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Original Article

Proinsulin and MAP3865c homologous epitopes are a target of antibody response in new-onset type 1 diabetes children from continental Italy

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Mycobacterium avium subspecies *paratuberculosis* (MAP) asymptomatic infection is speculated to play a role in type 1 diabetes (T1D) among Sardinian subjects. Data obtained analyzing a pediatric population from mainland Italy lends support to the hypothesis, which envisions MAP as an environmental factor at play in T1D pathogenesis. Aiming to investigate the likelihood of cross-recognition between linear determinants shared by self (proinsulin) and non-self (MAP) proteins, 59 children with new onset T1D and 60 healthy controls (HCs) from continental Italy were enrolled in the study. Serum samples were subjected to indirect enzyme-linked immunosorbent assay (ELISA) for the presence of antibodies (Abs) toward four homologues MAP/proinsulin epitopes. The rate of MAP infection (42.4% in T1D children and 5% in HCs; $p < 0.0001$) was estimated searching for Abs against MAP specific protein MptD. The homologous MAP2404c$_{70\text{-}85}$ and proinsulin (PI)$_{46\text{-}61}$ peptides were recognized by 42.4 and 39% of new-onset T1D children and only in 5% of HCs (AUC = 0.76, AUC = 0.7, $p < 0.0001$); whereas the prevalence of Abs against MAP 1,4-α-gbp$_{157\text{-}173}$ and PI$_{64-80}$ peptides was 45.7 and 49.1% in new-onset T1D children, respectively, compared with 3.3% of HCs (AUC = 0.74 and $p < 0.0001$ in both). Pre-incubation of MAP Ab-positive sera with proinsulin peptides was able to block the binding to the correspondent MAP epitopes, thus showing that Abs against these homologous peptides are cross-reactive. MAP/Proinsulin Ab mediated cross-recognition, most likely via molecular mimicry, maybe a factor in accelerating and/or initiating T1D in MAP-infected children. Indeed, it is known that anti-proinsulin and anti-Insulin autoantibodies are the earliest to appear.

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Type 1 diabetes (T1D) is a T-cell mediated autoimmune disease, resulting in the destruction of insulin secreting pancreatic β-cells. T1D is characterized as well by the emergence of islet-specific autoantibodies (aAbs) in at-risk subjects, even years before overt disease manifestations (1). Among the molecular target antigens of β-cell autoimmunity there are: proinsulin (PI), glutamic acid decarboxylase (GAD), tyrosine phosphatase-like islet cell antigen 2 (IA-2) and zinc transporter 8 (ZnT8). In the non obese diabetic (NOD) mouse model, the first islet-specific aAbs released by lymphocytic B-cells are directed against PI. Also in human, anti-insulin aAbs (IAA) are the first to appear, particularly in children, thus pointing at a triggering role for insulin in the early events of T1D development (2, 3). The insulin gene is located in the short arm of chromosome 11, and its transcriptions leads to preproinsulin (PPI) synthesis,

Table 1. Alignment of the amino acid sequences of the identified peptides.

Peptide	Sequence	Source
MAP2404c$_{70-85}$	RGFVVLPVTRRDVTDV	MAP2404c (UniProt accession no: Q73XA5)
PI$_{46-61}$	RGFFYTPKTRREAEDL	Human preproinsulin (UniProt accession no: I3WAC9)
	***. * ***: . *:	
MAP1,4-α-gbp$_{157-173}$	GTVELLGGPLAHPFQPL	1,4-α-glucan branching (UniProt accession no: V7NB01)
PI$_{64-80}$	GQVELGGGPGAGSLQPL	Human preproinsulin (UniProt accession no: I3WAC9)
	* *** *** * .:***	

MAP, *Mycobacterium avium* subspecies *paratuberculosis*; PI, proinsulin.
Sequence alignment obtained using Clustal w2. Result shows completely conserved residue with an asterisk (*) strongly similar properties with a colon (:) and weakly similar properties with a dot (.).

which contains a 24-amino acid long N-terminal leader sequence. After synthesis in the ribosomes, PPI is transported to the endoplasmic reticulum, where the leader sequence is cleaved generating the prohormone PI. PI consists of three distinct domains: α chain, β chain, and C-peptide. The prohormone is then transported to the Golgi where it is packaged into secretory vesicles and is finally cleaved to generate bioactive insulin (α chain, β chain) and the C-peptide.

It is speculated that *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection might be associated with T1D, multiple sclerosis, and Hashimoto's thyroiditis diseases in Sardinia (4–10). Moreover, concerning T1D some evidence linking MAP to the disease has been obtained analyzing children from continental Italy (8, 9). In *silico* analyses revealed stretches of homologies between PI and two MAP proteins (MAP2404c and MAP 1, 4-α-glucan branching protein). Indeed, it was highlighted the presence of highly conserved regions, where the percentage of aminoacid identity between homologues MAP/PI epitopes ranges from 50 to 65% (Table 1). The finding of the aforementioned regions of sequence homology also hints at MAP as a possible trigger of T1D in MAP infected individuals.

Noteworthy, the prevalence of IAA inversely correlates with the age at onset in children newly diagnosed with T1D (8). It would be interesting to discover if the appearance of anti-MAP Abs follows the same trend in new-onset T1D children. Hence, the objective of this study was to investigate the prevalence of anti-MAP/PI Abs in a pediatric population (59 new-onset T1D and 60 controls) from continental Italy, which had been previously tested to assess the presence of anti MAP/ZnT8 Abs (9). Moreover, the prevalence of Abs to insulin, GAD65, IA-2 and ZnT8 were assessed by radioligand and radioimmuno assays, and the rate of MAP infection was estimated searching for Abs against MAP specific protein MptD. Remarkably, the reported cross-recognition between Abs targeting MAP/PI homologues peptides may hint at MAP as a possible trigger of T1D in this new-onset T1D cohort of children from continental Italy.

Methods

Serum samples

Fifty-nine serum samples from new-onset T1D children (32 males, 27 females; mean age 9.4 ± 4.4 yr); diagnosed in-line with the American Diabetes Association criteria (11); and 60 sex-and age-matched control (HCs) sera from healthy individuals of the same region (32 males, 28 females; mean age 9.3 ± 3.5 yr; range) attending the Pediatric Diabetes Unit of Tor Vergata University Hospital of Roma were analyzed. Demographic characteristics and human leukocyte antigen (HLA) genotyping of the new-onset T1D children are reported in Table 2. The same cohort of patients was previously screened for the presence of Ab against homologous MAP3865c/ZnT8 epitopes (9). All samples were obtained before starting exogenous insulin therapy. Blood samples were drawn in Vacutainer Serum tubes (BD, Franklin Lakes, NJ, USA). Frozen aliquots were stored at −80°C and used within 6 months. The experimental protocol was approved by the Human Ethics Committee of the Pediatric Diabetes Unit of Tor Vergata University Hospital of Roma, Italy. Informed written consents were obtained from the guardians of all children.

Peptides

Peptides MAP2404c$_{70-85}$ [RGFFYTPKTRREAEDL] and MAP 1,4-α-glucan branching protein (gbp)$_{157-173}$ [GTVELLGGPLAHPFQPL] along with their respective homologous peptides PI$_{46-61}$ [RGFVVLPVTRRDVTDV], PI$_{64-80}$ [GQVELGGGPGAGSLQPL], reported in Table 1 were synthesized at >85% purity (Pepmic Co., Ltd, Suzhou, China). Peptides purity was assessed by high performance liquid chromatography (HPLC).

Enzyme-linked immunosorbent assay (ELISA)

Indirect enzyme-linked immunosorbent assay (ELISA) to detect Abs specific for the selected peptides were performed as follow. Ninety-six well plates were coated with 50 μL of peptides dissolved in 0.05 M carbonate–bicarbonate buffer, pH 9.5 (Sigma-Aldrich,

Table 2. Demographic characteristics and HLA haplotype of type 1 diabetes new onset children

Patient ID	Gender	Age at onset	Days after diagnosis	HLA
D.1	F	8.25	30	DQ8 (DQB1*0302)
D.2	F	14.66	30	NA
D.3	M	10.75	184	DQ8 (DQB1*0302)
D.5	F	15.25	92	NA
D.6	M	8.33	61	DQ2 (DQA1*0501-DQB1*0201)
D.7	F	11.33	61	DQ8 (DQB1*0302)
D.8	M	5.08	30	NA
D.9	M	7.83	92	DQ8 (DQB1*0302)
D.10	F	3.25	30	DQ2 (DQA1*0501-DQB1*0201)
D.11	F	24.25	0	DQ2 (DQA1*0501-DQB1*0201)
D.13	M	3.16	237	DQ8 (DQB1*0302)
D.14	F	9.58	250	DQ2 (DQA1*0501-DQB1*0201)
D.15	M	5.75	61	DQ8 (DQB1*0302)
D.16	F	15.25	236	DQ2(0201/0202)
D.17	M	15.25	250	DQ2(0201/0202)
D.18	F	5.83	0	DQ8 (DQB1*0302)
D.19	M	7.33	0	DQ2 (DQA1*0501-DQB1*0201)
D.20	F	6.5	92	DQ2 (DQA1*0501-DQB1*0201)
D.21	M	9.5	122	DQ2 (DQA1*0501-DQB1*0201)
D.22	M	10.58	122	DQ8 (DQB1*0302)
D.23	F	13.25	246	NA
D.24	M	5.08	122	DQ2 (DQA1*0501-DQB1*0201)
D.25	M	14	67	DQ2 (DQA1*0501-DQB1*0201)
D.26	M	15.42	61	DQ2 (DQA1*0501-DQB1*0201)
D.27	M	14.16	60	DQ8 (DQB1*0302)
D.28	M	6.66	0	DQ2 (DQA1*0501-DQB1*0201)
D.29	M	12.16	184	DQ2 (DQA1*0501-DQB1*0201)
D.30	F	3.08	120	DQ8 (DQB1*0302)
D.31	M	3.08	77	DQ2 (DQA1*0501-DQB1*0201)
D.32	F	7.83	122	DQ2 (DQA1*0501-DQB1*0201)
D.33	M	10.16	122	DQ2 (DQA1*0501-DQB1*0201)
D.34	F	13.08	184	NA
D.35	F	12	122	DQ8 (DQB1*0302)
D.99	F	12.83	30	NA
D.111	M	11.33	166	DQ2 (DQA1*0501-DQB1*0201)
D.113	M	4.66	61	DQ2 (DQA1*0501-DQB1*0201)
D.202	F	4.75	0	DQ8 (DQB1*0302)
D.208	F	4.92	171	DQ8 (DQB1*0302)
D.217	M	9.42	107	DQ2 (DQA1*0501-DQB1*0201)
D.228	M	10.82	92	DQ8 (DQB1*0302)
D.234	M	12.83	118	DQ2 (DQA1*0501-DQB1*0201)
D.253	M	10.92	137	DQ2 (DQA1*0501-DQB1*0201)
D.255	F	7.42	122	DQ2 (DQA1*0501-DQB1*0201)
D.262	F	1.83	119	DQ8 (DQB1*0302)
D.E2	F	10	37	DQ8 (DQB1*0302)
D.E3	M	1.91	0	DQ2 (DQA1*0501-DQB1*0201)
D.E4	F	4	186	DQ2 (DQA1*0201/05-DQB1*02/0301)
D.E5	M	11.83	210	DQ8 (DQB1*0302)
D.E6	M	15.16	105	DQ2 (DQA1*0201-DQB1*02)
D.E7	F	2.33	204	DQ2
D.E9	M	18.58	30	DQ2 (DQA1*05-DQB1*02)
D.E10	M	13.33	88	NA
D.E11	F	16.33	35	DQ8 (DQB1*0302)
D.E12	F	3.44	61	DQ8 (DQB1*0302)
D.E13	M	11.25	90	DQ2 (DQA1*0501-DQB1*0201)
D.E14	F	9.66	0	DQ2 (DQA1*05-DQB1*02)
D.E15	M	5.16	210	DQ2 (DQA1*0501-DQB1*0201)
D.E16	F	17	0	DQ8 (DQB1*0302)
D.E17	M	5.66	0	DQ8 (DQB1*0302)

HLA, human leukocyte antigen.

St. Louis, MO, USA) at a concentration of 10 μg/mlll and incubated at 4°C overnight. After removing the excess of unbound antigen, 100 μL of blocking solution [phosphate buffer saline (PBS), 5% non-fat dried milk] was added to each well, the wells were further incubated at room temperature for 1 h, and then washed twice with PBS containing 0.05% Tween 20 (PBS-T). A 100-μL volume of 1:100 dilutions of human sera in PBS-T was added in duplicate and incubated for 2 h at room temperature. After five washes in PBS-T, the wells were incubated with 100 μL of alkaline phosphatase-conjugated goat anti-human immunoglobulin G polyclonal Ab (1:1000; Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 1 h. Finally, after three washings, a 200-μL volume of para nitrophenylphosphate (Sigma-Aldrich, St. Louis, MO, USA) substrate solution was added as substrate for alkaline phosphatase. The optical density (OD) was read at 405 nm using VERSATunable Max microplate reader (Molecular Devices, Orleans Drive Sunnyvale, CA, USA). Negative and positive controls were included in each essay performed. The OD values were normalized to a strong positive control, which reactivity was set at 10 000 arbitrary units (AU)/mL.

The results obtained were analyzed by receiver operating characteristic (ROC) curves, and the cut off values were calculated setting the specificity at 95% (i.e. Ab + HCs ≤5%). Inter-assay coefficient of variation (CV) for the four peptide based ELISAs ranged from 7.2 to 8.4%. The statistical significance of the essay performed was determined by Mann–Whitney test via GRAPHPAD PRISM 6.0 software (San Diego, CA, USA).

Competitive inhibition assays

Competition inhibition assays were performed using the sera of two new onset T1D children positive for MAP-Abs and the sera of two HCs that were negative for MAP-Abs. Sera were pre-incubated overnight at 4°C with saturating concentrations (10–20 μM, titrated for each individual serum) of MAP peptides, the corresponding pro-insulin peptides, irrelevant peptide (control peptide PGQPFGPGGPYSPG) or no peptide. Sera were then submitted to ELISA on plates coated with MAP2404c$_{70-85}$ or MAP1,4-α-gbp$_{157-173}$ at the concentration of 10 μg/mL.

Autoantibody assays

Antibodies to the ZnT8 C-terminal region (268–369, 325R, or 325 W) present in the sera were measured by Protein A-radioimmunoprecipitation assays as described previously (12). Cutoff thresholds for determining positive or negative Ab status was set at the 99th percentile of 100 non-diabetic control, and was equal to Ab levels >30 U/mL. Inter-assay CV was 14% and an intra-assay CV was 11%.

Autoantibodies to insulin, GAD$_{65}$, and IA-2 were measured by radioligand assays using commercial kits (CentAK® IAA RT, CentAK® anti-GAD65, and CentAK® anti-IA2, Medipan, Germany) and according to the manufacturer's instruction. Results are expressed in AU, and thresholds for aAbs to insulin, to GAD65 and to IA-2 were set as >0.4, >0.9, and >0.75 U/mL, respectively.

Results

Indirect ELISAs were performed in order to assess the humoral responses mounted against MAP specific protein MptD and against four homologous MAP/PI epitopes in a pediatric cohort of 59 new-onset T1D and 60 HCs Italian children from central Italy. The newly identified peptides were highly recognized proving detectable reactivity. Results are depicted in Fig. 1.

The homologous MAP2404c$_{70-85}$ and PI$_{46-61}$ peptides (Fig. 1A–B) were recognized by 42.4 and 39% of new-onset T1D children, but only in 5% of HCs (AUC 0.76 and 0.7, respectively; $p < 0.0001$ for both). An even greater serum Ab reactivity was observed for the MAP 1,4-α-gbp$_{157-173}$ and PI$_{64-80}$ peptides (Fig. 1C–D), as 45.7 and 49.1% of new-onset T1D children were Ab-positive, respectively, compared with 3.3% of HCs (AUC 0.74 and $p < 0.0001$ for both).

Concerning MAP-specific protein MptD, the percentage fraction of Ab-positive children in new onset T1D children and HCs were 42.4 and 5%, respectively (AUC = 0.79 and $p < 0.0001$).

Competitive inhibition assays

The similar prevalence of Abs targeting MAP/PI homologous sequences in new-onset T1D children (45.7–47.4 and 42.4–44%, respectively; Fig. 1), lead us to surmise that Abs recognizing these epitopes could be cross-reactive. To verify the robustness of our hypothesis competition experiments were performed. Two anti-MAP2404c$_{70-85}$-positive and two-negative sera were pre-adsorbed overnight with saturating concentration of three different peptides, and then ELISA was performed on plates coated with MAP2404c$_{70-85}$. As shown in Fig. 2A, whereas a control peptide (no relevant sequence homology) barely decreased the signal, both the positive control (MAP2404c$_{70-85}$) and its homologous PI$_{46-61}$ peptide strongly inhibited MAP2404c$_{70-85}$ reactivity (61.4–64%). The same was true, even if at a lesser extent for MAP 1,4-α-gbp$_{15-173}$ reactivity, which was efficiently inhibited (38.7–49.9%) upon serum pre-adsorption with either MAP 1,4-α-gbp$_{157-173}$ or the homologous PI$_{64-80}$ (Fig. 2B). The experiment performed clearly showed that anti-MAP and anti-PI Abs targeting homologous stretches are cross-reactive.

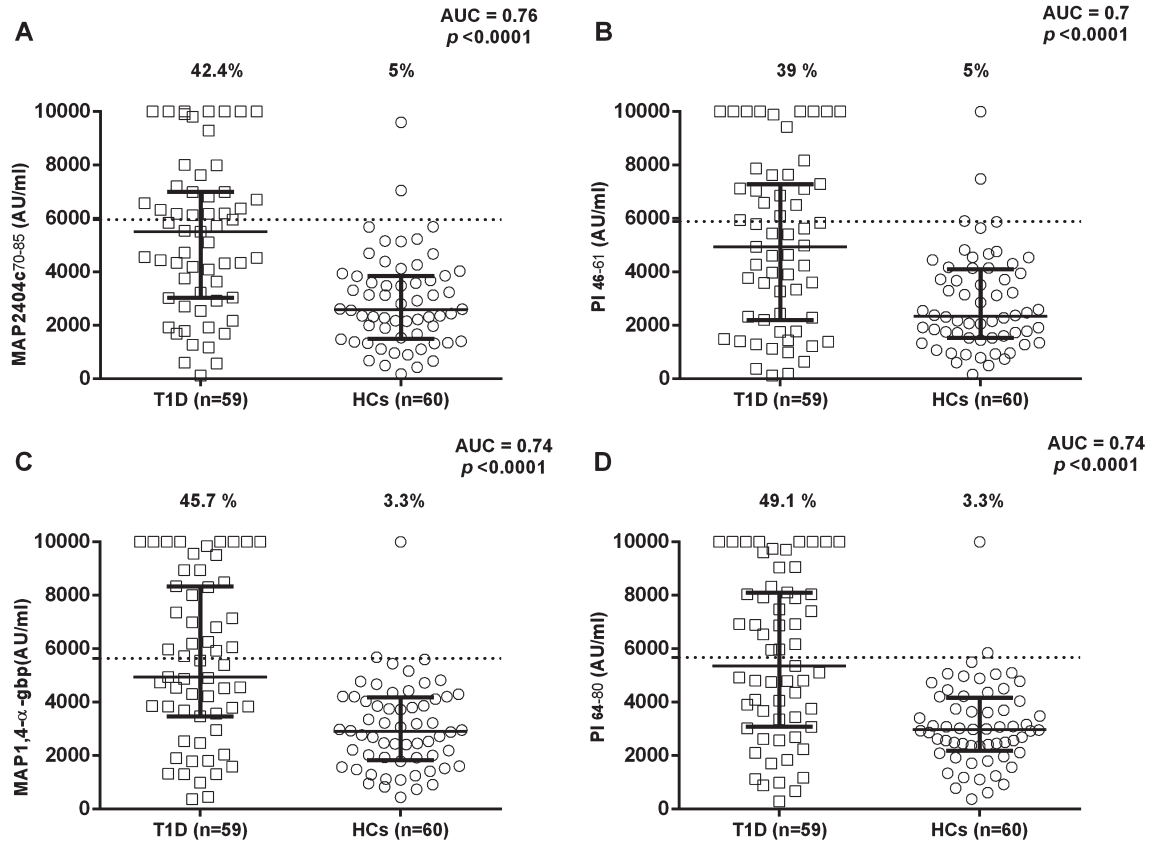

Fig. 1. Evaluation of the humoral response for different peptides in new onset type 1 diabetes (T1D), and healthy controls (HCs) children. Distribution of the enzyme-linked immunosorbent assay (ELISA) values of the IgG antibody response to *Mycobacterium avium* subspecies *paratuberculosis* (MAP)2404c$_{70-85}$ (panel A), proinsulin (PI)$_{46-61}$ (panel B), MAP 1,4-α-gbp$_{157-173}$ (panel C) and PI$_{64-80}$ (panel D) with T1D and healthy controls sera. The percent fraction of Ab + sera is indicated on top of each distribution, while bars indicate the corresponding median ± interquartile range. Area under the receiver operating characteristic (ROC) curve (AUC) and p value are displayed on the top right corner.

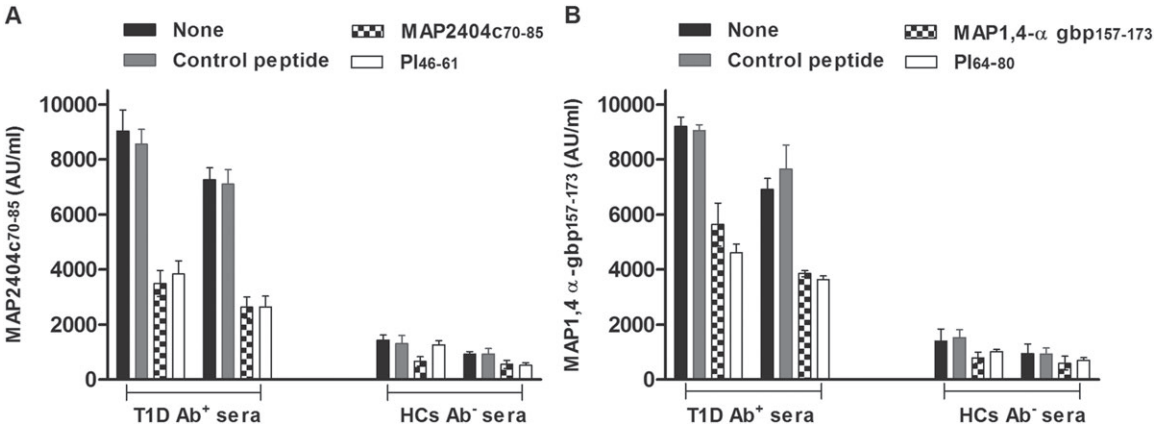

Fig. 2. Effect of *Mycobacterium avium* subspecies *paratuberculosis* (MAP)2404c$_{70-85}$ and MAP1,4-α-gbp$_{157-173}$ in type 1 diabetes (T1D) and health controls (HCs) sera. The peptides derived from the pre-insulin protein proinsulin (PI)$_{46-61}$ and PI$_{64-80}$ significantly block the binding of MAP2404c$_{70-85}$ and MAP 1,4-α-gbp$_{157-173}$ in the Ab + sera, respectively. Bars show means and standard deviation (SD) of triplicate wells and results are representative of two separately performed experiments.

Autoantibody assays

In this pediatric population of new-onset T1D children, the prevalence of aAbs to insulin, GAD65, IA-2 and ZnT8 were 57.7, 70, 68.4 and 63.3%, respectively.

Discussion

In light of our previous finding reporting an elevated sero-prevalence of MAP in patients with T1D from Sardinia and continental Italy (4–9, 13–15), we here

show that two epitopes belonging to different MAP proteins (MAP2404c and MAP 1,4-α-gbp) are targets of an Ab mediated response that cross-recognize the homologous PI sequences. MAP was called into question as one of the possible candidate initiator of T1D (13), a triggering role in β-cell mediated aAbs production has also been surmised for insulin (2). The identification of cross-reactive Abs targeting homologous MAP/PI regions could represent a small asset in understanding T1D pathogenesis. Therefore, the main goal of this study was to investigate whether MAP epitopes, which display sequence homology with the β-cell autoantigen PI, could be recognized in children at T1D onset. If this is the case, Abs against these epitopes might be added to the panel of existing biomarker to monitor T1D onset in at-risk pediatric populations. But how can MAP-infection precipitate T1D?

On one hand, MAP persistence in the gut may favor the spreading of inflammatory mediators responsible in turn of inducing local (β-cell) inflammation. MAP is the cause of Johne's disease in ruminants, an enteritis resulting in weight loss, diarrhea and cachessia bringing the animal to death (16). In humans MAP has been associated with Crohn's disease (17, 18), a chronic inflammatory bowel disease, although definitive evidence accounting for a causal role (as *Helicobacter pylori* and ulcer) is impossible to obtain because there is a large time lapse between infection and overt disease manifestation. However, MAP has been identified and isolated from human intestinal biopsies (17, 18).

Concerning the transmission of MAP from cattle to humans, milk is undoubtedly the vehicle for the diffusion of the bacterium mainly through the consumption of dairy products (19). Indeed MAP may persist for up to 1 yr in the environment, it is present in milk, water, and meat, and therefore human are constantly exposed to it (20). Consequently, if we consider the high prevalence of paratuberculosis reported worldwide (16) and the existence of a food-born chain of transmissions for MAP antigens from cattle to humans, an amount of heat-killed MAP antigen complex or live bacteria could be ingested orally by people daily. This persistent exposure of people to MAP might be able in turn of modulating host immunity.

Mechanistically, pancreas reactive immune cells may be activated by MAP because of molecular mimicry. It is plausible that the TH17 cells generated in the intestine produce cytokines capable of driving naïve T-cells toward pro-inflammatory lineages, it has been reported that enteric innate T helper type 17 (iTH17) responses, which occurred principally in the cecum, were dependent on the Nod-like receptors Nod1 and Nod2 (21). It was shown that specific commensal microbial species are capable of interacting with the innate immune system modifying T1D predisposition (22), moreover it was also found that mucosal damage may be necessary for the development of autoreactivity in the pancreas (23). Activated T and B-cells may migrate from the gut to pancreatic lymph nodes and from here attack microbial mimetic β-cells proteins.

Once, Abs against MAP/PI epitopes are generated, the epitope spreading phenomenon favors tissue destruction, and the release of other previously masked antigens, which in turn might become the target of other cross-reactive aAbs.

Remarkably, the percentage fraction of Ab-positive new-onset T1D children and HCs was very similar between the homologous pair of peptides (Fig. 1), thus suggesting that the reactivity against these homologues regions might be the result of Ab cross-recognition; this assumption was therefore confirmed performing competition assays.

In conclusion, our results support the existence of an association between Ab-mediated cross-recognition of homologous MAP/PI sequences, which may precipitate T1D in this cohort of new-onset T1D children from continental Italy. Future studies may include searching for MAP within the gut of high risk T1D children, in order to verify the presence of a MAP related enteropathy (17, 22, 23) and therefore the possibility to adopt an anti-MAP therapy.

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