

# Genetic-induced Variations in the GAD65 T-cell Repertoire Governs Efficacy of Anti-CD3/GAD65 Combination Therapy in New-onset Type 1 Diabetes

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To enhance efficacy of forthcoming type 1 diabetes (T1D) clinical trials, combination therapies (CTs) are envisaged. In this study, we showed that efficacy of a CT, using anti-CD3 antibody and glutamic acid decarboxylase of 65 kd (GAD65)-expressing plasmid, to reverse new-onset T1D was dependent upon the genetic background. Synergism between both treatments was only observed in the RIP-LCMV-GP but not in the nonobese diabetic (NOD) or RIP-LCMV-NOD models. Efficacy was associated with an expansion of bystander suppressor regulatory T cells (Tregs) recognizing the C-terminal region of GAD65 and secreting interleukin-10 (IL-10), transforming growth factor- $\beta$  (TGF- $\beta$ ), and interferon- $\gamma$  (IFN- $\gamma$ ). In addition, we found that frequency and epitope specificity of GAD65-reactive CD4<sup>+</sup> T cells during antigen priming at diabetes onset and Tregs detected after CT correlated. Consequently, NOD mice harbored significantly lower levels of GAD65-reactive CD4<sup>+</sup> T cells than RIP-LCMV-GP before and after treatment. Our results demonstrate that antigen-specific T cells available at treatment may differ between various major histocompatibility complex (MHC) and genetic backgrounds. These cells play a major role in shaping T-cell responses following antigen-specific immune intervention and determine whether a beneficial Tregs response is generated. Our findings hold important implications to understand and predict the success of antigen-based clinical trials, where responsiveness to immunotherapy might vary from patient to patient.

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## INTRODUCTION

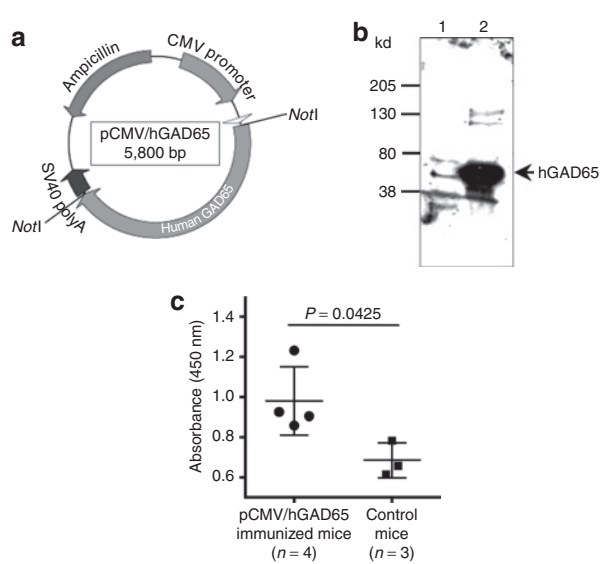
During pathogenesis of type 1 diabetes (T1D), the insulin-secreting  $\beta$  cells localized in the pancreatic islets of Langerhans are destroyed by an autoimmune attack.<sup>1</sup> To improve the efficiency of future clinical trials, a variety of combination therapies (CTs) are now being considered. The goal of CTs is to strengthen the

therapeutic response by targeting several pathways synergistically.<sup>2,3</sup> Expansion of islet-specific regulatory T cells (Tregs) will likely be the safest and most efficacious therapeutic option to establish long-term tolerance in diabetic patients. Vaccination using islet autoantigens (aAgs) can mediate protection from diabetes by expanding islet-specific Tregs.<sup>4,5</sup> This strategy is advantageous as it avoids general immunosuppression by acting site-specific within the pancreatic tissue and can dampen multiple autoaggressive responses by bystander suppression. Immunization with various islet aAgs has been shown to reverse T1D in animal models<sup>6–10</sup> and could preserve  $\beta$ -cell mass in humans.<sup>11–13</sup> Although glutamic acid decarboxylase of 65 kd (GAD65) is not considered to be the primary aAg in nonobese diabetic (NOD) mice and its precise role in human islets remains elusive,<sup>14–17</sup> GAD65-specific immunointerventions were efficacious to prevent T1D in mice,<sup>6,7,10,18–23</sup> but not in BioBreeding rats,<sup>24,25</sup> and provided initial encouraging results in recent-onset T1D in humans.<sup>11–13</sup> However, it is unclear whether the variability observed in the genetic background of patients with T1D might influence the presentation of GAD65 to the immune system and consequently affect the therapeutic efficacy. For instance, proliferative T-cell response to GAD65 was observed in ~50% of recent onset T1D patients and unexpectedly the majority of responders were HLA non-DR3/4 heterozygous patients.<sup>26</sup> Moreover, any antigen-specific intervention has at least the theoretical potential to exacerbate T1D.

Systemic anti-CD3 antibody therapy has the ability to permanently reverse new-onset T1D in mouse models,<sup>27,28</sup> when applied in humans a preservation of the  $\beta$ -cell function was seen for at least 2 years.<sup>29,30</sup> One of the mechanisms explaining this positive effect is the vigorous expansion of Tregs observed within few weeks after treatment.<sup>31–34</sup> We therefore reasoned that a CT employing anti-CD3 antibody and islet aAgs vaccination could invigorate *in vivo* expansion of islet-specific Tregs after new-onset T1D. Our previous studies showed that CT of anti-CD3 and proinsulin can indeed expand proinsulin-specific Tregs and increase protection from T1D into two animal models.<sup>34</sup>

Here, we studied the efficacy of low anti-CD3 antibody doses and GAD65-expressing DNA vaccine given alone or as a CT to reverse T1D. Synergy was evidenced with the RIP-LCMV-GP

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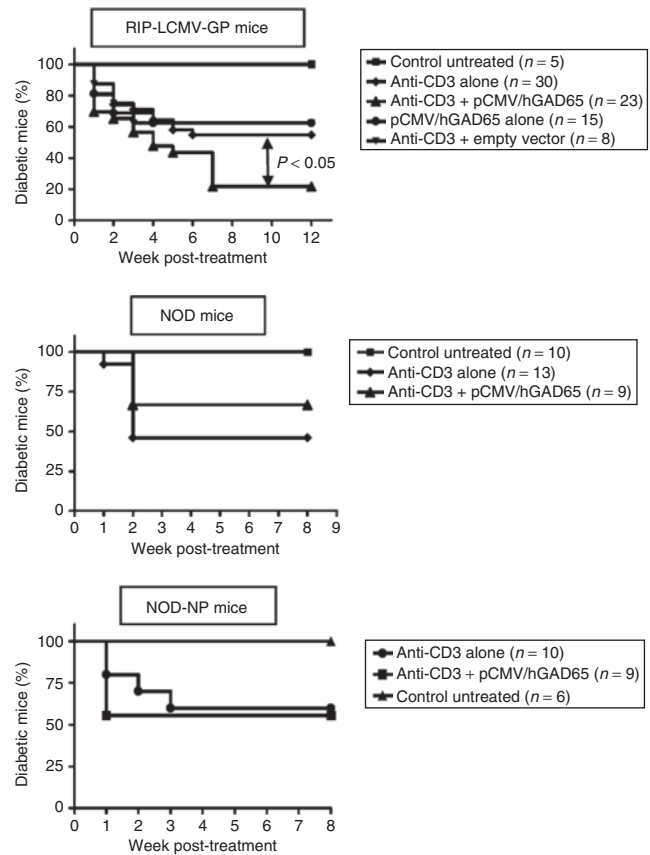
**Figure 1** Construction and functionality of human glutamic acid decarboxylase of 65kd (hGAD65)-expression plasmid (pCMV/hGAD65) used for vaccination. **(a)** The full sequence of the hGAD65 was cloned into a eukaryotic expression vector, downstream of the cytomegalovirus (CMV) promoter (pCMV/hGAD65). **(b)** Chinese hamster ovary (CHO) cells were transiently transfected with the plasmid and expression of hGAD65 was verified by western blotting. Total protein was extracted from nontransfected (lane 1) or pCMV/hGAD65-transfected (lane 2) CHO cells. Ten micrograms of each extract was loaded and run on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel. The presence of hGAD65 in the extract was detected using an anti-hGAD65-specific antibody. **(c)** C57BL/6 mice were immunized with the pCMV/hGAD65 DNA vaccine or left untreated. The data represent the titer of anti-hGAD65 antibodies in the serum of each individual mouse  $\pm$  SD.

but not with the NOD and NOD-NP mice. *Ex vivo* analysis revealed that efficacy in the RIP-LCMV-GP model was associated with an expansion of bystander suppressor Tregs recognizing the C-terminal region of GAD65 and secreting interleukin-10 (IL-10), transforming growth factor- $\beta$  (TGF- $\beta$ ), and interferon- $\gamma$  (IFN- $\gamma$ ). Analyze of GAD65-specific CD4<sup>+</sup> T-cell repertoire in both NOD and RIP-LCMV-GP mice revealed that frequency and epitope specificity at priming determine the fate of antigen-induced Tregs. All together, our data indicate that the therapeutic potential of anti-CD3 and GAD65 currently used in clinical trials for the treatment of new-onset T1D patients<sup>13,29,30</sup> can be increased when both molecules are combined. We showed that efficacy is driven by the expansion of GAD65-specific Tregs from the CD4<sup>+</sup> T-cell repertoire. The number and epitope specificity of these cells at treatment in RIP-LCMV-GP and NOD mice predicted Tregs expansion and consequently treatment efficacy.

## RESULTS

### Synergy of human GAD65–expressing plasmid with NM-anti-CD3 for reversing new-onset diabetes in RIP-LCMV-GP but not in NOD-LCMV-NP and NOD models

Functionality of the pCMV/hGAD65 DNA vaccine was verified *in vitro* by measuring the expression of human GAD65 (hGAD65) from Chinese hamster ovary cells transiently transfected and *in vivo* by assessing the anti-hGAD65 antibody titers from C57BL/6 mice



**Figure 2** Synergy between NM-anti-CD3 F(ab)<sup>2</sup> and human glutamic acid decarboxylase of 65kd (GAD65) vaccine administration enhances protection from diabetes in RIP-LCMV-GP but not in NOD and NOD-NP mice. Mice received various treatment regimens after recent-onset diabetes and their blood glucose was followed to assess any therapeutic effect. In the RIP-LCMV-GP model, the protective ability of the combination treatment (CT) reached 80% ( $n = 23$ ) as compared to 47% with anti-CD3 alone ( $n = 30$ ) or 40% with the GAD65 vaccine alone ( $n = 15$ ). At 12 weeks after treatment, the difference between the CT and the other groups was statistically significant using a Kaplan–Meier test ( $P < 0.05$ ). In the NOD and NOD-NP models, no synergism between anti-CD3 antibody therapy and GAD65 vaccination was observed. GP, glycoprotein; LCMV, lymphocytic choriomeningitis virus; NOD, non-obese diabetic; NP, nucleoprotein; RIP, rat insulin promoter.

immunized with pCMV/hGAD65 (Figure 1). Next, the ability of anti-CD3 and pCMV/hGAD65 CT to reverse new-onset T1D was studied in RIP-LCMV-GP (C57BL/6 background), NOD, and NOD-LCMV-NP mice (NOD background). RIP-LCMV-GP and NOD-LCMV-NP mice express the glycoprotein (GP) or nucleoprotein (NP) of the lymphocytic choriomeningitis virus (LCMV) specifically in the pancreatic  $\beta$  cells. Tolerance to GP and NP aAg is broken upon LCMV infection and T1D rapidly develops in both strains (1–2 weeks after infection). In contrast, NOD mice are genetically predisposed to T1D and disease spontaneously develops between 12 and 20 weeks of age in our NOD colony.

As shown in Figure 2, 80% of RIP-LCMV-GP mice treated with the CT were protected from T1D, as compared to ~45% protection observed when each therapy was administered independently. On the contrary, no synergism was observed for the NOD and NOD-NP mice, underlining that genetic backgrounds (NOD versus C57BL/6) rather than T1D models (spontaneous versus

**Table 1** Amino acid sequences and peptide pools of the human glutamic acid decarboxylase of 65 kd peptide library

Peptide no.	Amino acid sequence	Pool no.	Peptide no.	Amino acid sequence	Pool no.
1	MASPGSGFWSFGSEDGSGDS	1	31	LIKCDERGMIPSDLERRIL	6
2	FGSEDGSGDSENPGTARAWC	1	32	IPSDLERRILEAKQKGFVPPF	6
3	ENPGTARAWCQVAQKFTGGI	1	33	EAKQKGFVPPFLVSATAGTTV	7
4	QVAQKFTGGIGNKLCALLYG	1	34	LVSATAGTTVYGAFDPLLAV	7
5	GNKLCALLYGDAEKPAESGG	1	35	YGAFDPLLAVADICKKYKIW	7
6	DAEKPAESGGSQPPRAAARK	2	36	ADICKKYKIWMHVDAAWGGG	7
7	SQPPRAAARKAACACDQKPC	2	37	MHVDAAWGGGLLSRKHKWK	7
8	AACACDQKPCSCSKVDVNYA	2	38	LLMSRKHKWKLSGVERANSV	8
9	SCSKVDVNYAFLHATDLLPA	2	39	LSGVERANSVTWNPBKMMGV	8
10	FLHATDLLPACDGERPTLAF	2	40	TWNPBKMMGVPLQCSALLVR	8
11	CDGERPTLAFLQDVMNILLQ	2	41	PLQCSALLVREEGLMQNCNQ	8
12	LQDVMNILLQYVVKSFDRST	3	42	EEGLMQNCNQMHASYLFQQD	8
13	YVVKSFDRSTKVIDFHYPNE	3	43	MHASYLFQQDKHYDLSYDTG	9
14	KVIDFHYPNELLQEYNWELA	3	44	KHYDLSYDTGDKALQCGRHV	9
15	LLQEYNWELADQPQNLEEIL	3	45	DKALQCGRHVDVFKLWLMWR	9
16	DQPQNLEEILMHCQTTLKYA	3	46	DVFKLWLMWRAGTTGFEAH	9
17	MHCQTTLKYAIKTHGHPRYFN	4	47	AKGTTGFEAHVDKCLELAEY	9
18	IKTHGHPRYFNQLSTGLDMVG	4	48	VDKCLELAEYLYNIIKNREG	10
19	QLSTGLDMVGLAADWLTSTA	4	49	LYNIIKNREGYEMVFDGKPKQ	10
20	LAADWLTSTANTNMFTYEIA	4	50	YEMVFDGKPKQHTNVCFWYIP	10
21	NTNMFTYEIAPVFLLEYVT	4	51	HTNVCFWYIPPSLRTLEDNE	10
22	PVFLLEYVTLLKMKREIIGW	4	52	PSLRTLEDNEERMSRLSKVA	10
23	LKMKREIIGWPGGSGDGIFS	5	53	ERMSRLSKVAPVIKARMMMEY	11
24	PGGSGDGIFSPGGAISNMYA	5	54	PVIKARMMMEYGTMTVSYQPL	11
25	PGGAISNMYAMMIARFKMFP	5	55	GTTMTVSYQPLGDKVNFFRMV	11
26	MMIARFKMFPEVKEKGMAAL	5	56	GDKVNFFRMVISNPAATHQD	11
27	EVKEKGMAALPRLIAFTSEH	5	57	ISNPAATHQDIDFLIEEIER	11
28	PRLIAFTSEHSHFSLKKGAA	6	58	IDFLIEEIERLQGDL	11
29	SHFSLKKGAAALGIGTDSVI	6			
30	ALGIGTDSVILIKCDERGMK	6			

LCMV-induced T1D) are responsible for the protective immune responses.

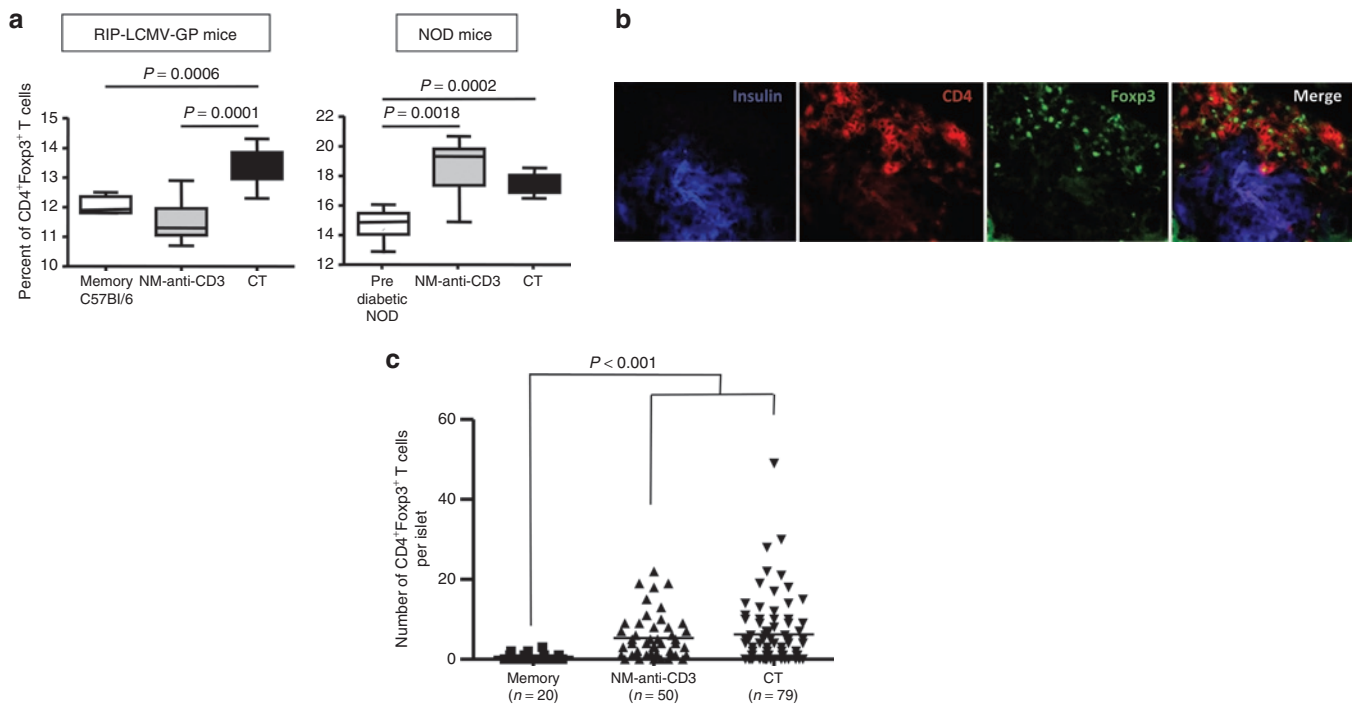
### CT expands GAD65-specific Tregs on the RIP-LCMV-GP C57BL/6 but not on the NOD background

Mechanistic experiments were carried out to address the question of whether CT would affect the number and specificity of Tregs. Four weeks after CT, the percentage of spleen- and pancreatic lymph node (PLN)-derived CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs significantly increased in both NOD and RIP-LCMV-GP models compared to nontreated animals (Figure 3a). However, when compared to NM-anti-CD3 therapy administered alone, the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs upon CT was only augmented in RIP-LCMV-GP mice (Figure 3a, left panel) correlating with the increased level of protection shown in Figure 1a. The increased number of Tregs after CT compared to NM-anti-CD3 alone was only visible in the PLNs and spleen but not in the pancreas itself as measured (Figure 3b,c). The total number of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs infiltrating the islet of Langerhans at this

time point did not significantly augment in the RIP-LCMV-GP model after NM-anti-CD3 or CT treatment. This observation supports the concept that regulation by Tregs occurs in the PLNs and maybe systemically and probably affects expansion of autoreactive CD8<sup>+</sup> T cells, rather than suppressing memory effector cells that have already reached the pancreatic islets.

### GAD65-specific Tregs expanded in the RIP-LCMV-GP model preferentially recognize the C-terminal region of hGAD65

To further characterize the specificity of GAD65-induced Tregs, splenocytes and PLNs from treated and protected mice were subjected to an *in vitro* proliferation assay with an hGAD65 peptide library covering the entire hGAD65 sequence (Table 1). Following NM-anti-CD3 therapy alone, RIP-LCMV-GP CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs strongly expanded when stimulated with the peptide pools 3, 10, and 11 (Figure 4a, right panel). When CT was used, additional amplification of Tregs was observed mainly with peptide pools 10



**Figure 3** Percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells increases after combination therapy (CT) only in the spleen and pancreatic lymph nodes but not in the pancreas of RIP-LCMV-GP mice. **(a)** The percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells in pooled splenocytes and pancreatic lymph node cells of protected RIP-LCMV-GP and NOD mice 4 weeks after therapy. In the left panel, memory C57BL/6 mice and protected RIP-LCMV-GP mice after NM-anti-CD3 alone (NM-anti-CD3) or CT were used. In the right panel, nontreated as well as anti-CD3 alone or CT-treated NOD were analyzed. The data represent an average of five to six mice per group. **(b)** Histological staining of pancreata from euglycemic RIP-LCMV-GP mice. Pancreata were harvested 4 weeks after therapy and 8- $\mu$ m tissue sections were cut and collected for immunohistochemistry. Sections were costained for insulin (blue), CD4 (red), and Foxp3 (green). **(c)** The number of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells surrounding or infiltrating the islets of Langerhans was quantified by microscopy in LCMV-infected C57BL/6 mice or RIP-LCMV-GP mice protected after NM-anti-CD3 or CT. GP, glycoprotein; LCMV, lymphocytic choriomeningitis virus; NOD, nonobese diabetic; RIP, rat insulin promoter.

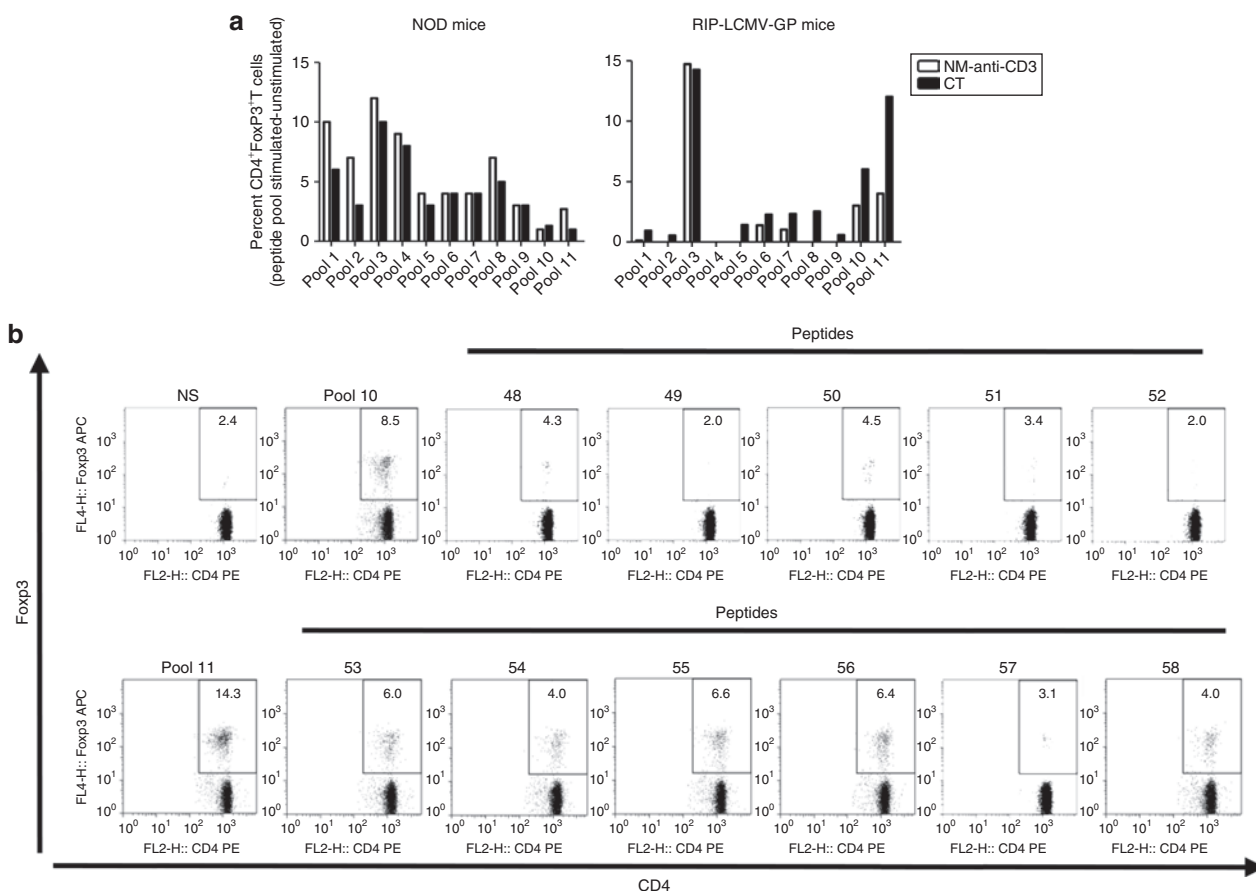
and 11 but also to a lesser extent with pools 5–8. The combined percentages of expanded Tregs reached 39% with the CT group but only 23% with the NM-anti-CD3 group. Therefore, the most robust amplification in RIP-LCMV-GP mice was observed with peptide pools covering the C-terminal region of hGAD65 following CT. Without stimulation Foxp3<sup>+</sup> Tregs represented 2.4% of the CD4<sup>+</sup> T cells in the RIP-LCMV-GP mice, whereas upon stimulation with pools 10 and 11, the percentage of GAD65-specific Tregs drastically increased to reach 8.5 and 14.3% of the CD4<sup>+</sup> T cells, respectively (Figure 4b). These GAD65-specific Tregs reacted primarily to peptides 53, 55, and 56 which encompassed the amino acid sequence 521–570 of hGAD65. In contrast, although NOD mice revealed strong Treg expansion after NM-anti-CD3 treatment for several peptide pools from the N-terminal hGAD65 sequence, no further increase was observed following CT with the hGAD65 DNA vaccine, which stands in striking contrast with the data obtained from RIP-LCMV-GP mice (Figure 4a,b). It is worth noting that peptide pool 3 encompasses GAD65-specific peptides which strongly expand Tregs after NM-anti-CD3 therapy in both animal models.

### Bystander control of diabetes by GAD65-specific Tregs

Adoptive transfer experiments were performed to examine the functionality of GAD65-specific Tregs. Purified CD4<sup>+</sup> T cells

from protected animals were transferred into prediabetic immunocompetent RIP-LCMV-GP recipient mice and diabetes development was monitored. We observed that only CD4<sup>+</sup> T cells isolated from donors protected by the CT were capable of mediating 50% protection from diabetes (blood glucose values <300 mg/dl) in recipients (Figure 5a,b, right panel). In contrast, the protection mediated by anti-CD3 alone was transferable in only 12.5% of recipient mice when CD4<sup>+</sup> T cells from anti-CD3-protected mice were injected into prediabetic RIP-LCMV-GP recipients. These data indicate that an insufficient amount of islet-specific Tregs is generated after NM-anti-CD3 alone to mediate infectious tolerance as previously observed in NOD and RIP-LCMV-GP models.<sup>28,35</sup> Moreover, the overall blood glucose values measured from recipient mice treated by infusion with CD4<sup>+</sup> T cells from the CT group never exceeded 550 mg/dl and showed an average of 350 mg/dl (Figure 5b). Collectively these experiments suggest that hGAD65-induced Tregs expanded upon CT are able to control autoimmunity and  $\beta$ -cell destruction but the rate of  $\beta$ -cell regeneration might determine whether an individual mouse can fully recover from diabetes and reach normoglycemia.

Next, Treg phenotype upon CT was further characterized. CD4<sup>+</sup>Foxp3<sup>+</sup> T cells coexpressed the markers CD25<sup>+</sup>, GITR<sup>+</sup>, CTLA-4<sup>+</sup>, and CD127<sup>low</sup> (Figure 5c, upper panels). Lastly, the frequency of hGAD65-specific IFN- $\gamma$ -, IL-4-, IL-10-, and TGF- $\beta$ -

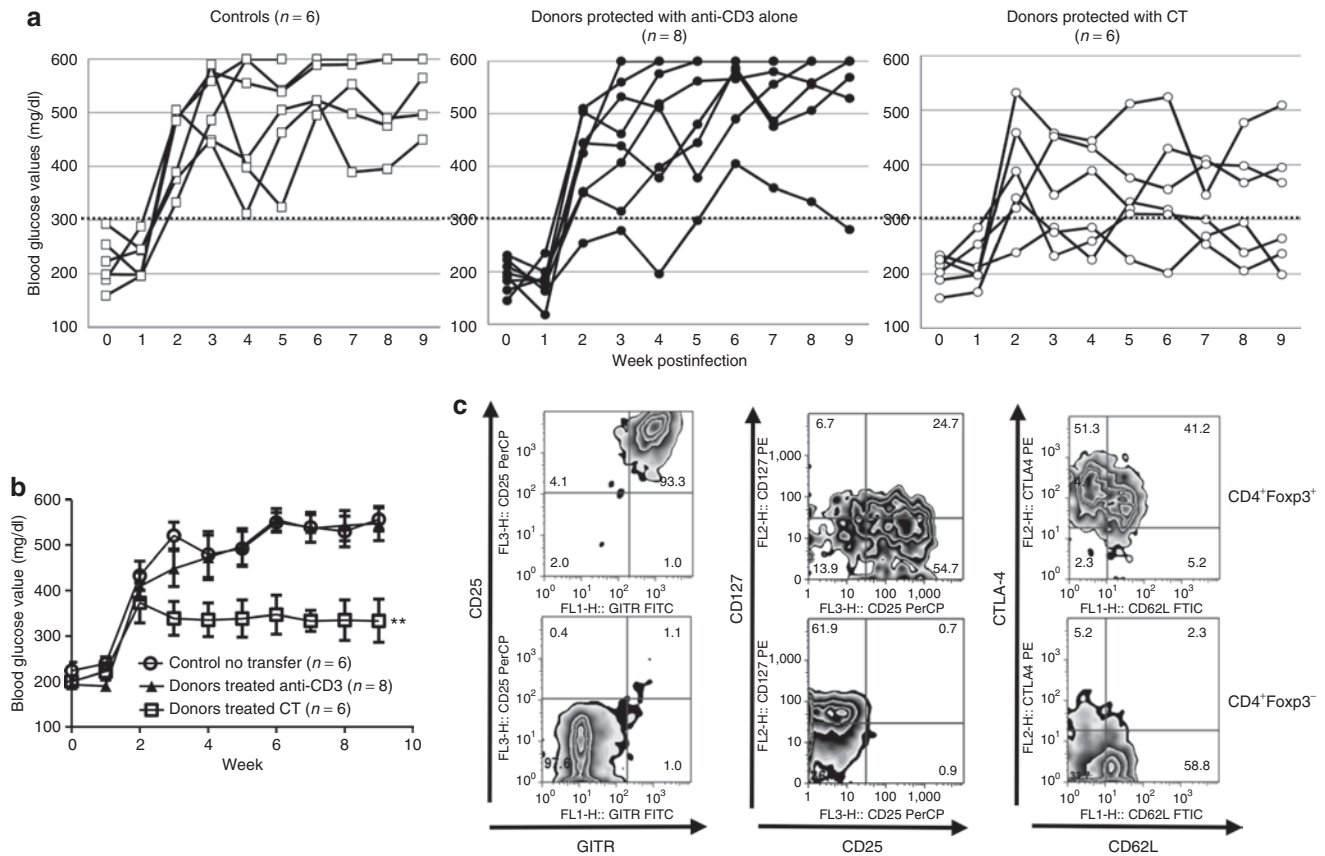


**Figure 4** Glutamic acid decarboxylase of 65kd (GAD65)-specific regulatory T cells (Tregs) expanded in the RIP-LCMV-GP mice after combination therapy (CT) primarily recognize the C-terminal region of human GAD65 (hGAD65). Splenocytes and pancreatic lymph node cells were derived from NOD- and RIP-LCMV-GP-protected mice 4 weeks after treatment with NM-anti-CD3 alone or in combination with pCMV/hGAD65 (CT). **(a)** Single-cell suspensions were stimulated *in vitro* with 11 pools of peptides derived from the primary amino acid sequence of hGAD65 and covering its entire sequence or were left unstimulated. On day 5 after stimulation, the number of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells (Tregs) was quantified by flow cytometry. The percent of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs expanded in RIP-LCMV-GP (right panel) or NOD (left panel) mice treated with NM-anti-CD3 (white bars) or CT (black bars) was calculated as follow: (percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells)<sub>peptide pool stimulation</sub> – (percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells)<sub>nonstimulated</sub>. **(b)** Splenocytes from RIP-LCMV-GP mice protected upon CT were stimulated with two pools of peptides (pools 10 and 11) or the single peptide from the pools 10 and 11 (covering the sequence 471–541 of hGAD65). On day 5 after stimulation, the percentage of Tregs was acquired by flow cytometry. Numbers shown in each histogram correspond to the percentage of Foxp3<sup>+</sup> in the CD4<sup>+</sup> T-cell population. Data are representative of four independent experiments. GP, glycoprotein; LCMV, lymphocytic choriomeningitis virus; NOD, nonobese diabetic; RIP, rat insulin promoter.

secreting CD4<sup>+</sup> T cells was measured from protected animals (Figure 6). Upon CT in the RIP-LCMV-GP mice, the number of IFN- $\gamma$ -, IL-10-, and TGF- $\beta$ -secreting CD4<sup>+</sup> T cells responding to hGAD65 significantly increased when compared to NM-anti-CD3 alone. The strong expansion of IFN- $\gamma$ -expressing GAD65-reactive CD4<sup>+</sup> T cells observed in RIP-LCMV-GP mice upon both NM-anti-CD3 treatment and CT may participate in long-term reversal of T1D by blocking IL-17-expressing effector T cells as previously observed in the NOD mice.<sup>36</sup> IL-4-secreting cells were also detected but not increased comparing CT- and NM-anti-CD3-treated RIP-LCMV-GP mice. In the NOD model, numbers of cytokine-expressing T cells were comparatively lower after *in vitro* stimulation with hGAD65 peptides and not significantly different between both treatment groups. However, the cells were not unresponsive to stimulation because after anti-CD3/anti-CD28 stimulation IL-4-, IL-10-, IFN- $\gamma$ -, and TGF- $\beta$ -producing cells strongly increased.

### Frequency and specificity of GAD65-reactive CD4<sup>+</sup> T cells at diabetes onset predict Tregs expansion after CT

Previous reports showed that antigen-specific T-cell frequency impacts T-cell response *in vivo*.<sup>37</sup> Therefore, one can argue that a significant difference in the frequency and/or specificity of GAD65-reactive T cells at treatment may directly influence the efficacy of GAD65-induced tolerance upon CT. To determine whether the size of uncommitted GAD65-reactive T-cell pool present at diabetes onset differed between RIP-LCMV-GP and NOD mice, newly diabetic mice were immunized with hGAD65 [200  $\mu$ g in incomplete Freund's adjuvant (IFA)] and tested for lymph node mononuclear cell recall responses. As shown in Figure 7a, GAD65-reactive T cells proliferated more robustly and in a dose-dependent manner in newly diabetic RIP-LCMV-GP than NOD mice. We concluded that the number of total uncommitted GAD65-specific CD4<sup>+</sup> T cells was significantly higher in



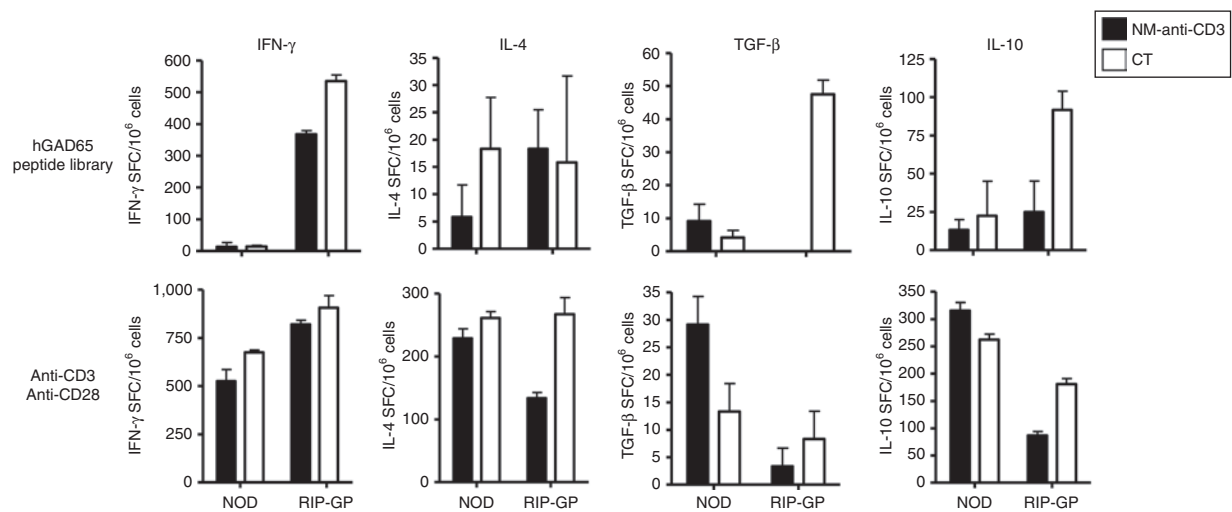
**Figure 5** Phenotype and functionality of glutamic acid decarboxylase of 65 kd (GAD65)-specific regulatory T cells (Tregs) in RIP-LCMV-GP mice. Adoptive transfer experiments were performed to assess the *in vivo* suppressive ability of GAD65-specific Tregs to prevent diabetes progression. CD8<sup>+</sup> T cell-depleted splenocytes from memory mice (control) and RIP-LCMV-GP mice protected upon treatment with NM-anti-CD3 alone or in combination with pCMV/hGAD65 (CT) were stimulated *in vitro* with human GAD65 (hGAD65) (10  $\mu$ g/ml). On day 6 after stimulation, CD4<sup>+</sup> T cells were purified and transferred into immunocompetent RIP-LCMV-GP mice (10<sup>6</sup> CD4<sup>+</sup> T cells/recipient) 5 days after LCMV infection. **(a)** The blood glucose values of each individual mouse were followed over a 9-week period in each group and **(b)** an average of these values is provided  $\pm$  SEM (\*\* $P < 0.05$ ). **(c)** Splenocytes obtained from RIP-LCMV-GP mice after combination therapy (CT) were stained for various regulatory and activation T-cell markers and analyzed by flow cytometry. The gates were set to detect CD4<sup>+</sup>Foxp3<sup>+</sup> (upper panel) or CD4<sup>+</sup>Foxp3<sup>-</sup> (lower panel) T cells. GP, glycoprotein; LCMV, lymphocytic choriomeningitis virus; NOD, nonobese diabetic; RIP, rat insulin promoter.

RIP-LCMV-GP animals at the time of antigen-specific therapy. Next, we addressed the question of whether frequency and specificity of uncommitted GAD65-reactive CD4<sup>+</sup> T cells differed between both backgrounds at the time of treatment. Newly diabetic RIP-LCMV-GP and NOD mice were immunized subcutaneously with the peptides (10  $\mu$ g/peptide in IFA) covering the entire sequence of hGAD65 (pools 1–11). Because the major histocompatibility complex (MHC) class II tetramers are not commercially available to track the GAD65-specific CD4<sup>+</sup> T-cell response, we developed an assay based on previously published studies.<sup>38,39</sup> Following *in vitro* stimulation with each peptide pools, the number of CD154-expressing CD4<sup>+</sup> T lymphocytes was assessed by flow cytometry. In this way, we were able to measure the number of GAD65-specific CD4<sup>+</sup> T cells that had been activated in response to *in vitro* stimulation with the GAD65 peptide pools. Consequently, this number reflects the quantity of GAD65-specific CD4<sup>+</sup> T cells activated in the mice after immunization with the same peptide pools. As shown in **Figure 7b,c**, the total number of GAD65-reactive CD4<sup>+</sup> T cells was three times higher in RIP-LCMV-GP than in the NOD mice (19,364 versus

6,444 CD4<sup>+</sup>CD154<sup>+</sup> T cells per 10<sup>6</sup> CD4<sup>+</sup> T cells). Moreover, the epitope specificity of GAD65-reactive CD4<sup>+</sup> T-cell repertoire resembles the specificity of GAD65-specific CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs expanded after CT in the RIP-LCMV-GP mice (**Figure 4a**, right panel). In particular, the number of CD4<sup>+</sup> T cells reacting against the GAD65 peptide pools 10 and 11 before treatment was among the highest observed at onset. Overall, the reactivity of GAD65-specific CD4<sup>+</sup> T-cell repertoire was shifted toward the C-terminal region in the RIP-LCMV-GP mice as observed for CD4<sup>+</sup> Tregs after CT (**Figure 4a**, right panel).

## DISCUSSION

In this article we demonstrate for the first time that hGAD65 treatment of new-onset T1D may be significantly increased by combination with low dose NM-anti-CD3, but that efficacy is dependent upon the genetic background that determines the magnitude and quality of the GAD65 Tregs response. We showed that protection was enhanced upon CT as compared to monotherapies on the C57BL/6 (RIP-LCMV-GP) but not on the NOD (NOD-LCMV-NP or regular NOD) background. Increased



**Figure 6** Glutamic acid decarboxylase of 65 kd (GAD65)-specific CD4<sup>+</sup> T cells express transforming growth factor- $\beta$  (TGF- $\beta$ ) and interleukin-10 (IL-10) regulatory cytokines and also interferon- $\gamma$  (IFN- $\gamma$ ). An enzyme-linked immunosorbent spot assay was established to assess the number of IFN- $\gamma$ , IL-4, IL-10-, and TGF- $\beta$ -producing GAD65-specific CD4<sup>+</sup> T cells. CD8-depleted splenocytes recovered from NOD and RIP-LCMV-GP mice protected by NM-anti-CD3 (black bars) or combination therapy (white bars) were stimulated *in vitro* either by (i) each GAD65-derived peptide pools separately (the number of spots obtained with each peptide pool was added to obtain the total number of spots shown on the figure), (ii) a mixture of anti-CD3/anti-CD28 antibodies, or (iii) not stimulated. The spots were counted 2 or 4 days after stimulation for anti-CD3/anti-CD28 or the peptide pools, respectively. The data correspond to an average of triplicate wells subtracted from the background values obtained with media alone  $\pm$  SD. They are representative of two independent experiments with six to seven mice per group. GP, glycoprotein; LCMV, lymphocytic choriomeningitis virus; NOD, nonobese diabetic; RIP, rat insulin promoter.

efficacy in the RIP-LCMV-GP model was associated with an expansion of hGAD65-specific Tregs specific for epitopes located within the C-terminal region of GAD65. Immunodominance was associated with peptide pools 3, 8, 10, and 11. Of interest, pools 8 and 11 contain two GAD65 epitopes (p27 398-430 and p35 524-543) previously described as immunodominant in newly diagnosed patients.<sup>40</sup> Furthermore, it has been reported that HLA-A2 transgenic mice immunized with a GAD65-expressing plasmid induced GAD(114-123)- and GAD(536-545)-specific T cells.<sup>41</sup> These two sequences are found within the peptide pools 3 and 11, respectively. On the NOD background, GAD65-specific Tregs expanded upon NM-anti-CD3 therapy were directed toward the N-terminal region of GAD65 (pools 1–4). As previously reported by the McDevitt's *et al.*,<sup>42</sup> these peptide pools contain the epitopes with the strongest binding capacity to the I-Ag7 MHC class II molecule.<sup>42</sup> Importantly, GAD65-specific Tregs were not further expanded when NOD mice were treated with the CT (**Figure 4a**).

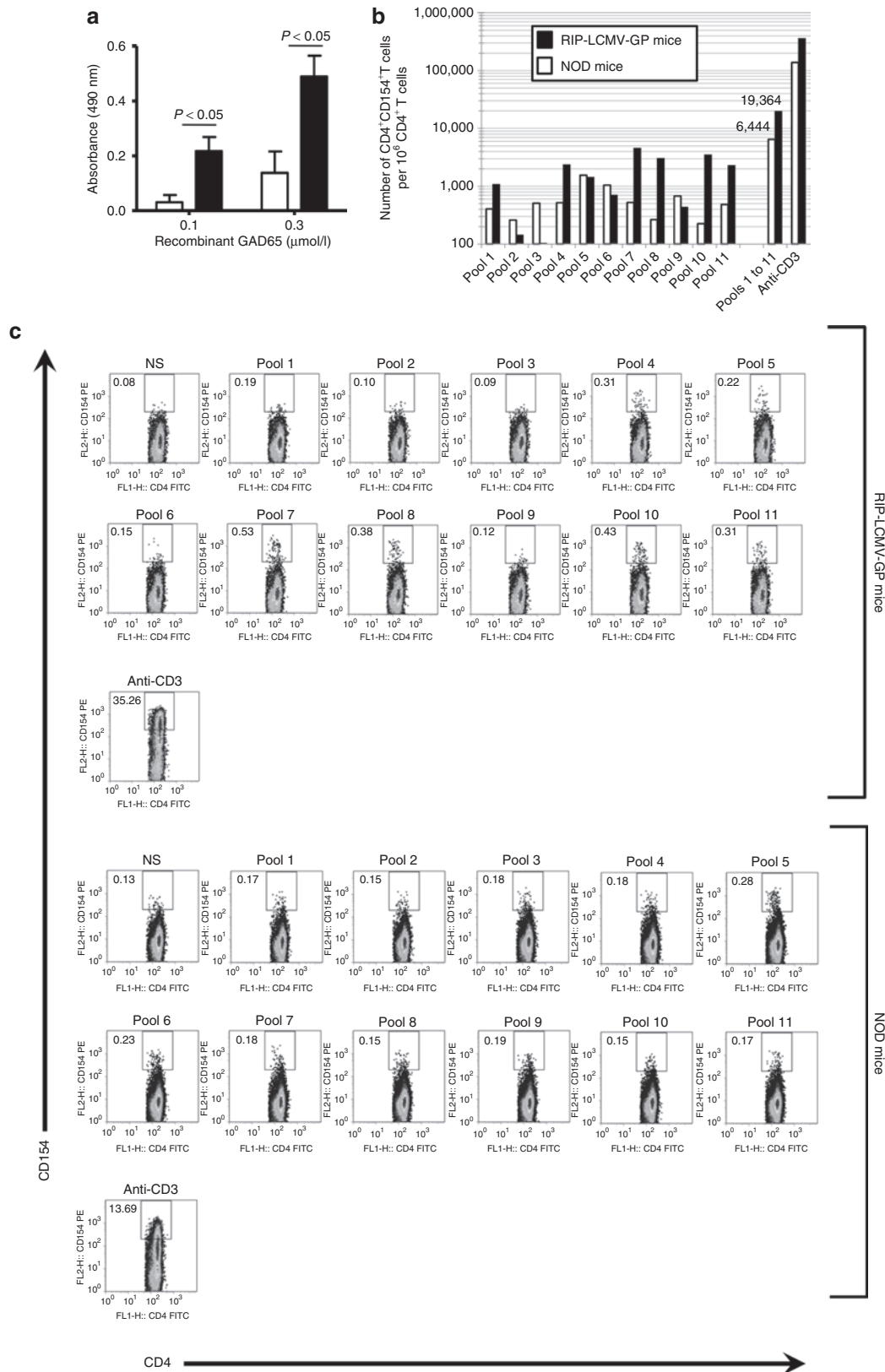
As previously shown, intramuscular DNA vaccination of NOD mice with a GAD65-expressing plasmid alone had only a minimal effect on GAD65-specific CD4<sup>+</sup> T-cell reactivity and disease progression.<sup>7</sup> Gene gun delivery of antigen-expressing DNA vaccine could ameliorate the clinical outcome by enhancing *in vivo* DNA transfection into resident antigen-presenting cells which produce high systemic levels of antigen, and by promoting type 2 CD4<sup>+</sup> T-cell responses.<sup>23</sup> Another work by Pop *et al.*<sup>18</sup> showed that syngeneic islet-graft survival, implanted into diabetic NOD mice, is only prolonged when GAD65-expressing vaccine was administered in conjunction with systemic IL-4 and IL-10 cytokine expressions. This again shows that CTs are often more effective to sustain long-term tolerance. They also suggested that tolerance toward islet grafts was governed by the type and frequency of GAD65-specific CD4<sup>+</sup> Tregs expanded after treatment.

Therefore, another major issue with antigen-specific tolerance is whether antigen-specific T cells need to be present at the time of antigenic administration to expand into Tregs or whether they can be produced/induced during the course of immunization. This is particularly relevant in humans where the diversity of HLA molecules can affect both the antigenic specificity of the T-cell repertoire and the ability to mount a T-cell response toward a particular antigen. T1D patients harbor a panel of HLA/MHC molecules<sup>43,44</sup> which was suggested to affect therapeutic outcome.<sup>45</sup> On the contrary, most preclinical studies using aAgs to induce tolerance were performed in a single animal model for T1D presenting a single MHC background. Thus, an important question is whether antigen-specific T cells capable of becoming Tregs in the context of various MHC molecules play a role in antigen-induced tolerance *in vivo*.

Here, we observed at diabetes onset a higher frequency of GAD65-reactive CD4<sup>+</sup> T cells in the RIP-LCMV-GP than in the NOD mouse model. This increased number of available T cells correlated with a stronger expansion of GAD65-specific Tregs after CT and consequently with the degree of efficacy after treatment. A global analysis of epitope specificity revealed continuity between the epitopes recognized by GAD65-reactive CD4<sup>+</sup> T cells before treatment and by GAD65-specific CD4<sup>+</sup> Tregs expanded after treatment. Even though CD4<sup>+</sup> T-cell reactivity was observed with most GAD65 peptide pools, the frequency of CD4<sup>+</sup> T cells recognizing the C-terminal region of GAD65 was higher in RIP-LCMV-GP than in NOD mice (**Figure 7b**) similarly to what we observed after CT for CD4<sup>+</sup> Tregs (**Figure 4a**). It is worth noting that systemic administration of anti-CD3 antibody could lead to a forceful expansion of GAD65-specific Tregs even when the frequency of uncommitted GAD65-reactive T cells was low. This was particularly evidenced by CD4<sup>+</sup> T cells specific for the peptide pool 3 (**Figures 4a** and **7b**).

In conclusion, we provide here important mechanistic insights into understanding the differential efficacy observed for GAD65-specific immunotherapy tested in two animal models on two different genetic backgrounds. Our data suggest that

variability in the genetic background (here comparing NOD and C57BL/6 mice) rather than the animal model (RIP-LCMV versus NOD) determines the number and specificity of the GAD65-specific Tregs repertoire available for priming by antigen-based



interventions and consequently affects the therapeutic efficacy. These observations highlight the need to use more than one genetic (and MHC) background to evaluate the therapeutic potential of antigen-based therapies. Our data show that even in conjunction with NM-anti-CD3 antibody (inducing potent Treg proliferation *in vivo*), antigen-specific Tregs cannot be expanded by CT if the antigen is not administered to an individual with suitable responsive immune system. To improve the efficacy of such interventions, one could envision the preparation of several customized CTs, tailor-made to fit with the patient's disease status. Individual immune monitoring will be needed to achieve this. Our findings may help design more effective therapies to expand antigen-specific Tregs *in vivo*, when administered after new-onset for the treatment of autoimmune disorders, if individual immune monitoring were to be implemented.

## MATERIALS AND METHODS

**Mice and virus.** H-2<sup>b</sup> RIP-LCMV-GP (C57BL/6) transgenic mice have been previously described.<sup>46</sup> LCMV strain Armstrong (Arm) was used to trigger diabetes in this model by infecting 8- to 12-week-old RIP-LCMV-GP mice with a single intraperitoneal dose of 10<sup>4</sup> plaque forming units. NOD/LtJ mice were purchased from Jackson Laboratory (Bar Harbor, ME). NOD-NP mice were previously described.<sup>47</sup> Diabetes was defined as two consecutive blood glucose values >250 or 300 mg/dl in NOD or RIP-LCMV-GP models, respectively. This study was approved by the La Jolla Institute for Allergy and Immunology Animal Care and Use Committee.

**Treatments.** After new-onset diabetes, ~50% protection was obtained by intravenous injection with suboptimal doses of non-Fc binding anti-CD3ε F(ab')<sub>2</sub> (NM-anti-CD3) obtained from BioExpress (West Lebanon, NH). Suboptimal doses of 40 μg/day were given for four or five consecutive days to RIP-LCMV-GP or NOD (and NOD-NP) mice, respectively. NM-anti-CD3 was given alone or in combination with an hGAD65-expressing plasmid (pCMV/hGAD65). Endotoxin-free pCMV/hGAD65 plasmid (prepared with the EndoFree plasmid giga kit; Qiagen, Valencia, CA) of 0.5 mg/ml was administered intramuscularly on days 0, 4, and 11 after recent-onset (100 μg/leg/injection). Control groups were treated using the same regimens with the pCMV/hGAD65 alone, with NM-anti-CD3 in combination with an empty pCMV plasmid, or remained untreated.

**Immunohistofluorescence.** Snap-frozen sections of pancreas were stained for insulin, CD4, and Foxp3. Day 0, primary antibodies biotin anti-CD4 RM4.5 from BD Biosciences (San Jose, CA; 4 μg/ml) and FITC-anti-Foxp3 (clone FJK-16s) from eBioscience (San Diego, CA; 2 μg/ml) were used in phosphate-buffered saline–3% bovine serum albumin overnight at 4 °C. Day 1, CD4 and Foxp3 were revealed, respectively, by using Alexa Fluor 594-conjugated streptavidin and anti-fluorescein goat IgG fraction Alexa Fluor 488-conjugated kit (Molecular Probes, Carlsbad, CA). After fixation (2% paraformaldehyde), insulin was detected with guinea pig

anti-swine insulin (DakoCytomation, Glostrup, Denmark) followed by Cy5-conjugated donkey anti-guinea-pig (Jackson ImmunoResearch).

**In vitro expansion of GAD65-specific Tregs.** Splenocytes and PLN cells from protected animals were cultured in complete RPMI 1640 medium. An hGAD65 peptide PEPscreen library of 20-mer peptides overlapped by 10 amino acid residues from ProImmune (Oxford, UK) was used for *in vitro* stimulations (5 days) with pooled peptides (Table 1) containing 10 μg/ml of each peptide. The percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells was quantified by fluorescence-activated cell sorting analysis.

**Enzyme-linked immunosorbent spot assay.** Pooled splenocytes and PLN cells from memory, anti-CD3 alone, or CT protected or diabetic mice were used. For some experiments, CD4<sup>+</sup> or CD8a<sup>+</sup> T cells were depleted by positive selection using CD4<sup>+</sup> or CD8a<sup>+</sup> isolation kits, respectively, from Miltenyi Biotec (Auburn, CA). Enzyme-linked immunosorbent spot was performed using the mouse IL-4, IL-10, and IFN-γ enzyme-linked immunosorbent spot pairs from BD Biosciences or the recombinant mouse TGF-β RII/mouse Fc chimera and polyclonal biotinylated anti-TGF-β1 pair purchased from R&D Systems (Minneapolis, MN).

**Adoptive transfers.** Pooled splenocytes and PLN cells from protected or untreated LCMV memory C57BL/6 mice (control) were stimulated *in vitro* with peptide pools 10 and 11 (10 μg/ml/peptide for 5 days). CD4<sup>+</sup> T cells were purified and transferred into immunocompetent male recipient RIP-LCMV-GP mice day 5 after LCMV infection (10<sup>6</sup> CD4<sup>+</sup> T cells/recipient).

**T-cell proliferation assays.** Newly diabetic NOD and RIP-LCMV-GP mice were immunized at the base of the tail with 100 μg of recombinant hGAD65 in IFA (Invitrogen Life Technologies, Carlsbad, CA). The mice were maintained euglycemic using an insulin pellet injected under the skin (LinShin, Toronto, Ontario, Canada). Nine days later, lymph node mononuclear cells were plated in 96-well plates at 3 × 10<sup>5</sup> cells/well in HL-1 (NOD) or RPMI 1640 (RIP-LCMV-GP) medium supplemented with antibiotics and 1% fetal calf serum, in the presence of hGAD65 (dose range: 0.1–0.3 μmol/l). Medium alone was used as the negative control. Proliferation after 96 hours of culture was measured using the cell proliferation kit II (XTT) (Roche Applied Science, Indianapolis, IN).

**Frequency and epitope specificity of uncommitted GAD65-reactive T cells.** To maintain normoglycemia and avoid major side effects due to high blood glucose levels, a small pellet releasing insulin was implanted under the skin of each mouse as recommended by the manufacturer (LinBit pellets, LinShin). Newly diabetic RIP-LCMV-GP and NOD mice were immunized subcutaneously with the pools of peptides covering the entire sequence of hGAD65 (10 μg/peptide) emulsified in IFA. Nine days later, cells were recovered from the draining lymph nodes and used in a recall response experiment to determine the number and specificity of uncommitted GAD65-reactive T cells. The protocol was adapted from the method previously described<sup>38,39</sup> to track CD154 expression on activated T cell upon antigen-specific stimulation.

**Figure 7** Frequency and specificity of uncommitted glutamic acid decarboxylase of 65 kd (GAD65)-reactive CD4<sup>+</sup> T cells in RIP-LCMV-GP and NOD mice. **(a)** Newly diabetic RIP-LCMV-GP and NOD mice were immunized with 100 μg of recombinant human GAD65 (rhGAD65) emulsified in incomplete Freund's adjuvant (IFA) at the base of the tail. Nine days later, cells recovered from the draining lymph nodes were tested for recall responses after 96 hours of stimulation over a dose range of rhGAD65. Proliferation was measured using the XTT dye after 12 hours of incubation (as described in the Materials and Methods section). Data shown are the mean absorbance at 490 nm ± SD of duplicate wells subtracted from the background values obtained with media alone (*n* = 4–5 mice/group). **(b,c)** Newly diabetic RIP-LCMV-GP and NOD mice were immunized subcutaneously with a mix of the hGAD65 peptide pools covering the whole sequence of hGAD65 (10 μg/peptide) emulsified in IFA. Nine days later, cells were recovered from the draining lymph nodes and used in a recall response experiment to determine the number and specificity of GAD65-reactive T cells. The number of CD4<sup>+</sup> T cells responding to each pool of peptides covering the entire sequence of hGAD65 was measured by flow cytometry. After a short-term recall response with each peptide pool or anti-CD3 antibody, the number of CD154-expressing CD4<sup>+</sup> T cells upon stimulation (corresponding to the uncommitted antigen-specific CD4<sup>+</sup> T-cell repertoire) was enumerated by flow cytometry **(c)**. The data **(b)** are representative of seven mice per group and show the number of CD4<sup>+</sup>CD154<sup>+</sup> T cells per 10<sup>6</sup> CD4<sup>+</sup> T cells. The total number of hGAD65-specific CD4<sup>+</sup>CD154<sup>+</sup> T cells for each animal model is given (pools 1–11). GP, glycoprotein; LCMV, lymphocytic choriomeningitis virus; NOD, nonobese diabetic; RIP, rat insulin promoter.

**Statistical analysis.** Data analysis was performed using GraphPad Prism, version 4 (GraphPad Software, San Diego, CA). In scatter plots, the horizontal bar represents the median of each data set. Significance was determined using the Kruskal–Wallis test. For survival analysis, survival curves were computed using the method of Kaplan–Meier. For other experiments, significance was determined using a two-tailed Student's *t*-test. A *P* value of <0.05 was considered statistically significant.

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