to be defined. Deletion of peripheral T lymphocytes may be the result of apoptosis through activation of the Fas (CD95)–FasL pathway. Fas function(s) is important in the development and maintenance of the immune system. Several critical parameters of both humoral and cellular responses are controlled by Fas after initiation of an immune response. Fas-mediated apoptosis may be essential in controlling the expansion of activated/primed T cell clones. A regulatory role requiring Fas up-regulation on activated lymphocytes is to suppress unintended activation of autoreactive clones, thereby protecting against initiation of an autoimmune response; therefore, activation-induced cell death by Fas–FasL pathway may restore peripheral tolerance [11–16].

One of the manipulations that prevents diabetes in NOD mice is infection with mycobacteria (e.g. bacille Calmette–Guerin (BCG) bacilli) or immunization of mice with mycobacteria-containing adjuvant (Freund's complete adjuvant (FCA)) [17–19]. Previous work from this laboratory has shown that infection of NOD mice with *Mycobacterium avium*, done before the mice show overt diabetes, also results in permanent protection of the animals from diabetes [20]; this protective effect was associated with increased numbers of $CD4^+$ T cells and $B220^+$ B cells [21]. In this work we have investigated whether the *M. avium*-induced protection of NOD mice from diabetes was associated with changes in the expression of Fas and FasL by immune cells, as well as alterations in cytotoxic activity, interferon-gamma (IFN-γ) and IL-4 production and activation of T cells of infected animals. Our data indicate that protection of NOD mice from diabetes is a Th1-type response that is mediated by up-regulation of the Fas–FasL pathway and involves an increase in the cytotoxicity of T cells. These changes are consistent with induction by the infection of regulatory T cells with the ability of triggering deletion or

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anergy of peripheral self-reactive lymphocytes that cause the autoimmune disease of NOD mice.

MATERIALS AND METHODS

Mice

Breeding nuclei of NOD/Lt and NON/Lt mice were established in this research centre from animals purchased from the Jackson Laboratory (Bar Harbour, ME). NOD mice spontaneously develop type 1 diabetes, whereas NON mice are a related diabetes-resistant strain. Female NOD and NON mice were infected at 8 weeks of age, i.e. before overt diabetes was observed in NOD animals. All mice were confirmed to be negative for glucose in urine at this age. Colourimetric strips were used to monitor glycosuria (Combur-Test; Boehringer, Mannheim, Germany).

Mycobacterial infection

Mycobacterium avium strain ATCC 25291, serotype 2, was grown in liquid culture at 37°C in Middlebrook 7H9 broth (Difco Labs, Detroit, MI) containing 0·04% Tween-80. *Mycobacterium avium* is a mycobacterial species that causes human disease only in immune deficient individuals; a previous report from this laboratory showed that both NOD and NON mice are naturally resistant to this infectious agent [20]. The mycobacteria were harvested from liquid culture by centrifugation (6000 ***g***) and washed three times in PBS, as described before [22–24]. The bacteria were suspended in saline containing 0·04% Tween-80 and diluted to a concentration of 2×10^8 viable bacilli of *M. avium* per ml. Eight-week-old NOD and NON mice were infected intraperitoneally with 0·5 ml of the *M. avium* suspension in saline (i.e. 10^8 viable bacilli per mouse). The animals were killed 1 month after infection. Age-matched control NOD and NON mice were inoculated with 0·04% Tween-80 in saline. Eight mice were used in each experimental group.

Monoclonal antibodies

MoAbs used in the flow cytometry analysis of splenic lymphocytes of the mice were the following: PE-labelled anti-CD4, PE-labelled anti-CD3, FITC-labelled anti-CD8 and FITC-labelled anti-CD4, FITC-labelled anti-IgM, PE-labelled anti-Fas, PE-labelled anti-CD69, and biotinylated-goat anti-rabbit, all purchased from PharMingen Inc. (San Diego, CA); FITC-labelled anti-IgG and PE-labelled streptavidin were purchased from Southern Biotechnology (Birmingham, AL) and purified rabbit anti-mouse FasL was purchased from Calbiochem (La Jolla, CA).

Spleen cell suspensions

A standard procedure was used to prepare exhaustive cell suspensions from spleen [25]. Viable cells were counted by a trypan blue exclusion test.

Flow cytometric analysis of cell surface markers of splenic cells

Splenic cells (10^6) were incubated with 50 μl of each MoAb preparation for 20 min on ice and in the dark. The dilutions of MoAbs were the following: PE–anti-CD4, 1:400; FITC–anti-CD4 and FITC–anti-CD8, all 1:200; FITC–anti-IgM, FITC–anti-IgG, PE–anti-Fas, PE–anti-CD69, purified FasL, biotin goat anti-rabbit and PE–avidin, all 1:100; and anti-CD3, 1:50. After the cell pellets were washed three times in PBS–NaN_3 0·01%–fetal calf serum (FCS) 3%, their staining pattern was analysed using a Becton Dickinson FACSort flow cytometer interfaced to a Hewlett Packard computer. Dead cells and erythrocytes were excluded from the analysis using a combination of forward light scatter and propidium iodide (PI; Sigma Chemical Co., St Louis, MO) gating, as previously described [26].

Cytotoxicity assay

A non-radioactive assay was used to determine the cytotoxic potential of splenic lymphocytes obtained from infected and control mice, as described elsewhere [27]. Briefly, splenic lymphocytes were unspecifically stimulated overnight with concanavalin A (Con A) (Sigma). The blasts (effector (E) cells) originated this way were separated by a Percoll (Pharmacia, Uppsala, Sweden) gradient. Viable target (T) lymphocytes were stained with Calcein-AM (CAM; Molecular Probes, Eugene, OR) 100 nM (30 min incubation at 37°C). E and T cells were cultured together in RPMI 1640 medium (GIBCO BRL Life Technologies Ltd) containing 10% FCS, 200 mM L-glutamine, 10 mM HEPES (all from GIBCO BRL) and 50 μM 2-mercaptoethanol (2-ME; Sigma), for 3 h (37°C, 5% CO_2). Fifteen minutes before the end of the incubation, PI was added. After washing the cells three times in PBS, their staining pattern was analysed using a Becton Dickinson FACSort flow cytometer interfaced to a Hewlett Packard computer. The double stain with CAM and PI enabled the distinction of killed target cells. Determination of the percentage of specific cytotoxicity was done as previously described [27].

ELISPOT assay

Measurement of IFN-γ and IL-4 production by ELISPOT was performed as described elsewhere [28]. Before performance of the ELISPOT assay, 4×10^6 splenic lymphocytes were stimulated overnight, either with antigen (4 μg/ml *M. avium* sonicate) or with 3 μg/ml Con A (positive control), in RPMI 1640 medium containing 5% FCS (37°C, 5% CO_2). The antibodies used for detection were anti-mouse IFN-γ, R46A2, anti-mouse IFN-γ–biotin, AN18, anti-mouse IL-4, 1D11, and anti-mouse IL-4–biotin, 24G2 (all kindly provided by Dr R. Appleberg, I.B.M.C., Porto, Portugal) and streptavidin–alkaline phosphatase (Jackson ImmunoResearch).

Statistical analysis

The numerical data were statistically compared using Student's *t*-test. We have considered two numerical populations to be significantly different if $P<0{\cdot}05$ or $P<0{\cdot}01$; these differences are, respectively, labelled with one or two asterisks in the figures.

RESULTS

To investigate the immune mechanisms underlying *M. avium*-induced prevention of diabetes in NOD mice, 8-week-old NOD and NON mice were intraperitoneally infected with the mycobacteria. After 1 month of infection, when major and stable changes of the host response to *M. avium* infection had already occurred [21], we characterized several immune parameters of the mycobacterial infected mice. The present experiments confirmed our previous report that *M. avium* infection causes in NOD mice (but not in NON animals) elevation in the numbers of $CD4^+$ T cells and $B220^+$ B cells [21]; we also confirmed a significant increase in the number of IgG^+ B cells, both in NOD and NON mice. We have now investigated whether the protective effect from diabetes of the *M. avium*-infected mice was related to changes in the Fas–FasL pathway, the cytotoxic activity of T cells or production of IFN-γ and cell activation.

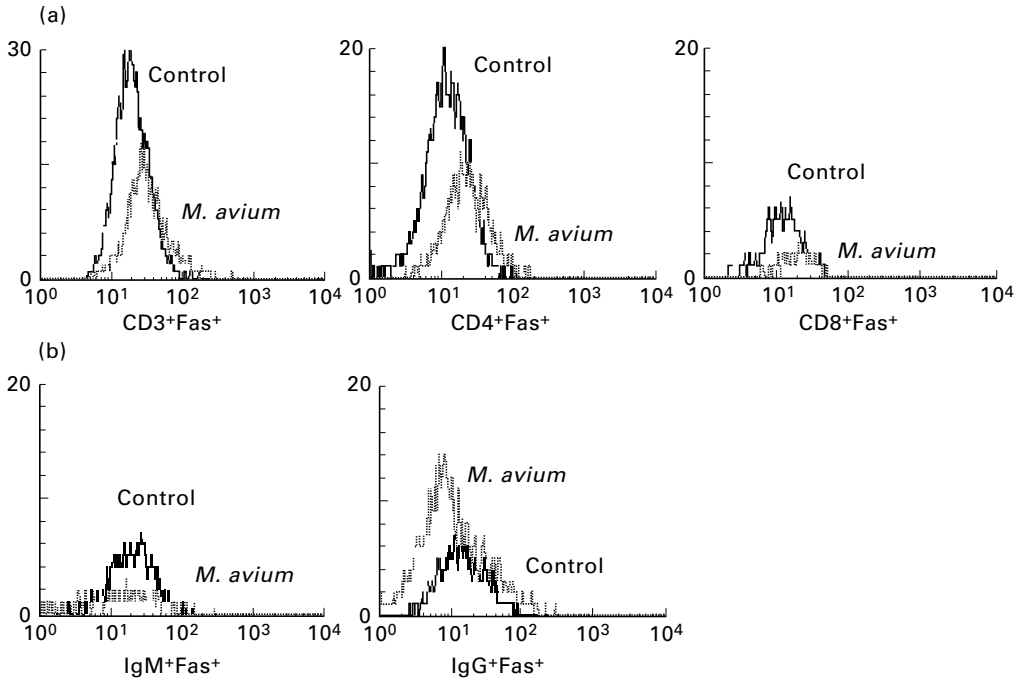

Fig. 1. *Mycobacterium avium* infection induces an increase in Fas expression on T cells of NOD mice. Fas expression on the surface of T and B lymphocytes of NOD mice was measured by flow cytometry after mycobacterial infection or saline injection. There was a pronounced increase in Fas expression of T lymphocytes (a) after *M. avium* infection of NOD mice. Fas expression of B cells (b) remained similar to control levels of expression. $n = 8$.

Expression of Fas and FasL

Flow cytometric analysis of splenic lymphocytes stained with either Fas or FasL MoAbs was performed. We observed that *M. avium* infection induced increased Fas expression in both $CD4^+$ and $CD8^+$ T cells. No significant changes in Fas expression were seen in IgM^+ B cells; infection decreased Fas expression in IgG^+ B cells (Fig. 1). Interestingly, we found that in control mice Fas expression is much lower in T cells of NOD mice than in T lymphocytes of NON animals (Fig. 2a), no difference being seen in Fas expression in B cells (Fig. 3). The expression of Fas in T lymphocytes of infected NOD mice reached levels similar to that observed on T cells of control NON mice (Fig. 2b). It has been documented that increase in Fas expression may not be enough for apoptosis induction of self-reactive cells; the phenomena may require FasL expression and interaction with Fas. Therefore, we also studied the expression of FasL in splenic T cells of the mice.

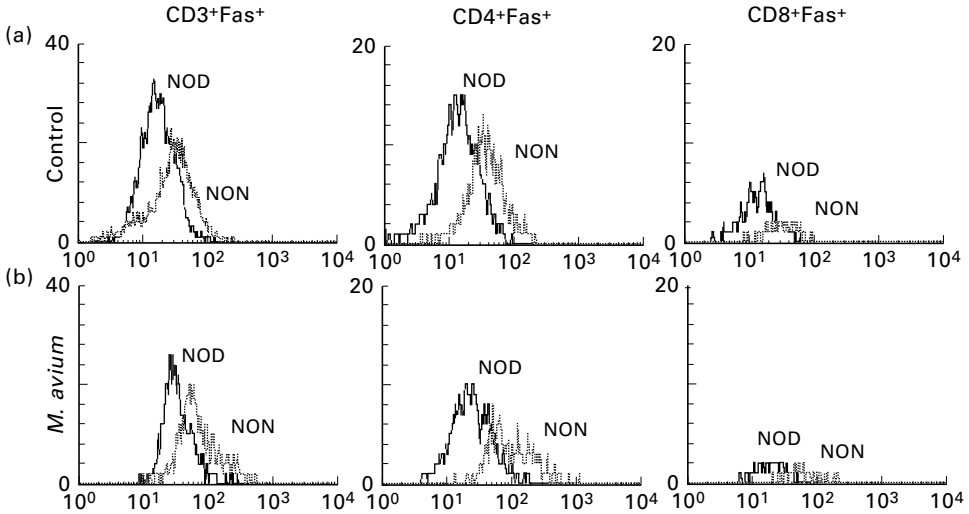

Fig. 2. NOD mice T lymphocytes showed decreased Fas expression when compared with its related diabetes-resistant strain, NON mice. NOD and NON mice were intraperitoneally infected with 10^8 *Mycobacterium avium* bacilli or injected with the vehicle of the bacteria. Fas expression on T lymphocytes was compared by flow cytometry. Control NOD mice showed deceased levels of Fas expression on T lymphocytes compared with control NON mice (a). Infection of NOD raised T lymphocyte Fas expression to levels similar to that observed in control NON mice (b). $n = 8$.

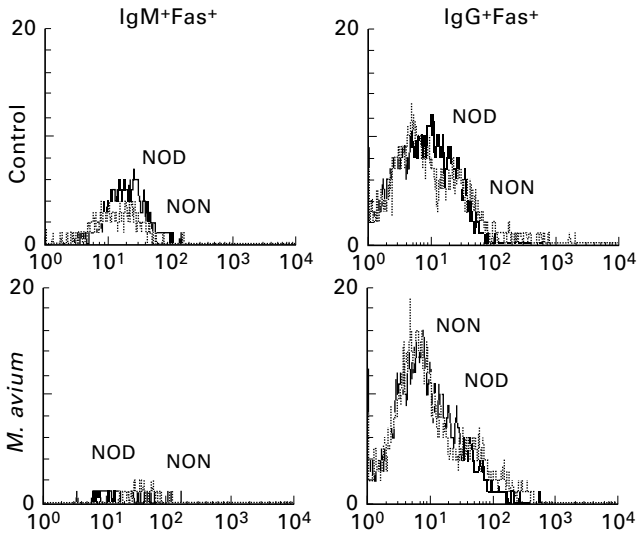

Fig. 3. Fas expression on B lymphocytes is similar in NOD and NON mice. NOD and NON mice were intraperitoneally infected with 10^8 *Mycobacterium avium* bacilli or injected with the vehicle of the bacteria. Fas expression on B lymphocytes was compared by flow cytometry. B lymphocytes from the two related strains showed similar levels of Fas. $n = 8$.

As documented in Fig. 4, mycobacterial infection led to a significant enhancement in the number of $FasL^+$ T lymphocytes, particularly of $CD4^+$ T cells.

Cytotoxicity of T cells

The cytotoxic potential of the activated T cells was assessed *in vitro* using lymphocytes as targets. We found that, in comparison with control mice, splenic lymphocytes from *M. avium*-infected NOD mice showed significantly increased cytotoxic activity against the target splenic cells (Fig. 5a). In contrast, infection did not result in an increased cytotoxic activity of T lymphocytes from NON mice (Fig. 5b).

IFN-γ and IL-4 production

An ELISPOT assay was performed to measure IFN-γ and IL-4 production by splenic lymphocytes of infected and control NOD mice. Splenic cells obtained from infected NOD mice showed increased IFN-γ production when compared with non-infected control mice. Interestingly, this effect was specific for the mycobacteria, as there were no differences in IFN-γ secretion between cells of infected and control mice when the cells were stimulated *in vitro* with Con A (Fig. 6a). IL-4 was detected in very small amounts in both groups of mice. Nevertheless, Con A-stimulated lymphocytes from control mice presented a significantly higher number of IL-4-producing cells than lymphocytes from infected mice (Fig. 6b).

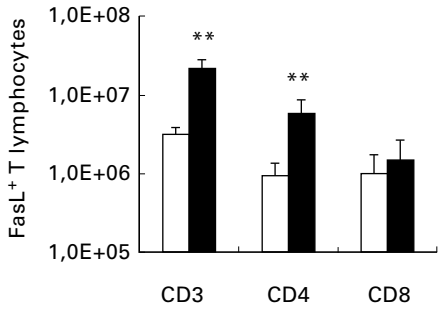

Fig. 4. Mycobacteria-induced changes in the number of $FasL^+$ T cells. FasL expression on the surface of T lymphocytes of NOD mice was measured by flow cytometry after mycobacterial infection or saline injection. The number of $FasL^+$ cells was derived from two values: the percentage of $FasL^+$ lymphocytes and the total number of splenic $CD3^+$, $CD4^+$ and $CD8^+$ T cells. *Mycobacterium avium* infection induced a significant increase in the number of $CD4^+$ T cells which express FasL. $1{,}0E + 08 = 10^8$. $n = 8$. □, Control; ■, *M. avium*.

Expression of the early activation marker CD69

Mycobacterium avium infection of NOD mice induced a significant increase in the number of T cells (both $CD4^+$ and $CD8^+$) and B cells (both IgM^+ and IgG^+) expressing the CD69 surface antigen (Fig. 7). In contrast, infected NON mice only showed increased numbers of $CD69^+$ IgG^+ B cells and only a small increase in $CD3^+$ T cells (that was not significant for $CD4^+$ or $CD8^+$ T cells) (Fig. 8).

DISCUSSION

We have shown before that *M. avium* infection of NOD mice prevents the development of autoimmune diabetes and is associated with numerical changes in subpopulations of lymphocytes [7,8]. Interestingly, Baxter *et al*. reported that mycobacterial infection induces systemic lupus erythematosus (SLE)-like autoantibodies in NOD mice [29]. We report now that T cells of these diabetes-resistant NOD mice show enhanced expression of Fas and FasL molecules, increased cytotoxic activity, and augmented production of IFN-γ and decreased secretion of IL-4.

Clement & Stamenkovic [30] have recently shown that susceptibility of lymphocytes to Fas-mediated apoptosis is correlated to Fas expression on the cellular surface of those cells. Indeed, they showed that low/high levels of Fas expression correlate with reduced sensitivity to death, whereas an intermediate Fas expression

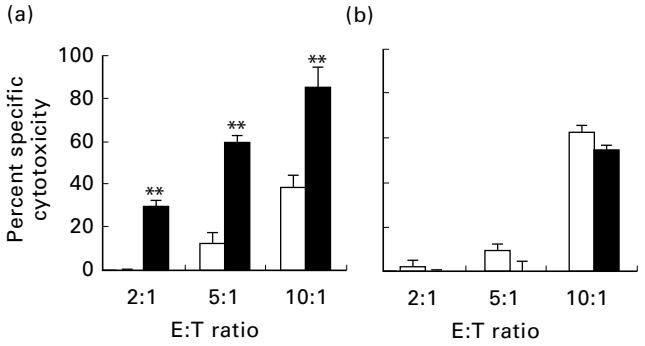

Fig. 5. T lymphocytes of infected NOD mice showed increased cytotoxic potential. NOD and NON mice were intraperitoneally infected with 10^8 *Mycobacterium avium* bacilli or injected with the vehicle of the bacteria. Lymphocytes obtained from either group of mice were used as effector cells in a cytotoxicity assay against splenic lymphocytes of untreated mice. T cells from infected NOD mice showed significantly increased cytotoxic potential (a); in contrast, there were no significant differences between cytotoxic activity of T cells from infected or control NON mice (b). Results refer to differences between groups of mice. $n = 8$. □, Control; ■, *M. avium*.

sensitizes cells to apoptosis. Fas cytotoxic signals may therefore be dependent on a critical level of receptor surface expression. The importance of Fas in the regulation of mature T cells and the persistence of memory is accentuated by the involvement of Fas in the maintenance of peripheral tolerance and by its possible deregulation by a soluble form of Fas molecule in autoimmune disease [31,32]. This view is reinforced by the recent observations that activation-induced cell death is mediated by Fas–FasL interactions [11,13,31,32]. Interestingly, we found that expression of Fas on T lymphocytes of diabetes-prone NOD mice is much lower than the observed Fas expression on T cells of mice from NOD's related non-autoimmune strain, the NON mice. Moreover, we observed that upon *M. avium* infection there is an up-regulation of Fas on T lymphocytes from NOD mice to levels that are similar to the ones observed in non-infected NON mice. Thus, one may argue that the constitutively low expression of Fas on T lymphocytes of NOD mice may lead to deficient deletion of autoreactive cells upon activation and, consequently, to the development of autoimmune diabetes. The protective effect of *M. avium* infection may therefore be the result of raising Fas expression of lymphocytes to levels that sensitize autoreactive T cells to undergo programmed cell death. We also observed that the mycobacterial infection induced increased numbers of FasL-expressing T cells in NOD mice. These data may indicate that the protective effect of *M. avium* infection may be favoured by the induction of peripheral deletion of autoreactive T cells via Fas–FasL. Furthermore, these molecules, along with related ones (TNF/TNFR1) have already been implicated by other authors in IDDM β-cell death [33,34].

The augmented cytotoxicity observed in infected NOD mice is consistent with a Th1-dominated immune response and could involve both Th1 $CD4^+$ T cells (through Fas–FasL pathway) and $CD8^+$ T cells (through perforin/granzyme and/or Fas–FasL pathways). The importance of these two subpopulations of T lymphocytes in establishment and maintenance of peripheral tolerance through deletion of self-reactive cells is already very well established [7,30,35]. Consistent with a Th1-dominated response is our finding of increased IFN-γ and decreased IL-4 production by lymphocytes of infected mice. Other reports also describe a role for Th1 T cells in IDDM suppression: Akhtar *et al*. [36] documented the existence of Th1 β-cell-reactive T cell clones that have acquired a protective effector function at least in part by down-modulating immune responses; Han and co-workers [37] also identified $CD4^+$, IFN-γ-producing, suppressor/regulatory T cells. They postulated that transforming growth factor-beta (TGF-β) also induced expansion of clones responsible for diabetes prevention, a

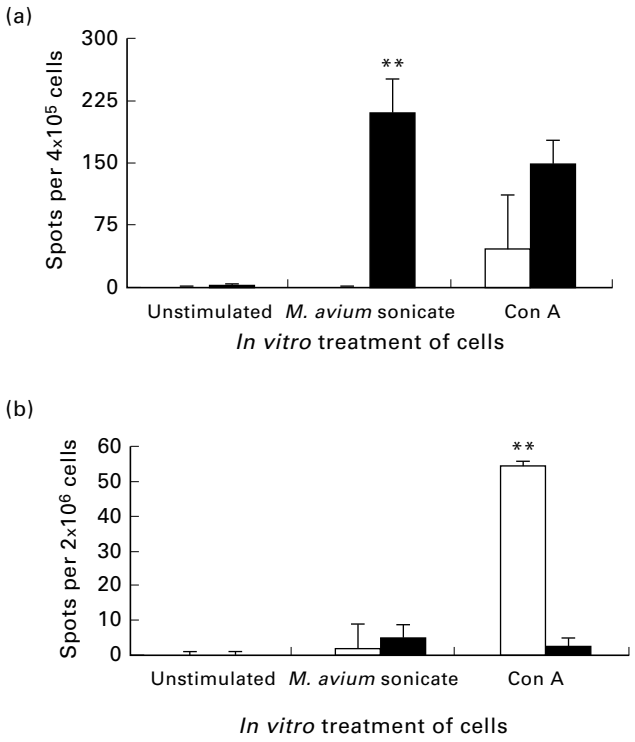

Fig. 6. Response of NOD mice to mycobacteria includes IFN-γ production. IFN-γ and IL-4 production was measured by ELISPOT after *in vitro* stimulation of lymphocytes of either infected or control, saline-injected NOD mice, with sonicated mycobacteria. Concanavalin A (Con A) stimulation was used as a positive control. (a) There was a significant increase of IFN-γ-producing cells of infected mice after lymphocyte stimulation with sonicated *Mycobacterium avium.* No significant differences were observed between infected and control mice when Con A stimulation was performed. (b) There was an increase in the number of IL-4-producing cells of control mice after stimulation with Con A; in contrast, lymphocytes of infected mice showed almost no IL-4 production, whatever the *in vitro* stimulation performed. $n = 8$. □, Control; ■, *M. avium.*

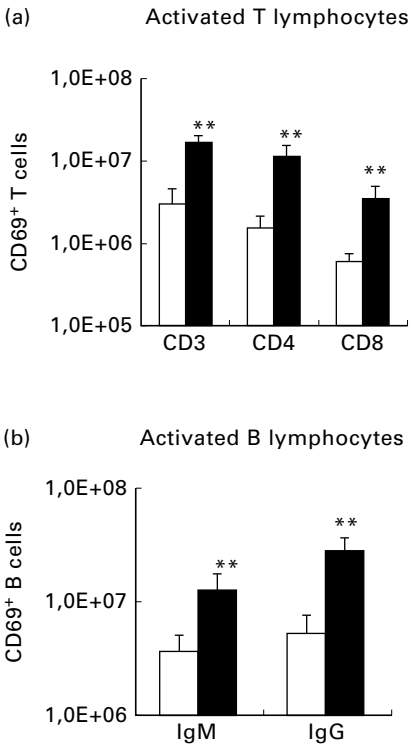

Fig. 7. Activation state of lymphocytes from NOD mice after mycobacterial infection. NOD mice were intraperitoneally infected with 10^8 *Mycobacterium avium* bacilli or injected with the vehicle of the bacteria. Cell acquisition was performed gating each subset of lymphocytes ($CD3^+$, $CD4^+$, $CD8^+$, IgM^+, IgG^+) and the percentage of $CD69^+$ cells was determined. The number of $CD69^+$ lymphocytes was derived from the percentage of $CD69^+$ cells and the numbers of splenic $CD3^+$, $CD4^+$, $CD8^+$, IgM^+ and IgG^+ lymphocytes. Mycobacterial infection induced significant increases in the numbers of activated, CD69-expressing T cells (both $CD4^+$ and $CD8^+$) and activated $CD69^+$ B cells (expressing either surface IgM or IgG). $1{,}0E + 08 = 10^8$. $n = 8$. □, Control; ■, *M. avium.*

hypothesis sustained by other investigations [38]. Finally, a beneficial role for IFN-γ in islet regeneration has also been postulated [7].

FasL expression has been correlated with the Th1 phenotype and it has been postulated that $FasL^+$ Th1 (but not $FasL^+$ Th2) cells are capable of lysing Th2, Th0 and Th1 cells by a Fas-mediated pathway [38]. Thus, Th1 lymphocytes may utilize Fas-mediated cell death to suppress Th2/Th0 as well as Th1 activity. β-cell destruction in IDDM is usually attributed to Th1-dominated response, whereas Th2 T cells are regarded as protective. However, there are other reports that disagree with the simple prediction that Th1 is pathogenic and Th2 is protective in diabetes of NOD mice. Wogensen and co-workers [39], for instance, have shown that transgenic expression of IL-10 in β-cells, which promotes a Th2-like cytokine pattern of islet-infiltrating cells, accelerated the onset of IDDM and led to an increased prevalence of the disease. Additionally, a recent work indicated that the inflammatory foci in NOD mice are dominated by a Th2 cytokine response [40]. Thus, β-cell destruction and T cell suppression of autoimmunity may involve a more complex interplay between Th1 and Th2 cells than is currently conceived. The Th1 $CD4^+$ T cell subsets described here may be a part of the regulatory network that under normal circumstances plays a role in maintaining peripheral tolerance to self.

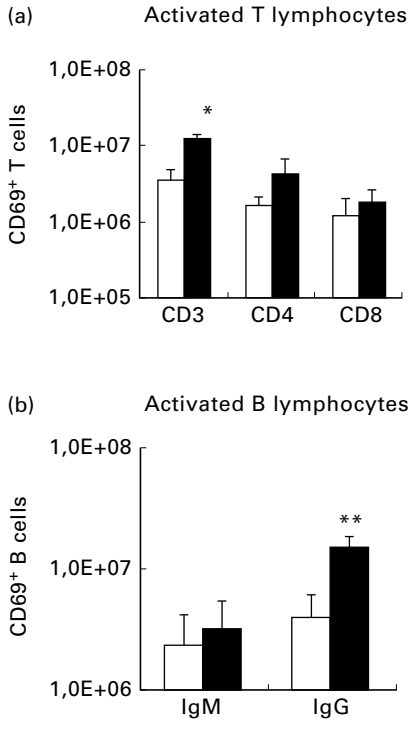

Fig. 8. Activation state of lymphocytes from NON mice after mycobacterial infection. Cell acquisition was performed gating each subset of lymphocytes ($CD3^+$, $CD4^+$, $CD8^+$, IgM^+, IgG^+) and the percentage of $CD69^+$ cells was determined. The number of $CD69^+$ lymphocytes was derived from the percentage of $CD69^+$ cells and the numbers of splenic $CD3^+$, $CD4^+$, $CD8^+$, IgM^+ and IgG^+ lymphocytes. NON mice were intraperitoneally infected with 10^8 *Mycobacterium avium* bacilli or injected with the vehicle of the bacteria. *M. avium* infection induced a significant increase in the number of CD69-expressing IgG^+ B cells, no significant differences being seen in either T cells or IgM^+ B cells. $1{,}0E+08 = 10^8$. $n = 8$. □, Control; ■, *M. avium.*

We propose that the protective effect of *M. avium* infection against diabetes of NOD mice may be achieved by peripheral deletion of autoreactive T lymphocytes via Fas–FasL. The event may result from molecular mimicry between mycobacterial antigen and pancreatic autoantigens, leading to deletion of lymphocytes reactive to β-cells as a consequence of the immune response directed to mycobacteria, or simply deletion of bystander autoreactive lymphocytes. The regulatory role of Fas up-regulation on activated lymphocytes could then be to suppress unintended activation of autoreactive clones, thereby protecting against the initiation of the autoimmune aggression of the pancreatic islets of NOD mice. In conclusion, our data suggest a mechanism to account for the protective effect of infection against the spontaneous autoimmune disease of NOD mice: infection induces deletion of peripheral autoreactive β-cell-specific T lymphocytes via Fas–FasL interactions effected by Th1-type T cells of enhanced cytotoxic activity.

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