Regulatory vs. inflammatory cytokine T-cell responses to mutated insulin peptides in healthy and type 1 diabetic subjects

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Type 1 diabetes (T1D), the autoimmune form of diabetes, results from T cell-mediated destruction of insulin-producing β-cells within pancreatic islets (1). The disease is dramatically increasing in incidence, doubling in the last two decades (2, 3), and now predictable with the measurement of antibodies directed against insulin and other proteins found in β-cells (4). Major efforts at disease prevention have been undertaken using preparations of insulin (s.c., oral, and intranasal) to induce tolerance and delay the onset of clinical symptoms (5–7). Measuring insulin-specific T-cell responses from the peripheral blood has been challenging but would allow for assessment of therapeutic response, which has been a major obstacle in these trials. In our study, we sought to detect peripheral T-cell responses to insulin in T1D patients and nondiabetic controls using a modified insulin B-chain peptide.

Insulin is a major self-antigen for both T and B cells in murine and human T1D, with insulin B-chain amino acids 9–23 (B9–23) being a key epitope presented by major histocompatibility complex class II (MHCII) molecules to CD4 T cells targeting pancreatic β-cells (8–10). There is strong evidence from the nonobese diabetic (NOD) mouse model of spontaneous autoimmune diabetes that the NOD MHCII molecule, IAαβ, is required for development of T1D and that pathogenic CD4 T cells recognize insulin B9–23 presented in an unfavorable position or register (Reg3) in the IAαβ peptide binding groove (9, 11, 12). A unique polymorphism in the IAαβ β-chain at position 57 changing the conserved Asp(D) to Ser(S) favors the binding of peptides that place an acidic amino acid at the p9 position in the peptide binding groove. In Reg3, the B22 Arg(R) of B9–23 is a very poor match for this pocket, but mutating the R to Glu(E) creates an insulin mimotope peptide (B22E) that binds to IAαβ almost exclusively in Reg3. The B22E mimotope stimulates B9–23-specific CD4 T cells about 100-fold better than the WT peptide, and fluorescent IAαβ tetramers made with the altered peptide detect CD4 T cells in the pancreas and pancreatic lymph nodes of prediabetic NOD mice (12). In addition, this mimotope but not the WT B9–23 peptide is capable of inducing tolerance at low doses and completely preventing diabetes onset in the NOD mouse (13).

In humans, particular HLA alleles are also associated with T1D, explaining more than 50% of the genetic risk for disease development (14). For example, the HLA-DQ8 (DQB*03:02) and DQ2 (DQB*02:01 and DQB*02:02) alleles greatly increase the risk of disease development, with ∼90% of all T1D individuals having one or both alleles (14–16). Strikingly, the polymorphic HLA-DQ6 (DQB*06:02) allele provides dominant protection from diabetes development (14). DQ is the homolog of mouse IAα, and the β-chains of DQ2 and DQ8 share with IAαβ the unique non-D amino acid polymorphism at position 57 that favors an acidic amino acid at the p9 position of their binding grooves (17). We hypothesized that diabetogenic insulin-reactive CD4 T cells in diabetes | CD4 T cells | autoimmunity | self-tolerance | insulin

Significance

Certain class II major histocompatibility alleles confer disease risk for type 1 diabetes (T1D). Insulin-specific and other autoantibodies often precede T1D development, but major efforts at disease prevention using insulin preparations (subcutaneous, oral, and intranasal) to induce tolerance have not been effective. Measuring insulin-specific T-cell responses from the peripheral blood has been a challenging feat but would allow for assessment of therapeutic response in these trials. In our study, we report CD4 T-cell responses to a mutated insulin B-chain peptide in new-onset and established T1D as well as control subjects dependent on HLA-DQ genotype. Our results have important implications for the application and monitoring of insulin-specific therapies to prevent diabetes onset.


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T1D patients may also recognize B:9–23 bound to DQ2 or DQ8 in the unfavorable Reg3. If so, the B:9–23 (B22E) mimotope might detect these T cells much better than the WT peptide. Therefore, we examined T cells in the peripheral blood of new-onset T1D individuals and then, those with established disease for their responses to the mimotope vs. WT B:9–23 peptide. We observed vigorous responses to the mimotope but not the WT insulin peptide in numerous T1D patients. Analysis of the T-cell receptors (TCRs) in CD4 T cells proliferating in vitro to response to the mimotope peptide revealed the presence of a highly enriched oligoclonal population. Surprisingly, we also observed responses to the mimotope peptide in control subjects, especially those in which β57D DQ alleles were present, opening the possibility that this peptide may stimulate both inflammatory and regulatory CD4 T cells.

Results

Subjects. The subjects in this study are listed in Fig. S1A. The subjects with new-onset T1D (n = 28) and established T1D (n = 7) were recruited from the Barbara Davis Center for Diabetes Clinics. Nondiabetic controls (n = 27) were healthy adult volunteers negative for all islet autoantibodies. The study protocol was approved by the Institutional Review Board, and written informed consent was obtained from all study participants. A detailed description of the new-onset and control subjects is given in Table S1. The new-onset T1D subjects had a very short duration of diabetes with the mean time from diagnosis being only 15 d; 26 of 28 (93%) T1D individuals had diabetes less than 3 wk before cytokine enzyme-linked immunosorbent spot (ELISPOT) assays were performed. All of the new-onset and control subjects were HLA-genotyped. The nondiabetic control subjects were slightly older than the T1D patients and included more individuals having risk DQ alleles (i.e., those lacking β57D) than expected in the general population to allow for comparison with T1D subjects, most of which had at least one DQ allele lacking β57D. The seven subjects with established T1D are described in Table S2. They had diabetes ranging from 1.5 to 29 y and already known HLA-DQ genotypes before performing T-cell proliferation assays on all subjects and TCR sequencing for three subjects.

Insulin Peptides. The native amino acid sequence of insulin B:9–23 is listed in Fig. S1B along with those of two altered B:9–23 mimotopes. These peptides were designed for the TID studied in the NOD mouse (9, 11, 12) and the structural features shared between IAβ and human DQ8 and DQ2 (18–20). Both had a Glu substituted for Arg at B22 to enhance binding in Reg3 by replacing the disfavored p9 anchor amino Arg with a highly favorable Glu. The first (B22E) had no other changes, but the second (B21G,22E) also had the Glu at B21 changed to Gly. In the mouse, B:9–23-specific CD4 T cells fall into two categories, A and B. Both react to the peptide bound to IAβ in Reg3; however, type A T cells prefer the B22E mimotope, and type B prefer the B21G,22E mimotope (12).

Detection of Robust IFN-γ Responses to an Insulin Mimotope. We determined the cytokine ELISPOT IFN-γ responses of unfractionated peripheral blood mononuclear cells (PBMCs) measured after short-term in vitro stimulation with no antigen vs. the WT insulin B:9–23 peptide or the two insulin mimotopes (Materials and Methods). The raw data for new-onset T1D patients (n = 28) and control subjects (n = 27) are listed in Tables S3 and S4, respectively, and shown graphically in Fig. 1. Among 55 subjects, the background responses without peptide stimulation were low (five or fewer spots per 10^6 input cells), except in 3 of the T1D subjects.

Among T1D subjects, the strongest IFN-γ responses (Fig. 1A, C, and D) were seen to the B22E mimotope, with 20 of 28 responding with more than five ELISPOTS per 10^6 cells. The magnitude of the responses varied from barely above background to well over 100 spots per 10^6 cells, with a geometric average of about 11. The frequency of individuals responding to the WT B:9–23 peptide (7 of 28) and the B21G,22E mimotope (7 of 28) was much lower, and only one response was above 100 spots per 10^6 cells (P < 0.01 comparing B22E responders with WT or B21G,22E) (Fig. 1A). Unexpectedly, the control subjects also mounted an IFN-γ response to the peptides (Fig. 1B–D and Table S4). Again, responses to the B22E mimotope (17 of 27) were much more frequent than those to the WT B:9–23 (2 of 27) or the B21G,22E mimotope (6 of 27; P < 0.01 comparing control B22E responders with WT or B21G,22E) (Fig. 1B). Only three T1D subjects and two controls had a high responder frequency with >100 spots per 10^6 PMBCs, indicating a frequency of ~1 spot per 10,000 PMBCs. In general, the average magnitude of the response to the insulin peptides and Pentacel, a childhood vaccine, was similar in the T1D and control groups, with the exception that T1D subjects lacking DQ β57D on both alleles responded significantly better to the B22E mimotope than the corresponding subjects in the control group (Fig. 1C) (P = 0.02).

Using the strongly stimulating insulin B22E mimotope, we were able to follow the persistence of the IFN-γ response at 6 and 16 wk after initial diagnosis in a new-onset T1D subject (18 in Table S3). The B22E mimotope response was consistently seen at both later time points (Fig. S2). During this time, the subject remained unresponsive to WT insulin B:9–23 and the B21G,22E mimotope. A positive control response to Pentacel was observed for each ELISPOT assay.

Relation Between HLA-DQ Genotype and IFN-γ/L10 Responses in Control Subjects. In addition to the IFN-γ response, we were able to track IL-10 responses of the control subjects and a few of the new-onset T1D patients (Fig. 2 and Table S3) in response to the insulin peptides. These data might offer an explanation for the unexpectedly strong IFN-γ response seen in the control subjects, despite the absence of T1D. As in the IFN-γ responses, the background IL-10 ELISPOT response in the absence of antigen stimulation was mostly five or fewer. As seen in Fig. 2B and
response, the B22E mimotope
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| 0 2 comparing B22E with B21G,22E). In
DQ alleles
The IL-10 ELISPOT responses of the (Fig. 3
Proliferation of unfractionated PBMCs with insulin peptides from
(subjects 1, 2, and 4) and TCR-
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ELISPOT assays, the B22E mimotope resulted in pro-
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response to the B22E mimotope. Notably, these six were among
B22E mimotope, but six of eight patients had a detectable IL-10
subjects lacking
the subjects with at least one nonrisk DQ allele having
mimotope peptides with no additional stimulus. Similar to the
unfractionated PBMCs were labeled with carboxyfluorescein
(meaning of the DQ antibody, indicating that the T-cell response to
after proliferation to the insulin B22E mimotope in the pres-
sequences in each sample are shown in Table S5. There was little
overlap in sequences among the subjects and between the
unstimulated vs. CFSE
T cells for any subject (Fig. S3).

In Fig. 4, we show the cumulative contribution of repeated sequences to the total unique sequences from those that repeat a particular number of times. In addition, the predicted contributions based on a cumulative Poisson distribution calculated from the average number of repeats for that sample are shown. All of the sequence sets from the unstimulated T cells (Fig. 4, Lower) matched the predicted distributions reasonably well, indicating a fairly random set of sequences in PBMCs with only a few sequences dramatically overrepresented, which was not case for stimulated T-cell samples (Fig. 4, Upper). These sequence sets, particularly for subjects 1 and 4, showed a bimodal distribution deviating dramatically from that predicted for a

Table S4, many of the control subjects had a robust IL-10 re-
response. As in the case of the IFN-γ response, the B22E mimotope
yielded the highest frequency of responses (24 of 26) compared
with the WT B:9–23 (11 of 26) or B21G,22E (16 of 26) peptide,
with more than five ELISPOTS per 10⁶ cells (P < 0.01 comparing
B22E with WT and P = 0.02 comparing B22E with B21G,22E). In
the response to the B22E mimotope, there was a correlation be-
tween the magnitudes of IFN-γ vs. IL-10 (Fig. 2C), and strikingly,
the subjects with at least one nonrisk DQ allele having β57D
mounted a stronger response to both IL-10 and IFN-γ than those
subjects lacking β57D on both DQ alleles (Fig. 2 D and E). We
were only able to obtain IL-10 data for eight of the T1D patients.
There was only one IL-10 response to the WT peptide or B21G,
B22E mimotope, but six of eight patients had a detectable IL-10
response to the B22E mimotope. Notably, these six were among
the poorest in the IFN-γ response (Table S3).

Proliferation of CD4 T Cells to the Insulin Peptides. To complement
our ELISPOT experiments from new-onset T1D subjects, we also examined T1D patients with established disease that had
known HLA-DQ genotypes that included two β57D” DQ alleles
(Table S2). Fig. 3 shows the proliferation results after bulk
unfractionated PBMCs were labeled with carboxyfluorescein
succinimidyl ester (CFSE) and cultured for 7 days in the pres-
ence of no antigen, Pentacel, WT B:9–23 peptide, or the B22E
mimotope peptides with no additional stimulus. Similar to the
IFN-γ ELISPOT assays, the B22E mimotope resulted in pro-
liferation of CD4 T cells much more than the WT peptide as
judged by the appearance of cells that had a loss of CFSE
fluorescence (Fig. 3A), which was true for all of the tested T1D
patients (Fig. 3 B and C). To determine whether these prolif-
erative responses were DQ-restricted, an anti-DQ blocking
antibody was added to the culture during proliferation of CD4
T cells. The antibody was able to block the proliferative response of
CD4 T cells from subject 5 to the B22E mimotope in a dose-
dependent fashion (Fig. 3D), and all of the tested T1D subjects
bearing at least one DQ8 allele had fewer CD4” CFSE” cells
after proliferation to the insulin B22E mimotope in the pres-
ence of the DQ antibody, indicating that the T-cell response to
the peptide is at least partially DQ-restricted (Fig. 3E).

Enrichment of TCR Sequences Among the T Cells Stimulated by the
B22E Mimotope. To examine the diversity of CD4 T cells pro-
liferating in response to the B22E mimotope, we sequenced TCRs
in the CD4 T cells from several of the established T1D subjects
(1, 2, and 4) described in Table S2. We sorted unstimulated
CFSE” T cells and the corresponding CFSE”-proliferating CD4
T cells for TCR-α (subjects 1, 2, and 4) and TCR-β (subjects
2 and 4) sequencing. The statistics for the productive V(D)J
sequences in each sample are shown in Table S5. There was little
overlap in sequences among the subjects and between the
unstimulated vs. CFSE
T cells for any subject (Fig. S3).

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The rationale for our experiments here was that, if the B9–23 peptide contained CD4 T-cell epitopes important in human T1D, the shared β57D polymorphism among mouse IAβ-2 and human T1D DQ risk alleles predicts that the B22 Arg to Glu mutation should dramatically improve presentation of the peptide to these T cells. To test this idea, we examined PBMCs from 28 new-onset T1D subjects for an IFN-γ response to the WT vs. mutant B9–23 peptide using an ELISPOT assay. Nearly all of the patients had at least one β57D DQ allele. In agreement with our prediction, most of these subjects had a positive response to the mutant insulin peptide but responded much less well to the WT peptide, which was true whether they had one or two β57D DQ alleles. A few of these subjects responded to the mutant peptide as strongly as they did to the Pentacel vaccine control antigen, but on average, their response was a more modest 10% of the response to Pentacel. We also tested an insulin peptide that had a second mutation of B21 Glu to Gly, because in the NOD mouse, the side chain of this p8 Glu interferes with recognition by some T cells. This double-mutant peptide was much less well-recognized in these human patients.

We also examined patients with more established T1D and found that they too had stronger proliferative DQ-restricted responses in vitro to the mutant than the WT peptide. In several of these experiments, we were able to isolate the proliferating CD4 T cells and show that their TCR-α and -β repertoires were very much skewed compared with the starting population, indicating...
an enrichment of antigen-reactive T cells. Although thousands of unique sequences were identified in the expanded T cells, five sequences accounted for a large percentage (4–20%) of the total sequences obtained. Furthermore, although each patient showed a unique α- and β-CDR3 signature among the most expanded T cells, there was an overenrichment of T cells bearing members of the TRAV-38 Vα family, reminiscent of the skewing in the use of the TRAV-5 Vα family among NOD CD4 B9–23-reactive T cells involved in T1D, despite heterogeneity in their Vα CDR3 sequences and Vj use (21, 22).

Because we used bulk populations of T cells, we have not formally shown in our experiments that the weak responses to the WT peptide and the strong responses to the mutant peptide involved the same T cells. However, in the course of these studies, several of our group collaborated with Kwok and coworkers (23) at the Benaroya Institute in Seattle. Kwok and coworkers used a different approach, in which T cells from established T1D patients with one or two DQ8 alleles were stimulated with WT or B22E mutant insulin peptides for a short time in vitro and then, single-cell-sorted with DQ8 tetramers loaded with either peptide to establish a set of CD4 T-cell clones. Regardless of their source for these clones, they responded much more strongly to the mutant than to the WT peptide. Furthermore, using the same techniques that were used to establish the register 3 nature of the NOD insulin epitopes, this study confirmed that the mutant peptide was recognized bound in register 3 to DQ8.

One difference between the experiments we report here and those with Kwok and coworkers is that we used the full-length B9–23 peptide, whereas Kwok and coworkers (23) used a peptide truncated toward the register 3 core, B11–23. However, the B22E mutation strongly promotes binding in register 3 to IAα and DQ8, leaving B9 and B10 well outside the MHC binding groove and far away for the usual TCR footprint. Therefore, we conclude that, taken together, these two studies point out the presence of a previously underappreciated response to insulin in T1D patients and provide useful tools to pursue the study of this response in the future. They also further reinforce the idea that the strong association of T1D in mice, rats, and humans with a similar MHCII polymorphism may be because of a similar usual mechanism of MHCII allele-specific autoimmune presentation (23).

The most unexpected results in our experiments were those of patients with non-T1D subjects, in T1D subjects having one or two β57D+ DQ alleles. In this case, we had enough of each sample to perform both IFN-γ and IL-10 ELISPOT assays (for example, cytokines associated with inflammatory vs. regulatory T cells, respectively). Surprisingly, for both cytokines, many of these individuals also made strong responses to the mutant but mostly poor responses to the WT insulin peptide. Compared with the T1D patients, the main difference in the pattern of response was the DQ genotype of the strong responders. Whereas among the T1D patients, there was no significant difference in the IFN-γ response of patients with one or two β57D+ DQ alleles, in the control subjects, both the IFN-γ and IL-10 responses were significantly lower in those with two β57D+ DQ alleles than those with one or even two β57D+ DQ alleles.

Our data do not offer any direct explanation for this unexpected but potentially important observation. At this point, we can only speculate on possibilities that can be tested in future experiments. Perhaps the simplest explanation is that the DQ β57D+ response to the register 3-presented insulin peptide is suppressed in these control subjects, explaining the lack of T1D and the poor response in the patients containing only DQ β57D+ alleles. The IFN-γ and IL-10 responses in the other control subjects may require the MHC alleles that lack the DQ β57D+ polymorphism. Because these responses are strongly enhanced by the B22 Arg to Glu mutation, this mutation should be involved in peptide processing or the binding and/or register selection by these other MHC molecules, perhaps by improving binding in the relevant register or inhibiting binding in other irrelevant registers. In the case of MHCI, the mutant peptide could favor the processing of a CD8 T-cell epitope. It is known that, within the insulin B9–23 peptide sequence, amino acids 10–18 activate CD8 T cells from T1D patients (24). It is also worth noting that our experiments involved short-term immediate ex vivo stimulation of the T cells, and therefore, it is likely that these responses, potentially driven by non-DQ β57D+ MHC molecules, most likely arose from memory T cells with previous antigen experience.

Most models for autoimmunity suggest that, in the non-autoimmune steady state, there is a balance between antigen-driven inflammatory and regulatory responses in the target organ that needs to be disrupted for autoimmunity to blossom out. The strong correlation between the IFN-γ and IL-10 responses to the mutant insulin peptide in the control subjects might reflect this balance that prevents T1D development. Other investigators have also shown the presence of autoreactive T cells in the peripheral blood of healthy donors that can be suppressed by their own regulatory T cells (25). Peakman and coworkers (26) reported a regulatory IL-10 response to the self-antigens insulinoma-associated antigen 2 (IA-2) and proinsulin, with a panel of IA-2 peptides able to distinguish diabetic from control subjects independent of HLA type. More recently, posttranslational modification of a proinsulin peptide (insulin B30–C13) with tissue transglutaminase was able to bind HLA-DQ8 and identified inflammatory IFN-γ responses from T1D subjects and regulatory IL-10 responses from some healthy controls (27). Other studies have documented responses to other insulin epitopes in human T1D. For example, the work by Kent et al. (28) identified CD4 T cells responding to DR4-presented epitopes from the insulin A-chain. Another recent study has described a series of CD4 T-cell clones isolated from the pancreas of a T1D patient that is reactive to C-peptide epitopes presented by DQ8 or DQ8/DQ2 heterodimers (29). Unfortunately, unlike in the case of NOD mouse, the tools are presently lacking to determine the relative importance of these different antigens in responses in the onset and progression of the disease in humans.

In conclusion, the data presented here show a modified insulin B-chain peptide capable of detecting robust autoreactive T-cell responses from the peripheral blood and suggest that T1D risk may be related to how an HLA-DQ genotype determines the balance of the T-cell inflammatory vs. regulatory responses to insulin. With the increasing incidence of T1D over the last two decades, especially in children less than 5 y of age (3), and the ability to predict diabetes development in individuals with two or more islet autoantibodies (4), diabetes prevention trials are underway (30). Antigen-specific therapy holds the potential for being able to safely and specifically induce tolerance to an administered self-antigen. The insulin B9–23 (B22E) mimotope has been used as antigen-specific therapy in the NOD mouse, in which small amounts of the mimotope are able to convert naïve T cells into Foxp3+ regulatory T cells and prevent diabetes onset (13). Therefore, because of its superagonist properties, the potential exists to use the insulin B22E mimotope as a tolerogenic vaccine in those at risk for T1D. In other studies, antibodies specific for the register 3 B9–23/IAα7 complex inhibit T-cell responses in vitro and delay T1D onset in vivo in NOD mice (31, 32), opening another potential avenue for antigen-specific therapy. Our results also raise the possibility that individuals with a β57D+ containing DQ allele may have the ability to produce a regulatory response to the insulin epitope. With the genetics of T1D shifting toward more new-onset patients with at least one of these alleles (33), insulin-specific immune therapies are an appealing approach for diabetes prevention, because there is now a robust means to assess proinflammatory and regulatory T-cell responses from the peripheral blood.
Materials and Methods

Subjects and Samples. Subjects were recruited from the Barbara Davis Center for Diabetes Clinics, and written informed consent was obtained after the nature and possible consequences of the study were explained to individuals. The clinical investigation in this study was conducted in accordance with the Declaration of Helsinki principles, with study approval provided by the Colorado Multiple Institutional Review Board. Peripheral blood was obtained for T-cell assays, islet autoantibodies, and HLA genotyping. Islet autoantibodies to insulin, GAD65, IA-2, and ZnT8 were measured from the serum by radioimmunoassay as previously described (34). HLA-DRB, DQA, and DQB genotyping was performed using linear arrays of immobilized sequence-specific oligonucleotides similar to previously described methodology (35).

Antigens. The insulin B9–23 and mimotope peptides listed in Fig. S18 were obtained from Genemed Synthesis Inc. at >95% purity. For a control antigen, we used Pentacel, a childhood vaccine containing five different immunogens, obtained from Sanofi Pasteur.

Human Indirect ELISPOT. ELISPOT analyses were conducted as previously described using the human IFN-γ ELISPOT Kit (UCyTech Biosciences) (29). Details are discussed in SI Materials and Methods.

CFSE Proliferation Assay. As described in SI Materials and Methods, PBMCs were isolated from whole blood, labeled with CFSE, and cultured with no antigen, Pentacel, the WT B9–23 peptide, or the B22E mimotope. After 7 days, the loss of CFSE from dividing CD4 T cells was determined by flow cytometry.

TCR Sequencing. We determined the TCRα sequences of sorted CFSE-labeled CD4 T cells from three of DQ2/DQ8 subjects (1, 2, and 4) listed in Table S2. TCRβ sequences were also determined for two of these subjects (2 and 4). For each subject, two sets of T cells were sorted: total unstimulated CD4 “CFSE” cells and CD4 “CFSE” cells after 1 week of in vitro stimulation with the B22E insulin mimotope. Total RNA was directly extracted from sorted cells using the RNeasy Mini Kit (Qiagen) for cells before proliferation and the PicoPure RNA Isolation Kit (Life Technologies) for those after proliferation. cDNA was prepared from each of the samples, and TCR Vβ and Vδ sequences obtained and analyzed as described in SI Materials and Methods.

Statistical Analysis. Total spot numbers from ELISPOT assays were analyzed with a nonparametric Mann–Whitney test (rank sum test). ELISPOT response rates to a given antigen were compared with a two-sided Fisher’s exact test. For CFSE proliferation assays, a Wilcoxon signed rank test compared samples from the same subject. For all statistical tests, a two-tailed P value of <0.05 is considered significant. Analyses were performed using GraphPad Prism 4.0 software.

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