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Extracellular lysosome-associated membrane protein-1 (LAMP-1) mediates autoimmune disease progression in the NOD model of type 1 diabetes

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Treatment (from 5 to 25 weeks of age) with a novel blocking monoclonal antibody, mAb I-10, directed against the plasma membrane (pm) form of LAMP-1, protected against development of autoimmune diabetes in the NOD mouse. A shorter course of treatment, *i.e.* from 5 to 12 weeks of age, significantly reduced the occurrence of insulitis as well as disease onset. Interfering with pm-LAMP-1 required continuous treatment as tolerance was not observed when treatment was stopped, and no higher proportion of cells with a T regulatory phenotype (*e.g.* $CD4^+CD25^+$) were induced. The mechanism appears to involve modulating a proinflammatory cytokine, as the proportion of pancreatic-infiltrating IFN-γ-positive cells was significantly reduced in the mAb I-10-treated group. These results demonstrate an unexpected role for pm-LAMP-1 in autoimmune disease progression, and suggest that further investigation should be performed to understand how this molecule modulates IFN-γ-driven responses.

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Introduction

The activation process towards an effector T cell is a multifactorial process regulated by cytokines, dose of antigen, maturation status of the antigen-presenting cell (APC) and the expression of costimulatory and adhesion molecules [1]. Compromising any of these aspects of APC-T cell interaction results in the emergence of phenotypes ranging from anergy, apoptosis and antigen-specific hyporesponsiveness [2] to the induction of suppressor or regulatory T cells (Treg) cells [3].

To identify novel molecules that modulate APC-T cell interactions, a panel of mAb were obtained by injecting murine primary lymph node (LN) DC into rats. mAb I-10 was shown to inhibit IFN-γ production in an antigen-presentation assay *in vitro*. The protein recognized by mAb I-10 is the plasma membrane (pm) form of lysosome-associated membrane protein-1 (LAMP-1). Indeed, a postranslational isoform of LAMP-1 expressed on the surface of activated macrophages has recently been shown to specifically elicit Th1 responses [4].

Our *in vitro* observations prompted us to evaluate the effects of blocking pm-LAMP-1 *in vivo*. Type 1 diabetes (T1D) is characterized by the T cell-mediated destruction of the insulin-producing β cells of the pancreas [5]. The non-obese diabetic (NOD) mouse spontaneously develops autoimmune diabetes and serves as a model for the human disease [6]. Initial insulitis, which involves

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Abbreviations: **T1D:** Type 1 diabetes · **LAMP-1:** Lysosome-associated membrane protein-1 · **MBP:** Myelin basic protein · **Treg:** T regulatory cells

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the recruitment of APC including macrophage and DC, is first detectable at 3–4 weeks of age and progresses over several weeks [6]. During this prolonged prediabetic period, these islet-infiltrating APC are proposed to take up endogenous proteins, traffic to the pancreatic LN and stimulate antigen-specific T cells [7]. The islet-reactive T cells then home to the pancreas where they cause a slow, progressive, and selective T cell-mediated destruction of insulin-producing cells, resulting in overt hyperglycemia by 4–6 months of age [6].

It has been demonstrated that a Th1 immune response is responsible for the destruction of pancreatic ß cells [8], and IFN-γ-producing T islet-infiltrating cells have been implicated in the effector phase of ß cell destruction [9]. Accordingly, T1D can be prevented in NOD mice by neutralizing the action of endogenous IFN-γ [10]. In addition, newly diagnosed T1D patients present a Th1-dominated T cells insulitis [11], making NOD mice a relevant model for studying human disease.

Using different treatment protocols of mAb I-10 administration, we have demonstrated that interfering with the ability of the extracellular form of LAMP-1 to be engaged *in vivo* suppresses disease development, but does not induce a longer lasting tolerance effect or generate Treg cells.

Results

mAb I-10 down-regulates Th1 responses *in vitro* and binds to cell surface LAMP-1

mAb were generated using primary murine LN DC as the immunogen to identify new molecules expressed on DC and involved in the modulation of antigen presentation. Each hybridoma supernatant was tested for efficacy using myelin basic protein (MBP) added to bone marrow-derived DC (BM-DC) and transgenic $CD4^+$ T cells (MBP-TCR transgenic T cells) co-cultures. As shown in Fig. 1A, mAb clone I-10, inhibited T cell proliferation and IFN-γ production. No increase in cell death was observed in comparison to treatment with the isotype-matched control mAb, as evaluated by trypan blue dye exclusion and annexin-V staining (data not shown). mAb I-10-labeled splenic and LN DC, macrophages, $CD4^+$ T cells, $CD8^+$ T cells, NK cells and B cells (Fig. 1B).

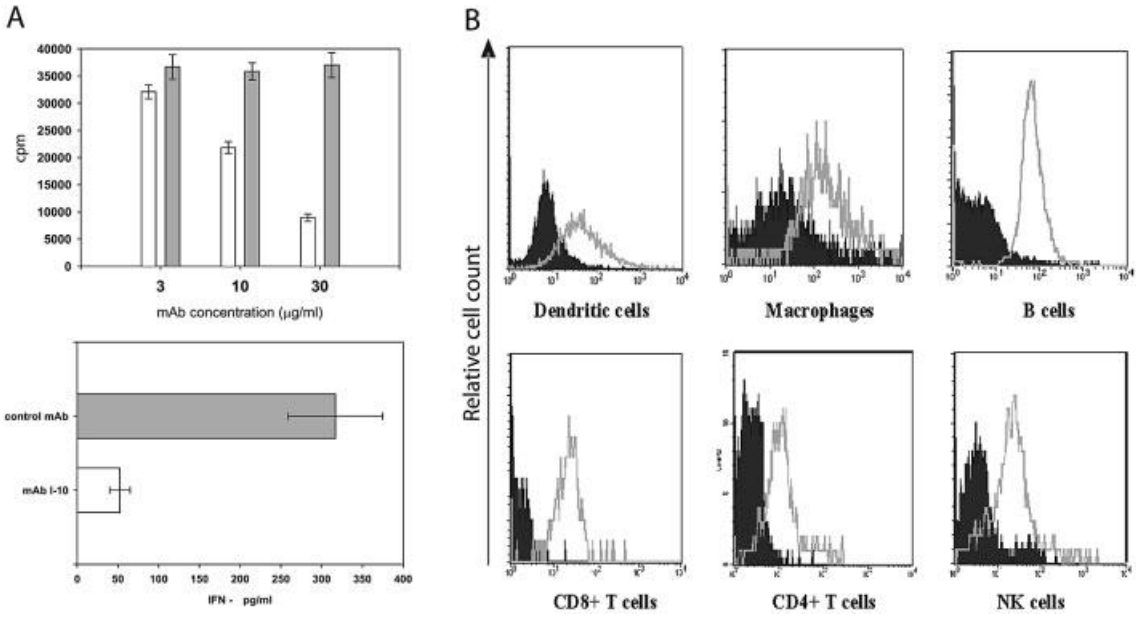
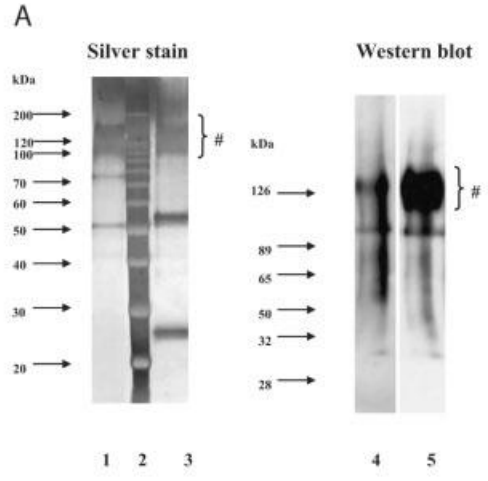


Fig. 1. mAb I-10 alters T cell responses in antigen-presentation assays and binds to APC and lymphoid immune cells. (A) Upper panel shows inhibition of CD4 T cell proliferation after mAb I-10 treatment *in vitro*. MBP-specific transgenic $CD4^+$ T cells (10^5 cells) were co-cultured with γ-irradiated (2.5 Gy) BM-DC (10^5 cells) in the presence of MBP (10 μg/ml) and increasing concentrations of mAb I-10 (white bars) or an irrelevant isotype-matched control mAb (gray bars). Proliferation was measured by $[^3H]$thymidine incorporation during the last 16 h of the 72-h culture. Lower panel shows decreased production of IFN-γ by specific $CD4^+$ T cells after mAb I-10 treatment. MBP-specific $CD4^+$ T cells were co-cultured with γ-irradiated (2.5 Gy) wild-type bulk splenocytes in the presence of MBP (10 μg/ml) and 3 μg/ml of mAb I-10 (white bar) or control mAb (gray bar). Culture supernatants were collected after 72 h and cytokine production was measured by flow cytometry using the CBA kit. (B) mAb I-10 antigen is expressed by APC, T cells and NK cells. Splenocytes (10^6 cells) from B10.PL wt mice were labeled with purified mAb I-10 or the isotype control mAb, followed by a PE-conjugated mouse anti-rat IgG2a mAb. The cells were then dual labeled with FITC-conjugated mAb to either CD11c (DC), CD11b (macrophages), CD19 (B cells), CD8, CD4 and NK1.1 (NK cells). Analysis was done by comparing mAb I-10 (empty histograms) and isotype control (filled histograms) PE fluorescence on each cell subpopulation as defined by a gate using FITC and SSC parameters. Similar results were obtained using cells from C57BL/6, BALB/c and NOD mice.

To identify the protein recognized by mAb I-10, DC plasma membrane extracts were subjected to affinity chromatography using mAb I-10. Captured proteins were then eluted and resolved by SDS-PAGE and silver stained. A diffuse band of between 120–150 kDa was identified (Fig. 2A, lanes 1 and 3, #) and further processed for sequencing by mass spectrometry. The sequences of five tryptic peptides were obtained and the protein was identified as murine LAMP-1 (Fig. 2B). Next, we analyzed the same affinity-purified material by Western blotting and compared our mAb I-10 (Fig. 2A, lane 4) with the commercially available rat anti-mouse LAMP-1 mAb (clone 1D4B; Fig. 2A, lane 5). Both mAb detected a band of approximately 126 kDa (Fig. 2A). Confocal microscopy combined with immunohistochemistry on LN sections using both mAb revealed that all the protein labeled by mAb I-10 co-labeled with 1D4B (data not shown). However, much more lysosomal labeling was observed with 1D4B, suggesting heterogeneity in this intracellular compartment of LAMP-1 combined with different epitopes being recognized by the two mAb.



B

MAAPGARRPL LLLLLAGLAH GASALFEVKN NGTTCIMASF SASFLTTYET ANGSQIVNIS
LPASAEVLKN GSSCGKENVS DPSLTITFGR GYLLTLNFTK NTTRYSVQHM YFTYNLSDTE
HFPNAISKEI YTMDSTTDIK ADINKAYRCV SDIRVYMKNV TVVLRDATIQ AYLSSGNFSK
EETHCTQDGP SPTTGPPSPS PPLVPTNPTV SKYNVTGNNG TCLLASMALQ LNITYLKKDN
KTVTRAFNIS PNDTSSGSCG INLVTLKVEN KNRALELQFG MNASSSLFFL QGVRLNMTLP
DALVPTFSIS NHSLKALQAT VGNSYKCNTE EHIFVSKMLS LNVFSVQVQA FKVDSDRFGS
VEECVQDGNN MLIPIAVGGA LAGLVLIVLI AYLIGRKRSH AGYQTI

Fig. 2. Identification and characterization of antigen recognized by mAb I-10. (A) SDS PAGE staining by silver (left) and Western blotting (right) of plasma membrane preparation of DC. After immunoprecipitation with mAb I-10, the eluted material was separated by 10% SDS PAGE and either silver stained (lane 1, non-reducing or, lane 3, reducing conditions), or analyzed by Western blotting using mAb I-10 (lane 4) or 1D4B (lane 5). Lane 2: Molecular weight markers; #, indicates region of membrane taken for in-gel digestion and subsequent sequencing. (B) Sequence of murine LAMP-1. Peptide sequences obtained after in gel digestion of the protein band captured by mAb I-10 (shown in A as #) are underlined.

In vivo treatment with mAb I-10 protect NOD mice from autoimmune diabetes

The observation that mAb I-10 reduced IFN-γ production by T cells *in vitro* (Fig. 1A) prompted us to evaluate the *in vivo* effects of this modulating mAb in the NOD model. A bi-weekly treatment from the age of 5 to 25 weeks resulted in a statistically significant persistent protection (70% and 10% of disease-free animals in mAb I-10 and mAb control-treated groups, respectively, $p<0.01$, Fig. 3A).

To address whether early treatment would alter disease, NOD mice were treated from the age of 5 to 12 weeks. Treated mice remained disease free, maintaining a healthy general appearance and also gained in body weight similarly to non-diseased, non-treated mice (data not shown). Histological examination of pancreatic ß cells revealed that, while most of the control mAb treated mice exhibited ongoing insulitis (Fig. 3B), which varied from peri-ductular infiltrate (grade 1) to intra-islet infiltrate associated with ß cell destruction (grade 4; histology not shown), the insulitis process was milder in NOD mice receiving mAb I-10 (Fig. 3B), mostly characterized by peri-ductular or peri-islet infiltrate (grades 1 and 2, respectively, not shown). We next questioned whether similar treatment would result in a lower incidence of clinical diabetes. Female 5-week-old NOD mice were randomly assigned to receive the treatment, as described above, for 7 weeks. The treatment was then stopped and the mice screened for diabetes development by bi-weekly examination of glycosuria. Interestingly, at 19 weeks of age, the incidence of disease in mAb I-10-treated mice was lower than in the control-treated recipients. However, active tolerance was not induced, as the difference did not reach statistical significance and, at 22 weeks of age, the incidence of clinical diabetes was similar between the two groups (Fig. 3C).

In contrast to treatment protocols involving very early stages of disease progression, reversal of established disease could not be obtained by blocking pm-LAMP-1. A late treatment schedule (*i.e.* from 12 to 25 weeks of age) did not alter disease (Fig. 3D).

Protection against diabetes due to blocking pm-LAMP-1 via mAb I-10 treatment does not depend on generation of regulatory cells

We next evaluated whether engagement of pm-LAMP-1 on DC altered the expression of costimulatory molecules. Immature BM-derived DC were incubated overnight with mAb I-10 or isotype control mAb. No changes in the levels of MHC class II, CD86 and CD40 expression were observed using flow cytometry (data not shown). The proportion of cells expressing these markers was also not altered.

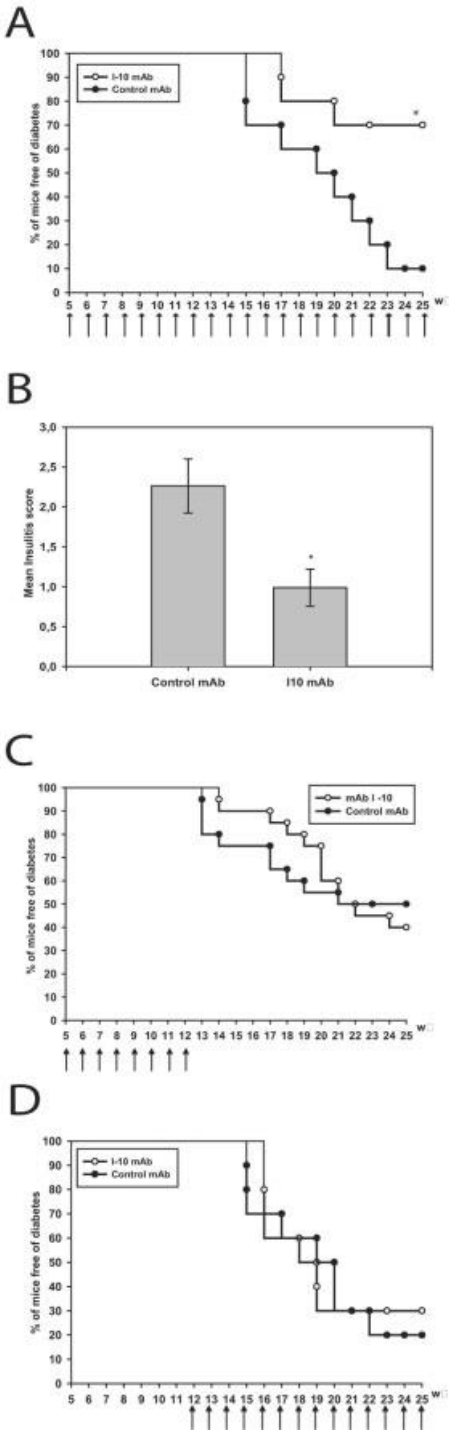


Fig. 3. *In vivo* treatment with mAb I-10 reduces insulitis and protects against T1D development in NOD mice. (A) Five-week-old female NOD mice (n=10/group) received either mAb I-10 or the isotype control rat IgG2a mAb at the dose of 150 μg i.p. twice a week until 25 weeks of age (black arrows), (B) Five-week-old female NOD mice (n=8/group) received either mAb I-10 or the isotype control rat IgG2a mAb as above until 12 weeks of age. Mice were then killed and pancreatic specimens collected. The histological analysis of insulitis and the mean insulitis score were calculated as described in Materials and methods section. (C) mAb I-10 prophylactic treatment delays but does not prevent T1D onset. (D) mAb I-10 treatment does not reverse an advanced stage of insulitis. $^*p<0.05$.

Treg cells have been shown to be important in the control of harmful immunopathological responses against certain self antigens [12], and are able to prevent the development of T1D in NOD mice [13, 14]. Therefore, we evaluated the proportion of cells with regulatory phenotypes in different cellular compartments after treatment. Spleen, pancreas-draining LN and pancreas-infiltrating cells were obtained from NOD mice treated with mAb I-10 or isotype control mAb at 10 weeks of age (during the antibody treatment period) or at 17 to 20 weeks of age (5 to 8 weeks after the interruption of treatment). Flow cytometry revealed similar numbers of $CD4^+CD25^+$, $CD4^+CD62L^{high}$, $CD45RB^{low}$ and $CD4^+CD152^+$ cells in mAb I-10 and control mAb-treated recipients (data not shown). Additionally, no differences were observed in the proportions of $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, $B220^+$ and $CD122^+$ cells (data not shown). To further evaluate the potential induction of Treg cells by mAb I-10, an accelerated form of diabetes inducible in NOD mice by transferring splenocytes from acutely diabetic NOD mice [15] was performed. Female 9-week-old NOD mice were assigned to three groups. A control group of five animals received i.v. 15×10^6 splenocytes from acutely diabetic NOD mice. Two other groups (ten mice each) received 15×10^6 splenocytes from acutely diabetic NOD mice plus 40×10^6 splenocytes from 14-week-old NOD mice treated previously (from weeks 5 to 12 of age) with either mAb I-10 or the isotype control mAb. As expected, 100% of animals receiving diabetogeneic cells alone were diabetic 2 weeks after the cell transfer. An equivalent proportion (80%) of recipient mice were diabetic 2 week after co-transfer of diabetogenic cells and cells from mAb I-10-treated or isotype control-treated mice (data not shown). In summary, these combined results suggest that protection against diabetes after mAb I-10 treatment does not depend on generation of regulatory cells.

mAb I-10 treatment is associated with a reduction of IFN-γ-secreting cells infiltrating the pancreas

We next evaluated whether mAb I-10 was down-regulating Th1 responses or inducing Th2 responses in NOD mice. At 17 to 19 weeks of age, cells were isolated from the spleen, pancreas-draining LN and pancreatic tissue of mice that had been treated with either mAb I-10 or isotype control mAb from 5 to 12 weeks of age. Similar cell numbers in corresponding tissues were observed: notably a similar number of lymphoid cells obtained from pancreatic tissues after collagenase digestion and Percoll density sedimentation in the two experimental groups [$1.2\pm0.2\times10^6$ and $1.3\pm0.1\times10^6$ cells from mAb I-10 or control-treated recipients, respectively, p=not significant (NS)]. Fol-

lowing isolation, cells were stimulated *in vitro* for 4 h with PMA/ionomycin. The resulting cytokine production was then evaluated by FACS analysis after intracellular labeling for IFN-γ, TNF-α, IL-4, IL-6 and IL-10. In splenic and pancreas-draining LN cell populations, no differences were observed in the proportions of cells positive for each cytokine tested. IL-4-, IL-6- and IL-10-positive cells were barely detectable in pancreas-infiltrating cell population in the two groups of mice, and the proportion of TNF-α-positive cells, while measurable, also did not differ between the two groups of mice (data not shown). However, a lower proportion of IFN-γ-positive $CD3^+$ T cells infiltrating the pancreas was consistently observed after mAb I-10 treatment (Fig. 4), but the mean fluorescence intensity (MFI) of IFN-γ, albeit reduced, did not reach statistical significance (MFI 100±15 *vs.* 143±18, from mAb I-10 and control-treated recipients, respectively, p=NS). These combined results suggest that mAb I-10 treatment is not associated with an induction of Th2 response, but instead with an interference of Th1 responses of insulitis-mediating cells.

Discussion

To identify molecules potentially altering T cell responses, we raised mAb using newly recruited LN DC as immunogens. We assayed for modifications of T cell function in an antigen-presentation assay and identified one mAb (I-10) that impaired T cell proliferation and IFN-γ production *in vitro*. The antigen targeted by mAb I-10 was identified as cell surface LAMP-1, a surprise at the time due to the relative low number of publications on plasma membrane expression of this integral lysosomal protein. However, Prasad and colleagues [4] have demonstrated that M150, a protein previously described to stimulate Th1-specific responses, is a posttranslational isoform of LAMP-1 expressed on the cell surface of activated macrophages. Their report, together with our *in vitro* data, show that various glycosylated forms of LAMP-1 exist and are differentially distributed in membrane compartments.

The known pathogenic role of IFN-γ in the NOD model [9, 10] prompted us to test the effects of mAb I-10 in this setting. mAb I-10 reduced insulitis and delayed diabetes onset if the treatment was initiated during the primary phase of insulitis. In addition, fewer Th1 T cells infiltrated the pancreas, as shown by the reduced proportion of T cells committed to secret IFN-γ in mAb I-10-treated mice (Fig. 4). Although slightly reduced, the capacity of $CD3^+$ pancreas-infiltrating cells to produce IFN-γ was not significantly different between mAb I-10 or isotype control treated mice. Importantly, we did not observe differences in the numbers or proportions of total $CD3^+$, $CD4^+$, $CD8^+$ and NK cells among the lymphoid cells isolated from pancreatic tissue after mAb I-10 or control treatments. These combined results suggest that mAb I-10 treatment does not alter the homing of effector cells to inflammatory site, or their capacity to secrete IFN-γ. Therefore, a possible explanation for T1D protection by mAb I-10 in NOD mice is a decrease in the generation of Th1 T cells, resulting in a lower number of such cells available to infiltrate the pancreas and mediate insulitis.

The function of cell surface LAMP-1 is not known. As LAMP-1 has a significant sequence homology with CD68, a scavenger receptor involved in lipoprotein capture by APC [16, 17], we hypothesized that mAb I-10 might interfere with a phagocytic-related function of cell-surface LAMP-1. This is probably not the case, as mAb I-10 treatment had no influence on the capture of

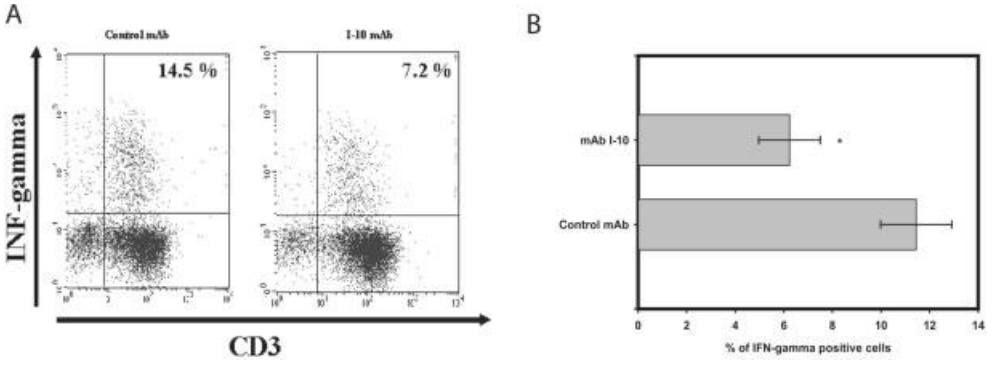


Fig. 4. mAb I-10 treatment is associated with a reduction of IFN-γ-secreting cells infiltrating the pancreas. Female NOD mice were treated from weeks 5 to 12 of age with either mAb I-10 or control mAb at the dose of 150 μg i.p. twice a week. Pancreatic tissue was obtained 5–7 weeks after interruption of treatment. The infiltrating lymphoid cells were isolated and then stimulated *in vitro* with PMA/ionomycin. IFN-γ production of $CD3^+$ T cells was evaluated by intracellular labeling and FACS analysis. (A) Representative dot plots from control mAb (left) and mAb I-10-treated recipients (right). (B) Combined results of mean % of IFN-γ $CD3^+$ T cells from two independent experiments are shown, n=6 mice/group, 17–19 weeks old; $^*p<0.05$.

hapten by tissue draining APC in an *in vivo* assay (data not shown). However, a specific effect on pancreatic antigen capture and loading *in vivo* remains a possibility.

Another explanation for the protective effect observed is the generation of regulatory Treg cells, shown to confer tolerance in T1D animal models [14, 18]. However, the similar proportion of cells expressing a Treg-related phenotype ($CD4^+CD25^+$, $CD4^+CD62L^{high}$, $CD45RB^{low}$, $CD4^+CD152^+$) in mice treated with either mAb I-10 or the control mAb, the lack of detection of Th2 cells following mAb I-10 treatment and the observation that the transfer of cells from mAb I-10-treated recipients did not protect naive recipients from diabetes development, strongly suggests that the mode of action of mAb I-10 is not related to an increase of Treg or Th2 cells. Furthermore, mAb I-10 does not block DC maturation *in vitro* (M.d.C.B. and M.K.V., unpublished observations) ruling out the possibility of an increase in the number of immature DC able to induce Treg cells [19].

The functional role of pm-LAMP remains unclear, but several reports suggest a relation to cellular activation and adhesion. Pm-LAMP expression is up-regulated in PBMC of scleroderma and systemic lupus erythematosus patients, and correlated with markers of immunological activation and clinical disease activity [20, 21]. Platelet activation is also correlated with expression of pm-LAMP-1, possibly playing a role in the adhesive and prothrombotic platelet phenotype [22]. Highly metastatic tumor cells express more pm-LAMP than those that are poorly metastatic [23], probably involving ligation of sialyl Le^x residues in LAMP and E-selectins expressed on vascular endothelium.

T and NK cells up-regulate pm-LAMP expression after *in vitro* activation [24]. The adhesion of activated cells to endothelial cells can be modulated by the cell-surface expression of LAMP and is inhibited by an anti-LAMP mAb [24], supporting the hypothesis of a role of pm-LAMP-1 in the extravasation of activated inflammatory cells. In our study, we found that mAb I-10 and isotype control treated recipients had similar total numbers of lymphoid cells infiltrating the pancreas, arguing against an effect of mAb I-10 on the homing of effector cells to the site of insulitis. It should, however, be noted that these data were obtained 5–7 weeks after treatment interruption, when the immunopharmacological effects of mAb I-10 might have disappeared. Indeed, the milder insulitis process observed at the end of the 7-week treatment period (Fig. 3A) supports a role in homing inhibition in mAb I-10-treated mice. As the effect of mAb I-10 was only seen if the treatment was initiated during the priming phase of diabetogenic T cells, we can also not exclude an interference on the homing of antigen-loaded APC to LN draining the pancreas.

Finally, another possibility is an interference in the interaction between antigen-loaded DC and naive T cells in LN draining the pancreas via blocking the interaction between galectin-3 and pm-LAMP-1 by mAb I-10. Galectin-3 is expressed by macrophages and activated T cells, endothelial cells and DC [25–27], and strongly binds to cell-surface LAMP-1 in neoplastic [28, 29] and macrophage cell lines [30], suggesting an interaction in tumor invasion and metastatic processes [29]. Thus, in our model, blocking the interaction of a ligand (pm-LAMP-1) of galectin-3 may decrease the adhesion of naive diabetogenic T cells to antigen-loaded DC in LN draining the pancreas, then altering a critical first step in optimal T cell priming [31]. In addition, galectin-3 promotes tumor metastasis and angiogenesis [32, 33]. In a murine model of cancer cachexia, mAb I-10-treated recipients presented less inflammatory cell infiltration and less neovascular formation (M.K.V., unpublished observations), reinforcing the hypothesis of a blockade of galectin-3 interactions with pm-LAMP-1 after mAb I-10 treatment.

In summary, we demonstrate that triggering cell-surface LAMP-1 with a novel mAb results in a down-regulation of Th1 responses both *in vitro* and *in vivo*. Further studies are necessary for elucidating the detailed mechanisms underlying these effects, but available experimental data suggest that the most likely mechanism involves cell adhesion in APC-T cell interactions, resulting in a suboptimal priming of Th1-committed T cells.

Materials and methods

Animals

NOD female mice were obtained from Charles Rivers (Calco, Italy). B10.PL wild-type (WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). MBP-TCR transgenic mice [34] were a generous gift from Dr. J. Lafaille, and were reared in our animal facilities under specific pathogen-free conditions. NOD and B10.PL WT animals were housed in a conventional mouse facility. All animals were used between 5 and 25 weeks of age. All animal experiments were approved by University of Catania and Swiss veterinary authorities.

Cell isolation

DC

BALB/c mice were subcutaneously immunized with OVA in alum, and 24 h later the draining LN were obtained for DC isolation as described elsewhere [35]. LN tissues were digested with 2.5 mg/ml of collagenase IV (Worthington, Freehold, NJ) and 1%/ml deoxyribonuclease (Sigma, St Louis, MO). LN cells were then centrifuged and resuspended in Iscove's modified Dulbecco's medium (IMDM, Life Technologies, Grand Island, NY) plus 5% fetal calf serum (FCS) and layered over continuous Percoll gradients (Pharmacia, Piscataway, NJ).

The gradients were centrifuged at 400×g for 30 min and the 1.060–1.065 g/ml low density band removed. After washing, the cells were resuspended in complete medium (IMDM containing 10% FCS, 2 mM glutamine, 100 U/μl penicillin, 100 μg/ml streptomycin and 50 mM 2-mercaptoethanol, from Life Technologies) and labeled with fluorescence-bound anti-CD11c and soluble mannose receptor before sorting for double-positive cells using flow cytometry (FACSVantage, Becton Dickinson, Le Pont de Claix, France). The resulting population was greater than 95% pure DC. BM-DC were generated as described [36]. BM cells from B10.PL mice were depleted of B cells, T cells, and MHC class II-positive cells using sheep anti-rat IgG magnetic Dynabeads (Dynal, Oslo, Norway) coupled with anti-CD4, anti-CD8, anti-B220 and anti-MHC class II mAb. The remaining mononuclear cells were plated in 100-mm tissue culture dishes in complete medium supplemented with 10% culture supernatant from J558L cells transfected with a GM-CSF construct (kindly provided by A Rolink, Basel Institute of Immunology, Basel, Switzerland). Every 2 days, 75% of the medium containing the nonadherent cells was discarded and fresh medium was added to the remaining adherent cells. On day 6, the nonadherent and loosely adherent cells were collected and flow cytometry analysis confirmed their phenotype of immature DC as determined by expression of $CD11c^+$ and low levels of MHC class II and CD86. The cells were then replated in complete medium without GM-CSF to induce maturation.

Splenocytes and LN cells

Splenocytes and LN cells used for phenotyping and *in vitro* or *in vivo* studies were obtained after organ disruption by enzyme digestion as above followed by two washes in PBS and resuspended in complete medium.

Pancreas-infiltrating leukocytes

Pancreas-infiltrating leukocytes were isolated according to a modification of a previously described method [37]. Individual NOD mouse pancreata were minced into small pieces in PBS containing 5% FCS and 1% glucose. Pancreatic pieces were then distributed in 15-ml plastic tubes and digested using a collagenase P solution (Boehringer Mannheim, Germany) (5 mg/ml PBS + 15% FCS; 3 ml/tube) by vigorous hand shaking in a water bath at 37°C for 10–12 min. When tissue appeared to be completed digested, the enzyme reaction was stopped by the addition of ice-cold IMDM. After three washes using cold IMDM, digested tissue was submitted to discontinuous Percoll density gradient centrifugation and T cells were obtained by harvesting the 1.087–1.083 and 1.083–1.079 density interfaces [35]. Cell numbers and viability were assessed by trypan blue dye exclusion.

DC membrane preparation

LN-derived DC were washed in cold PBS and resuspended in 3 mM imidazole (pH 7.4), 250 mM sucrose, 1 mM EDTA and protease inhibitors (1 mM PMSF, 0.2 mM TLCK, 5 mM iodoacetamide) and homogenized by several passages through a 22-gauge needle. Broken cells were fractionated by differential centrifugation steps (1,000×g for 10 min, 7,000×g for 10 min, 10,000×g for 12 min and 100,000×g for 60 min) and tracked by Western blotting using mAb I-10 (data not shown). This enriched 100,000×g pellet was solubilized overnight in 50 mM Tris-HCl (pH 7.5) buffer containing 0.5% Triton X-100, 0.5% NP-40, 10 mM CHAPS, 0.1 M NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, protease inhibitors and recentrifuged. Resulting supernatants were first washed on an Affigel-10 resin (Bio-Rad, Reinach, Switzerland) coupled with rat IgG2a (isotype matched). Flow through was subsequently applied onto Affigel-10 resin coupled with either mAb I-10 or mAb 1D4B (anti-LAMP-1, BD PharMingen). Affigel-10 purified protein eluates were then separated by SDS-PAGE (1D and 2D) and analyzed by Western blots. Corresponding silver-stained protein bands/spots were then processed for tandem mass spectrometry as described [38].

In vitro antigen-presentation assay

Single cell suspensions from the spleen or pooled from the inguinal, axillary and cervical LN were obtained from MBP-TCR transgenic mice. MBP-specific transgenic $CD4^+$ T cells were then purified by negative selection using sheep anti-rat IgG magnetic Dynabeads (Dynal) coupled with anti-CD8, anti-B220 and anti-MHC class II mAb. The resulting population was higher than 95% pure for $CD4^+$ Vβ8 TCR^+. Cells (10^5) were then co-cultured in triplicates with γ-irradiated (2.5 Gy) BM-DC (10^5 cells) from MHC-matched B10.PL mice in complete medium with 10 μg/ml MBP (Sigma) and increasing concentrations of mAb I-10 or an isotype-matched (IgG2a) control rat mAb [39] as indicated. For proliferation assays, 72-h cultures were pulsed with 1 μCi [^{3}H]thymidine and incubated for additional 16 h before harvesting and quantitated using a beta-counter (Coulter). For analysis of cytokine content, cell culture supernatants were collected at 72 h of culture and kept frozen at –80°C.

Quantification of cytokine production

IL-2, IL-4, IL-5, TNF-α and IFN-γ levels were measured using the mouse Th1/Th2 cytokine bead array kit (CBA, BD PharMingen) according to the manufacturer's instructions. Intracellular cytokine staining was done as described [40]. Splenocytes, pancreas-draining LN cells and pancreas-infiltrating leukocytes from each mouse (5×10^5 cells) were stimulated separately with PMA (50 ng/ml) and ionomycin (500 ng/ml) in complete medium for 4 h. Two hours prior to harvest, Brefeldin A (2 μg/ml) was added to the cultures. At harvest, cells were washed, pre-incubated with the 2.4G2 mAb to block FcγR binding, and stained with CyChrome-conjugated anti-CD3 (17A2) for 30 min at 4°C in PBS/0.5% BSA. Following two washes in PBS/0.5% BSA, cells were fixed and permeabilized in Cytofix/Cytoperm buffer (BD PharMingen) followed by labeling for 30 min with either FITC or PE anti-IFN-γ (XMG1.2), anti-IL-4 (11B11), anti-IL-6 (MP5–20F3), anti-IL-10 (JES3–16E3) and anti-TNF-α (MP6-XT22) mAb. FITC- and PE-conjugated rat IgG1 (R3–34) were used as isotype controls. All antibodies were from BD PharMingen. Cells were then washed twice with permeabilization buffer and resuspended in PBS for analysis by flow

cytometry using a FACSCalibur (BD Biosciences) and CellQuest Software.

Flow cytometry phenotyping

Flow cytometric analysis for cell surface antigen was performed using purified and FITC-, PE-, CyChrome- or APC-conjugated mAb (all purchased from BD PharMingen) directed against mouse CD3, CD4, CD8, CD11b, CD11c, CD14, CD19, CD25, CD40, CD45RB, CD54, CD62L, CD86, CD107a, CD122, CD152, Vβ8 TCR, MHC-II, NK1.1 and B220. Rat IgG1, Rat IgG2a, Rat IgG2b, mouse IgG2a and Armenian Hamster IgG were used as isotype controls. Cell suspensions were pre-incubated with the 2.4G2 mAb to block FcγR binding, and then incubated with the relevant mAb for 30 min at 4°C. Subsequently, cells were washed and fixed in Cytofix buffer before FACS analysis. To assess mAb I-10 antigen expression on different cellular populations, splenocytes and LN cells isolated from NOD, C57BL/6, BALB/c and B10.PL mice, were similarly pre-incubated with 2.4G2 and then incubated with mAb I-10 or an IgG2a isotype-control mAb for 30 min at 4°C. After two washes, cells were incubated with a PE-conjugated mouse anti-rat IgG2a mAb for 30 min at 4°C. The cells were then washed again, and free binding sites of the anti-rat IgG2a mAb were blocked with normal rat IgG (Caltag, Burlingame, CA) for 10 min. Finally, the cells were labeled with FITC-conjugated mAb to either CD11c (DC), CD11b (macrophages), CD19 (B cells), CD8, CD4 and NK1.1 (NK cells) for additional 30 min at 4°C, washed and resuspended in Cytofix buffer before analysis.

In vivo treatment with mAb I-10 and insulitis assessment

Female NOD mice were randomly assigned to receive either mAb I-10 or the isotype control rat IgG2a at a dose of 150 μg i.p. in 150 μl of PBS twice a week. Age at the beginning of treatment and duration of the treatment were as indicated. Mice were screened for diabetes development twice a week by testing for glycosuria, followed, when positive, by fasting glycemia using a Medisense Precision Q.I.D glucose meter (Abbott Laboratories, Maidenhead, UK). Mice were considered diabetic if glycemia was above 12 mmol/l for 2 consecutive days. In some experiments, at the end of the treatment period, pancreatic specimens were collected for histological analysis of insulitis as described [10]. For the semiquantitative evaluation of infiltration, only sections containing ten or more islets were selected, and at least ten islets were evaluated in a blind fashion by an observer unaware of the treatment given. The degree of mononuclear cell infiltration was graded as follows: 0, no infiltrate; 1, periductular infiltrate; 2, peri-islet infiltrate; 3 intra-islet infiltrate; and 4, intra-islet infiltrate associated with β cell destruction. The mean score for each pancreas was calculated by dividing the total score by the number of islets examined.

Statistical analysis

The occurrence of diabetes in the different experimental groups was plotted using the Kaplan-Meier method, *i.e.* nonparametric cumulative survival plot. The statistical comparison between the curves was performed using the logrank (Mantel-Cox) test, which provided the corresponding *p* values. In addition, when needed, results were analyzed using the Student's *t*-test.

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