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*J Immunol* 2010; 185:4590-4601; Prepublished online 22 September 2010; doi: 10.4049/jimmunol.0803994

http://www.jimmunol.org/content/185/8/4590

Supplementary Material

http://www.jimmunol.org/content/suppl/2010/09/20/jimmunol.0803994.DC1

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Induction of Homologous Rather than Heterologous Antigen-Specific CD4 T Cell Responses Is Critical for Functional CD8 T Cell Responses in Mice Transgenic for a Foreign Antigen

Nicolas Sabarth, Louise Chamberlain, Sara Brett, John Tite, and Jenny Craigen

The development of a successful cancer vaccine requires the ability to break immunological tolerance to self-Ags expressed on tumor-cells. The transgenic rat insulin promoter (RIP) OVA\textsuperscript{LOW} mouse model has been reported to be hyporesponsive for both OVA-specific CD4 and CD8 T cell responses. The experiments described in the current study show that this hyporesponsiveness can be overcome by inclusion of GM-CSF and the TLR7 agonist imiquimod as adjuvants in a DNA immunization regimen with OVA-encoding plasmids. High frequencies of OVA-specific CD8 and CD4 T cells, including a response to a CD4 T cell epitope seen only in the RIP OVA\textsuperscript{LOW} mice, were generated by this regimen. These responses were associated with the development of autoimmunity and increased protection to tumor challenge in the RIP OVA\textsuperscript{LOW} mice. Heterologous CD4 T cell help has been shown to improve functional CD8 T cell responses, and we confirmed that inclusion of the induction of the CD4 T cell epitope pan HLA-DR–binding epitope improved CD8 T cell responses compared with self-Ag alone. Addition of GM-CSF and imiquimod, however, resulted in dominance of the pan HLA-DR–binding epitope-specific response over the OVA-specific CD4 T cell responses, decreased OVA-specific CD8 T cell numbers and function in tolerant RIP OVA\textsuperscript{LOW} mice, and failure to induce diabetes. The results of this study suggest that the use of heterologous help needs to be evaluated carefully in the context of specific immunization regimes and that a preferable approach may be adjuvantization of DNA vaccines. The Journal of Immunology, 2010, 185: 4590–4601.

Most tumor Ags are self-Ags and therefore not recognized by the immune system due to the development of Ag-specific tolerance. However, many Ags differ in their expression level on tumor and normal cells, which may allow selective targeting of tumor cells using cancer vaccines to develop a specific response against tumor cells with minimal damage to normal cells. DNA vaccination may be a useful technology for delivery of therapeutic cancer vaccines, as it induces CD4 and CD8 T cell responses as well as Ab responses. It has the advantage over viral vector approaches, which can also induce such responses, in that it can be used for multiple immunizations without the development of antivector immune responses. Although DNA vaccination has shown to be extremely potent in rodents, unfortunately this has not translated into humans. Multiple approaches are currently being developed to improve the potency of DNA vaccination, including coexpression of cytokines and dendritic cell (DC) stimulatory factors as adjuvants.
there is little information available regarding the possibility of competition or immunodominance of foreign epitopes over endogenous CD4 T cell epitopes. Immunodominance has been reported frequently in the CD8 T cell compartment but rarely in the CD4 T cell compartment. An understanding of the dynamics of the CD4 T cell compartment in the presence of adjacents is an important factor in vaccine design.

The primary challenge for cancer vaccines is overcoming immunological tolerance. The transgenic rat insulin promoter (RIP) OVA mouse represents a well-characterized model of immunological tolerance (21). RIP OVA\textsuperscript{LOW} mice are engineered to express a secreted form of OVA in insulin-producing \beta cells of the pancreas and are therefore tolerant to this molecule (22). Methods utilizing adoptive transfer of OVA-specific CTL result in autoimmune destruction of the \beta cells and development of diabetes, which is therefore a measure of the induction of a functional immune response. In the current study, the RIP OVA\textsuperscript{LOW} mouse model was chosen as a model for tolerance breaking, with responses to OVA representing potential responses toward tumor Ags. The adjuvant combination of GM-CSF and IM was evaluated as an useful component of a cancer vaccine in which the strong CD4 T cell response led to induction of effective CD8 T cell responses and functional tolerance breaking. In addition, it was hypothesized that the use of these adjuvants in combination with a strong foreign Th epitope may add to the efficacy of the vaccine by inducing higher responses or achieving responses earlier in the immunization regimen. The effect of the adjuvant on tolerance breaking was therefore tested using constructs encoding OVA alone or encoding the PADRE epitope as a fusion in the same construct.

Materials and Methods

Mice

C57BL/6 mice were purchased from Charles River Laboratories (Margate, U.K.). RIP OVA\textsuperscript{LOW} mice were obtained from Dr. Brocker (Institute for Immunology, Ludwig-Maximilians-Universität, Munich, Germany) with the kind permission of Dr. Frank Carbone (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) and bred and maintained under specific pathogen-free conditions in the animal facilities of GlaxoSmithKline in Frythe, U.K. All experiments were conducted in accordance with United Kingdom animal experiment legislation. Urine glucose was monitored with urine test strips (Diastix, Bayer, Berkshire, U.K.) three times a week. A glucose level >14 mM was considered to be diabetic. The upper limit of detection was 110 mM.

DNA constructs

The p7313Empty (empty vector), p7313OVAcyt, and p7313GM-CSF plasmids were supplied by Peter Ertl, Neil Burden, and Volker Germaeschwski (GlaxoSmithKline). p7313Empty is designed to express an Ag in vivo under the control of a truncated human CMV immediate early promoter, containing CMV exon 1 as a 5' untranslated leader sequence. The Ag encoding sequence to is followed by a rabbit \(\beta\)-globin polyadenylation sequence. To allow propagation in \textit{Escherichia coli}, the backbone also contains a \(\beta\)C19-derived origin of replication, a ColE1-derived cer sequence to reduce plasmid multimerization, and a kanamycin resistance cassette TN903. The p7313OVAcyt plasmid expresses a deletion variant of chicken OVA, in which the secretion signal containing aa sequence 20–145 was removed, resulting in an OVA gene largely confined to the cytoplasm. The truncated OVA gene of p7313OVAcyt was derived from pVAC1. OVA and GM-CSF-CSF were constructed by synthesizing the genes using PCR and overlapping oligonucleotides and cloning them in p7313. The plasmid pOVA-P was generated by Paul Hamblin (GlaxoSmithKline). It is derived from pVAC1.OVA.(10) with the aa residues 320–340 of the OVA replaced by the synthetic heterologous Th cell PADRE (AKF-VAAWTLKAA). All plasmids were prepared for immunization using Endofree Maxiprep kits (Qiagen, West Sussex, U.K.). Coexpression of OVA and GM-CSF does not influence the expression of each plasmid in vitro (Volker Germaeschwski, personal communication, data not shown). The immunogenicity of truncated OVA was equivalent in the plasmid backbones p7313 and pVAC1 (data not shown).

Cartridge preparation for particle-mediated epidermal delivery and immunizations

Recipient mice were immunized by particle-mediated epidermal delivery (PMED) of DNA at 400 \(\mu\)g of helium pressure using the PowderJect gene gun (PowderJect Vaccines, Madison, WI). Two shots on the shaved abdomen typically delivered 1.0 \(\mu\)g DNA coated onto 2-\(\mu\)m gold particles and loaded on Tefzel cartridges as described previously (23). When IM was administered, it was applied topically to the site of immunization in the form of Aldara (Graceway Pharmaceuticals, Bristol, TN) cream (40 \(\mu\)g/mouse) 24 h after PMED unless otherwise stated. Mice were immunized according to the experimental protocol one to four times in intervals of 3 wk.

Tumor protection assay

Seven days after the second or fourth immunization, \(1 \times 10^8\) OVA-expressing EG.7 cells in 100 \(\mu\)l PBS were injected s.c. on the shaven ventral site of each mouse. Perpendicular tumor diameters were measured daily until one diameter exceeded 15 mm. Tumor volume was calculated using the formula \(V = 0.4 \times L \times W^2\), with \(V\) as volume, \(L\) as the longer, and \(W\) as the shorter diameter of the tumor.

Intracellular cytokine staining, ELISPOT, and flow cytometric analysis

Unless otherwise stated, 7 d after the last boost, a single-cell suspension of splenocytes was prepared, and the cytokine production was monitored as described previously (10) either by ELISPOT or by intracellular cytokine staining (ICS) followed by flow cytometry. In brief, for ELISPOT analysis, RBCs were lysed and remaining splenocytes aliquoted into 96-well filter plates previously coated with IFN-\(\gamma\) capture Ab. Cells were restimulated with either 50 nM CD8 T cell-restricted peptide SINIFELK (OVAs255–263), with 10 \(\mu\)M CD4 T cell-restricted peptide TETWSSNV-MEEKIKV (OVAs266–274) or with 10 \(\mu\)M other peptides of a OVA peptide library of 15-mers, overlapping in 11 aa. Cytokine secretion was visualized using biotinylated detection Ab, followed by streptavidin-alkaline phosphatase and adequate substrate. Individual spots were counted with an ELISPOT reader (Autoimmun Diagnostika, Strassberg, Germany). For flow cytometry of intracellular cytokines, splenocytes were stimulated with anti-CD28 and anti-CD94/95 Abs and the peptides SIINFEKL (50 nM), TETWSSNV-MEEKIKV (10 \(\mu\)M), EEKYNLTSVLM (OVAs289–299, 10 \(\mu\)M), or AKFVAAWTLKAA (PADRE, 10 \(\mu\)M), indicated peptides of an OVA peptide library (10 \(\mu\)M) or OVA protein (1 mg/ml). Addition of 10 \(\mu\)g/ml brefeldin after 1 h retained the cytokines in the cytoplasm. After overnight incubation, cells were stained with anti-CD4-PerCP and anti-CD8-allophycocyanin and postfixation and permeabilization (Caltag Laboratories, Burlingame, CA) stained with anti–IFN-\(\gamma\)-PE and anti-IL-2-FITC. Samples were analyzed on a BD FACSCalibur (BD Biosciences, San Jose, CA) using a four-color protocol.

Tetramer staining

Tetramer analysis was performed as described previously (10). In brief, blood samples were taken 7 or 14 d after each immunization and red cells lysed using a whole blood lysing kit (Beckman Coulter, Fullerton, CA). Remaining cells were incubated at room temperature with SIINFEKL-H\(2K\)-PE–conjugated tetramers for 20 min followed by anti-CD8-CyChrome for 10 min. Washed cells were analyzed on a BD FACSCalibur (BD Biosciences).

Statistics

The log-rank test using the software Statistica was applied to calculate the significance of group differences and was conducted by using the software GraphPad Prism (GraphPad, San Diego, CA).

Results

GM-CSF/IM enhances T cell responses and breaks tolerance in RIP OVA\textsuperscript{LOW} mice

To test the efficacy of PMED immunization with adjuvants in a cancer-like tolerance situation, we used the RIP OVA\textsuperscript{LOW} mouse strain, which is hyporesponsive to OVA as demonstrated by us (data not shown) and others (24, 25). RIP OVA\textsuperscript{LOW} mice were immunized using PMED to deliver either a plasmid encoding OVA alone.

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or a 1:1 ratio of plasmids encoding OVA and GM-CSF cocated onto gold beads (OVA/GM-CSF). When used, IM was delivered topically in the form of Aldara cream on the immunization site 24 h postimmunization (OVA/GM-CSF/IM). A longitudinal study was carried out to assess the kinetics of the CD4 and CD8 T cell responses in RIP OVA\textsuperscript{LOW} mice compared with syngenic C57BL/6 wild-type mice. Immunizations were carried out at 3-wk intervals. Blood samples were SIINFEKL-tetramer stained 7 d after each immunization (and 15 d post primary immunization). In C57BL/6 mice, inclusion of GM-CSF/IM resulted in a faster induction of CD8 T cell responses compared with Ag alone, and the strong CD8 T cell response was maintained throughout the course of the experiment (Fig. 1A). RIP OVA\textsuperscript{LOW} mice were shown to be tolerant to OVA as demonstrated by negligible CD8 T cell response even after three immunizations with OVA alone compared with responses seen in wild-type animals. In contrast, immunization with OVA/GM-CSF/IM elicited a good CD8 T cell response after two immunizations, which was further increased after further immunizations (Fig. 1A).

After the end of the immunization protocol (four immunizations), splenocytes were analyzed using ICS for Ag-specific IFN-\gamma and IL-2 (Fig. 1B). A good correlation was found between CD8 T cells in blood detected by tetramer and CD8 T cells in the spleen as measured by ICS. In wild-type mice, CD8 T cell responses to OVA alone were enhanced significantly by addition of GM-CSF and IM. In the RIP OVA\textsuperscript{LOW} mice, inclusion of GM-CSF/IM also induced strong CD8 T cell IFN-\gamma responses, reaching approximately half the levels found in wild-type mice. In contrast, in both strains, the CD4 T cell response to immunization with OVA alone was negligible, but this was increased in both strains to high levels in the presence of GM-CSF/IM, with RIP OVA\textsuperscript{LOW} responses reaching approximately half the level of CD4 T cell responses seen in wild-type mice. Interestingly, the CD4 T cell response was characterized by a predominance of dual IFN-\gamma/IL-2 producers.

Glucosuria was monitored every 2 to 3 d in the RIP OVA\textsuperscript{LOW} mice to assess the onset of autoimmunity caused by the destruction of \( \beta \) cells in the pancreas. The results showed that the inclusion of GM-CSF/IM was essential to induce glucosuria (Fig. 1C). The onset of diabetes occurred after the third or fourth immunization despite presence of CD8 T cell responses in the blood after the second immunization.

The RIP OVA\textsuperscript{LOW} mice were also used to investigate the requirement for the delay in application of IM following PMED immunization. The RIP OVA\textsuperscript{LOW} mice were immunized with OVA alone, OVA/GM-CSF, or OVA/GM-CSF with IM application immediately postimmunization or after 1, 3, 6, or 24 h. The highest CD8 T cell response was generated by allowing a 24-h delay before IM application (Fig. 1D). Similar data were generated using glucosuria as readout (data not shown).

In conclusion, immunization with OVA by PMED in combination with GM-CSF and IM delivered at 24 h postimmunization induced strong CD4 and CD8 T cell responses in wild-type mice and reversed the hyporesponsiveness of both CD4 and CD8 T cells in tolerant RIP OVA\textsuperscript{LOW} mice resulting in induction of autoimmunity.

**FIGURE 1.** GM-CSF/IM breaks tolerance in RIP OVA\textsuperscript{LOW} mice. RIP OVA\textsuperscript{LOW} mice and C57/BL6 mice were immunized four times with Empty/GM-CSF/IM OVA or OVA/GM-CSF/IM. A, Blood samples were taken throughout the study at indicated time points, stained with SIINFEKL-H-2\textsuperscript{Kb} tetramer and anti-CD8 mAb, and analyzed by flow cytometry gating on CD8\textsuperscript{+} cells. B, IFN-\gamma and IL-2 production in CD4 or CD8 T cells was monitored by ICS of splenocytes stimulated ex vivo with TETWSSNV-MEERKIKV peptide (CD4 T cells) or SIINFEKL peptide (CD8 T cells), respectively. Samples were analyzed by flow cytometry gating on CD4 or CD8 T cells, respectively. C, RIP OVA\textsuperscript{LOW} mice were monitored throughout the study at indicated time points by measurement of the urine glucose level. The data shown represent the average glucose level (\( n = 7–8 \) mice). D, GM-CSF/IM-enhanced OVA immunization induces CD8 T cell responses in RIP OVA\textsuperscript{LOW} mice only if applied 24 h apart. RIP OVA\textsuperscript{LOW} mice were immunized four times with OVA/GM-CSF/IM, with IM application immediately or 1, 3, 6, or 24 h after OVA/GM-CSF delivery by PMED. Blood samples were taken throughout the study 7 d after each (out of four) immunization, stained with SIINFEKL-H-2\textsuperscript{Kb} tetramer and anti-CD8 mAb, and analyzed by flow cytometry gating on CD8\textsuperscript{+} cells. The mean \( \pm \) SEM of 3–5 mice per group is indicated in A and D.
Synergistic action of GM-CSF and IM is essential for CD4 T cell response and breaking functional tolerance

The individual contribution of the adjuvants GM-CSF and IM in breaking tolerance in RIP OVA\textsuperscript{LOW} mice was assessed by analysis of blood, splenocytes, and urine 7 d after the fourth immunization. Mice were immunized at 3-wk intervals with OVA using PMED in combination with either GM-CSF, IM, or both.

Data collated over several experiments showed that addition of the GM-CSF/IM combination resulted in a strong, statistically significant effect on frequency of SIINFEKL-tetramer\textsuperscript{+} CD8 T cells above that found with either adjuvant alone (Fig. 2\textsuperscript{A}). This was accompanied by a positive trend in CD8 T cell cytokine production, but this did not reach statistical significance between the IM and GM-CSF/IM groups (Fig. 2\textsuperscript{B}, 2\textsuperscript{C}). The synergy between GM-CSF and IM was clearer in the CD4 T cell compartment, where the combination induced a robust, significant CD4 T cell response in most animals compared with sporadic responses observed with either adjuvant alone (Fig. 2\textsuperscript{E}, 2\textsuperscript{F}).

The measurement of the urine glucose throughout the experiment provided clear evidence of a functional consequence of the GM-CSF/IM combination on T cell activity (Fig. 2\textsuperscript{D}). At the end of the experiment, 25 out of 29 mice had developed diabetes in the OVA/GM-CSF/IM group compared with 4 out of 16 OVA/IM-immunized mice. The development of diabetes was also earlier in the OVA/GM-CSF/IM group.

RIP OVA\textsuperscript{LOW} mice show a shift in their CD4 T cell epitope dominance hierarchy toward a new CD4 T cell epitope

To investigate the specificity of the T cell repertoire in RIP OVA\textsuperscript{LOW} mice, a peptide library consisting of 15-mers overlapping by 11 aa covering the entire sequence of OVA was screened by using ELISPOT (Fig. 3) and ICS (Fig. 4). The splenocytes screened were from either C57/BL6 or RIP OVA\textsuperscript{LOW} mice immunized with OVA/GM-CSF/IM four times at three weekly intervals. Peptides 64–67 induced a strong IFN-\gamma secretion in splenocytes of C57/BL6 mice and to a minor degree in RIP OVA\textsuperscript{LOW} mice as revealed by ELISPOT (Fig. 3). In contrast, peptides 72 and 73 stimulated IFN-\gamma secretion in splenocytes of RIP OVA\textsuperscript{LOW} mice, but this was only a minor component of the response in C57/BL6 mice. When analyzed by ICS, peptides 64 and 65 (Table I), which contain the SIINFEKL motif, elicited IFN-\gamma/IL-2 secretion by CD8 T cells in both mice strains, although the frequency was decreased in RIP OVA\textsuperscript{LOW} compared with C57/BL6 mice (Fig. 4\textsuperscript{A}, 4\textsuperscript{B}). The known CD4 T cell motif TEWTSSNVMEERKIKV covered by peptides 66 and 67 triggered a strong CD4 T cell response in C57/BL6 and a weaker response in RIP OVA\textsuperscript{LOW} mice (Fig. 4\textsuperscript{C}, 4\textsuperscript{D}). However, CD4 T cells that recognize the sequence EEKYNLTSVLM [the amino acids in common from peptides 72 and 73 (Fig. 4\textsuperscript{C}, 4\textsuperscript{D}, Table I)] had an elevated frequency in RIP OVA\textsuperscript{LOW} compared with C57/BL6 mice. No other peptides apart from the aforementioned

**FIGURE 2.** GM-CSF and IM are acting synergistically in breaking functional tolerance in RIP OVA\textsuperscript{LOW} mice. RIP OVA\textsuperscript{LOW} mice received four immunizations with Empty/GM-CSF/IM OVA, OVA/GM-CSF, OVA/IM, or OVA/GM-CSF/IM. **A,** Blood samples were taken 7 d after the last boost, stained with SIINFEKL-H-2K\textsuperscript{b} tetramer and anti-CD8 mAb, and analyzed by flow cytometry gating on CD8 T cells. **B,** C, **E,** and **F,** Splenocytes were stimulated using peptides and monitored by ICS following flow cytometry gating on CD4 or CD8 T cells, respectively. IL-2 and IFN-\gamma production by CD8 T cells stimulated with SIINFEKL peptide is shown in **B** and **C.** IL-2 and IFN-\gamma production by CD4 T cells stimulated with TEWTSSNVMEERKIKV peptide is shown in **E** and **F.** For **A–C,** E, and **F,** statistical significance was assessed by a one-way ANOVA with Tukey-Kramer posttest. ns = p > 0.05; *0.05 \(\geq\) p \(\geq\) 0.01; **0.01 \(\geq\) p \(\geq\) 0.001; ***p < 0.001. **D,** Percentage of diabetic mice was determined throughout the study with a level of \(\geq\)14 mM urine glucose considered to be indicative of diabetes. Statistical significance between groups was assessed by log-rank test. Figure combines the data from four different experiments.

GM-CSF/IM provides tumor protection that coincides with development of autoimmunity

Experiments were carried out to assess the utility of GM-CSF and IM in protection against tumor challenge in RIP OVA<sup>LOW</sup> mice and C57/BL6 mice. Two immunizations of C57/BL6 mice with OVA alone followed by a challenge with the OVA-expressing tumor cell line EG.7 7 d later resulted in 100% protection (Fig. 5A). RIP OVA<sup>LOW</sup> mice were immunized four times with OVA in the presence or absence of GM-CSF/IM and challenged after either the second or fourth immunization with EG.7 cells. In contrast to wild-type mice, two immunizations of RIP OVA<sup>LOW</sup> mice did not protect against tumor challenge or delay tumor growth even in the presence of the adjuvant combination (Fig. 5B), nor did they induce diabetes. However, four immunizations with the addition of both adjuvants delayed tumor growth and prevented tumor growth significantly in four out of seven RIP OVA<sup>LOW</sup> animals (Fig. 5C). In contrast, protection against tumor challenge or delayed tumor growth was not seen after four immunizations with OVA (Fig. 5C).

In contrast to OVA alone, immunization with OVA in the presence of GM-CSF/IM also induced diabetes, detected after the third immunization (Fig. 5D). Interestingly, it was found that those animals surviving the tumor challenge had consistently high levels of glucosuria prior to tumor challenge, whereas those not surviving the tumor challenge had consistently lower urine glucose levels throughout the experiment (Fig. 5E). Hence, autoimmunity and tumor immunity correlated, suggesting both processes are based on similar mechanisms. We also observed that the urine glucose
McGhie et al. The Journal of Immunology 4595

FIGURE 4. RIP OVA$^{\text{LOW}}$ mice shift their CD4 T cell epitope dominance hierarchy toward a new CD4 epitope. C57/Bl6 (A, C) and RIP OVA$^{\text{LOW}}$ mice (B, D) were immunized four times with OVA/GM-CSF/IM. An OVA peptide library of 15-mers, overlapping in 11 aa, was screened by an IFN-γ ELISPOT in three independent experiments using pooled splenocytes isolated 7 d after the last immunization (Supplemental Material). Results were confirmed by ICS of splenocytes stimulated ex vivo with peptides of an OVA peptide library covering position 245–307 of the OVA sequence. Sequence of peptides 64–67 and 72 and 73 are indicated in Table I. Samples were analyzed by flow cytometry gating on CD4 and CD8 cells and IFN-γ and IL-2 production by CD4 (A, B) or CD8 (C, D) T cells monitored. The mean ± SEM of duplicate measurements is indicated.

level in the group of surviving mice dropped after the tumor challenge, possibly by a diversion of the responding cells to the tumor site from the pancreas. This reverted 30 d later and remained high even 90 d after the last immunization (Fig. 5D).

Thus, repeated immunizations of RIP OVA$^{\text{LOW}}$ mice with OVA in combination with GM-CSF and IM protected against tumor challenge accompanied by development of autoimmunity.

GM-CSF/IM combined with the Th epitope PADRE abrogates OVA-specific CD8 T cell responses and autoimmunity induction in RIP OVA$^{\text{LOW}}$ mice

We have shown in this study that the use of GM-CSF and IM has a dramatic impact on CD4 T cell responses as well as CD8 T cell responses (Fig. 1B). Others have shown that inclusion of heterologous Th cell epitopes, such as the synthetic PADRE, may be an effective strategy for enhancing Ag-specific CD8 T cell responses for therapeutic vaccination for cancer. Having established that there was a correlation between induction of glucosuria in the RIP OVA$^{\text{LOW}}$ mice and functional CD8 T cell responses in tumor protection, we were interested to use this model to further dissect the role of CD4 T cells in CD8 T cell function. We compared the effect of inclusion of the PADRE epitope with the use of the GM-CSF/IM adjuvant combination, and also the combination of both, in our autoimmunity model to further dissect the role of the Ag-specific CD4 on CD8 T cell function.

Immunization schedules were set up in RIP OVA$^{\text{LOW}}$ mice in which OVA was replaced by OVA-P either at prime only, prime and first boost, or throughout the four immunizations in the schedule over a 75-d period. To determine the effects of the adjuvants, each schedule was carried out either in the presence (Fig. 6B, 6D) or absence of GM-CSF and IM (Fig. 6A, 6C). SIINFEKL-tetramer$^{+}$ CD8 T cell responses were monitored from day 14 throughout the schedule, whereas glucosuria was monitored from 7 d post first boost.

In terms of OVA-specific CD8 T cell responses, the main finding was that in the absence of adjuvants, inclusion of PADRE in the immunization schedule had a positive impact in a dose-specific manner (Fig. 6A). However, in the nonadjuvant groups, the T cell responses were relatively low level and translated into only low level and sporadic glucosuria. Where glucosuria was found, it was in the groups receiving PADRE, demonstrating a positive impact of the heterologous CD4 help on frequency and function of OVA-specific CD8 T cell responses in this model (Fig. 6C).

In the presence of adjuvants, the converse was found, with maximal OVA-specific CD8 T cell responses and glucosuria found in the groups immunized either without PADRE or with PADRE at prime only. Significantly lower responses and no significant glucosuria were observed in the group that received PADRE at all four immunizations compared with high levels and sustained glucosuria in the OVA/GM-CSF/IM group and the group that had PADRE included at prime only ($p < 0.005$; Fig. 6B, 6D, Supplemental Tables I, II). Interestingly, it was found that inclusion of PADRE at prime only in this situation resulted in a significantly earlier development of glucosuria compared with the group without PADRE (Supplemental Table II), supporting the idea that in the priming stage of a CD8 T cell response, the Ag specificity of the CD4 help is not important. Although we were not able to measure CD4 T cell responses throughout the schedule, the data suggest that enhancement of OVA-specific CD4 T cell responses was beneficial for tolerance breaking and maintenance of OVA-specific CD8 T cell responses, whereas heterologous PADRE-specific CD4 responses, apart from at prime only, had a negative impact if used repeatedly.

Table I. OVA peptide sequences recognized by T cells of RIP OVA$^{\text{LOW}}$ mice

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence§</th>
<th>Position of Motive in OVA Sequence</th>
<th>Response in RIP OVA$^{\text{LOW}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>64</td>
<td>LEQLESIINFEEKL</td>
<td>257–264</td>
<td>−</td>
</tr>
<tr>
<td>65</td>
<td>ESINFEKLTETWSS</td>
<td>268–275</td>
<td>−</td>
</tr>
<tr>
<td>66</td>
<td>NFKEKLTETWSSNVME</td>
<td>265–274</td>
<td>−</td>
</tr>
<tr>
<td>67</td>
<td>LTEWTSSNVMEERKI</td>
<td>275–290</td>
<td>−</td>
</tr>
<tr>
<td>72</td>
<td>RMKMEEKYNLISVLM</td>
<td>289–299</td>
<td>+</td>
</tr>
<tr>
<td>73</td>
<td>EERKNLISVLMAMGI</td>
<td>290–300</td>
<td>+</td>
</tr>
</tbody>
</table>

*An OVA peptide library of 15-mers overlapping in 11 aa was screened by an IFN-γ ELISPOT in three independent experiments and results confirmed by ICS. The peptide number of the library is indicated.

§Underlined amino acids reflect the motive.

−, decreased frequency of IFN-γ/IL-2–secreting T cells; +, increased frequency of IFN-γ/IL-2–secreting T cells.
Heterologous CD4 help does not result in the same frequency and quality of CD8 T cell response as homologous help

These data have demonstrated that there is a discordance between levels of CD8 T cell responses measured by SIINFEKL-tetramer staining and the function of the cells and that the major factor impacting this negatively is the continued presence of PADRE in the immunization regimen. Further analysis of T cell specificity of splenocytes was carried out at the end of the same series of immunizations shown in Fig. 6. Levels of CD4 T cells specific for either PADRE or OV A were measured, as were levels of IFN-γ-expressing OVA-specific CD8 T cells.

The strength of the PADRE-specific CD4 T cell responses in individual animals (both C57/BL6 and RIP OVA<sub>LOW</sub> mice) was shown in these experiments (Fig. 7A, 7D), with levels reaching &gt;20% of total CD4 T cells in which PADRE was delivered four times. In RIP OVA<sub>LOW</sub> mice, it was found that in the presence of GM-CSF/IM, the insertion of the PADRE epitope into the OVA sequence diminished the OVA-specific CD4 T cell response [measured using both TEWTTSSNVMEERKIKV (Fig. 7E) and EEEKYNTLSVLM (data not shown) peptides] severely compared with immunization without PADRE. Even an initial immunization with OVA-P (1× OVA-P/GM-CSF/IM, 3× OVA/GM-CSF/IM) had a negative effect on the OVA-specific CD4 T cell response, and further OVA-P vaccinations decreased the response accordingly. These data indicate immunodominance of the PADRE epitope on the CD4 T cell response in RIP OVA<sub>LOW</sub> mice. A less expected result was found in analysis of the CD8 T cell response, in which dose-dependent inhibition of OVA-specific CD8 T cells was also seen (Fig. 7F). This was apparent following inclusion of PADRE at prime and first boost, but became significant when more than one PADRE immunization was given. This explains the lack of glucosuria observed in the animals receiving this immunization schedule.

A similar analysis in C57/BL6 mice showed a similar pattern in dominance of CD4 T cell responses (Fig. 7B), but did not have a significant effect on CD8 T cell responses (Fig. 7C). Hence, the secondary effect of the PADRE dominance on CD8 T cell responses was dependant on the animal strain and its tolerance context.

To investigate a possible involvement of the humoral immune response in tolerance breaking, serum samples of RIP OVA<sub>LOW</sub> mice taken 7 d after the last boost were analyzed by ELISA. PADRE immunization induced a higher OVA-specific IgG titer than immunization in the absence of PADRE (Fig. 8). Hence, the Ab response did not correlate with the CD4 and CD8 T cell responses and development of autoimmunity, indicating that breaking tolerance was independent of the humoral response.

In summary, the PADRE epitope improved frequency of CD8 T cell response in RIP OVA<sub>LOW</sub> and C57/BL6 mice, if given without adjuvants, although this was only associated with onset of a very low level sporadic onset of diabetes. By using GM-CSF/IM in combination as an adjuvant regimen along with the PADRE epitope, a strong PADRE CD4 T cell response was induced, dominating the OVA-specific CD4 T cell response in both animal strains. This prevented the development of the homologous OVA-specific CD4 T cell response and in the tolerant RIP OVA<sub>LOW</sub> mice but also had the consequence of reduction of both the OVA-specific CD8 T cell response and significant reduction in levels of diabetes induced. This study highlights the importance of the specificity of the CD4 T cell response on function of CD8 T cell cells and is important to consider in future therapeutic vaccine design.
Discussion

The immune system tolerates tumor Ags because these are self-Ags. Tolerance can be achieved by suppressive mechanisms, processes of peripheral tolerance, or by the process of central tolerance, which deletes high-avidity T cells, leaving lower avidity T cells behind. If the remaining lower avidity T cells are triggered sufficiently by cancer vaccination, they can target tumor Ags, and hence tolerance is broken.

The requirement for Th1 immunity for successful induction of tumor therapy in tumor-bearing hosts is well accepted (26). Clinical evidence of this includes enhanced efficacy of adoptive T cell therapy when the transferred cells contained CD4 T cell clones in addition to CD8 T cell clones (27). Murine adoptive transfer models have been informative in demonstrating the importance of Th1 Ag-specific cells in induction and maintenance of CTL responses. Demonstrated roles for CD4 T cells in tumor immunity include induction and maintenance of CTL responses but also support for functional characteristics such as tumor infiltration (28, 29).

To evaluate the role of CD4 T cells in tolerance breaking and the efficacy of cancer vaccination, RIP OVA\textsuperscript{LOW} mice are useful models. RIP OVA\textsuperscript{LOW} mice are hyporesponsive to OVA as observed by us and others, and to some extent this is caused by the absence of high avidity CD8 T cells (24, 25). DNA immunization of RIP OVA\textsuperscript{LOW} mice with OVA by PMED was not able to break CD4 or CD8 T cell tolerance as assessed by monitoring OVA-specific T cell responses and damage to pancreatic β cells expressing OVA in the RIP OVA\textsuperscript{LOW} mouse strain. Vaccination with OVA/GM-CSF or OVA/IM elevated the CD8 T cell response considerably but neither improved the CD4 T cell response or triggered glucosuria. Only OVA immunization with the combination of GM-CSF and IM broke tolerance both in the CD4 and CD8 T cell compartment and induced autoimmunity. Combinations of TLR ligands with other adjuvants have been tested previously (e.g., Montainde ISA720 and the TLR9 ligand CpG were used to enhance HCV protein immunization resulting in an additive but not synergistic effect on CD4 and CD8 T cell activation, as seen in this study) (30). GM-CSF protein and CpG in combination with tumor Ags elevated CD4 and CD8 T cell and antitumor responses (19, 31). The combination of GM-CSF and IM is likely to have multiple effects: GM-CSF is a chemoattractant for DCs including the myeloid DCs (3, 4). IM is a ligand of TLR-7 (7, 8), which is expressed on both murine CD8 myeloid and plasmacytoid DC subsets (32). Hence, IM can induce maturation of both DC subsets (33). However, plasmacytoid DCs would not be expected to be concentrated in the tissue where Ag has been delivered (34). Plasmacytoid DCs secrete IFN-α upon activation, but our studies have shown that codeelivery of IFN-α via PMED did not enhance immune response compared with OVA alone (data not shown). This suggests that the adjuvant effect of IM in our system is not solely dependent on IFN-α production by plasmacytoid DCs. Moreover, we demonstrated previously the upregulation of
costimulatory molecules on CD11c+ cells by IM application (10). Similarly, a TLR7 agonist was shown to trigger myeloid DCs to secrete IL-12 and express activation markers (35). Hence, IM application of the motif EEKYNLTSVLM were found in RIP OVALOW mice. The TEWTSSNVMEERKIKV-recognizing T cells are possibly of higher affinity and therefore suppressed or partially eliminated in RIP OVALOW mice by central tolerance. This allows the CD8 T cell response to escape immune dominance and proliferate in response to their respective self-Ag (38, 39). Hence, immune tolerance in RIP OVALOW mice is not only due to alterations in the CD8 T cell, but also in the CD4 T cell compartment.

Unexpectedly, the OVA-specific CD4 T cell repertoire was skewed in RIP OVALOW mice compared with C57/BL6 mice. The TEWTSSNVMEERKIKV and an increased frequency of CD4 T cells recognizing the normally immunodominant motif TEWTSSNVMEERKIKV and an increased frequency of CD4 T cells recognizing the motif EEKYNLTSVLM were found in RIP OVA-LOW mice. The TEWTSSNVMEERKIKV-recognizing T cells are possibly of higher affinity and therefore suppressed or partially eliminated in RIP OVALOW mice by central tolerance. This allows the EEKYNLTSVLM-recognizing T cells of presumably lower affinity to escape immune dominance and proliferate in response to adjuvant-enhanced OVA immunizations. Similar shifted hierarchies of immunodominant epitopes due to differences in T cell avidity have been observed with hen egg-white lysozyme transgenic mice or myelin basic protein-expressing/deficient mice postimmunization (13, 36, 37) by maintaining longevity and fitness of memory CTLs (14). Because we were using a prime-boost immunization schedule, CD4 T cell help might be of particular importance for CD8 T cell memory.

Several groups have demonstrated that a foreign Th epitope could be a useful component of a therapeutic cancer vaccine, and the current study has demonstrated the beneficial effects of an adjuvant that enhances both CD4 and CD8 T cell immunity. The idea that it may be possible to combine the two approaches to produce an optimal vaccine design was intriguing, as such a vaccine...
would induce strong Th1 help earlier in the vaccination schedule than would be achieved if relying on the response to tumor Ags. Constructs incorporating the foreign Th epitope PADRE in the OVA DNA construct were generated to test the concept, and the results were unexpected. Without adjuvantization, the OVA-specific CD8 T cell response was elevated by PADRE in both mouse strains, presumably due to the help provided by PADRE-specific CD4 T cells. The OVA-specific CD4 T cell response was low in both RIP OVA$^{\text{LOW}}$ and wild-type C57BL/6 mice, and the PADRE epitope had little impact on this. However, the enhanced CD8 T cell response was not sufficient to induce diabetes in RIP OVA$^{\text{LOW}}$ mice, suggesting a functional defect. However, in the presence of GM-CSF/IM, heterologous CD4 T cell help via PADRE dominated the CD4 T cell response at the expense of the OVA-specific CD4 T cell response in both mouse strains. The OVA-specific CD8 T cell response was robust and was not influenced in wild-type mice by PADRE if applied together with the adjuvant combination, indicating that in the absence of a target organ, the vaccine was sufficient to support generation and expansion of CD8 T cell responses in this model. However, most strikingly, in RIP OVA$^{\text{LOW}}$ mice, PADRE in combination with GM-CSF/IM had a strong detrimental effect on the OVA-specific CD8 T cell frequency, and, as a consequence, diabetes induction was abrogated.

PADRE has been used previously with additional adjuvants, resulting in enhanced Ab titer, CTL activity, IFN-γ secretion, lymphocyte proliferation, or tumor immunity (40–44). Only the combination of PADRE and CpG or cholera toxin in an HIV peptide-based vaccine reduced the IgA response (45). However, we observed an increase in OVA-specific IgG titer after OVA-P immunization. This divergent effect of PADRE and adjuvantization on humoral and cellular immunity resembles clinical data, which indicate a role for enhanced humoral immunity, in combination with suppressed cellular immunity, in the pathogenesis of several human cancer types (46). PADRE also dominated the CD4 T cell response when MUC1 transgenic mice received GM-CSF/IM-enhanced DNA immunization with the tumor Ag MUC1 (data not shown). This suggests that PADRE immunodominance in combination with GM-CSF/IM is not unique to the OVA system, but that the relative balance of homologous versus heterologous responses may be related to the strength of heterologous T cell epitope as well as the adjuvant used to enhance it.

Immunodominance of one epitope over another in a given Ag has been described for the CD4 T cell compartment and related to the kinetic stability of MHC class II–peptide complexes and thus the epitope density on the priming APCs (47). Because both the PADRE epitope and the CD4 T cell epitopes TEWTSSNV-MEERKIKV or EEKYNLTSLVM bind to the murine MHC class II allele I-A$^b$ (20, 48), different kinetic stability of resulting complexes might explain the observed immunodominance in wild-type and RIP OVA$^{\text{LOW}}$ mice. Without adjuvantization, few OVA-specific CD4 T cells were induced, and potential immunodominance of PADRE might not have become apparent. In the presence of GM-CSF/IM, however, APC maturation and Ag presentation by APCs is likely to be increased, and the superior kinetic stability of the MHC class II–PADRE complexes starts to outcompete the OVA binding to MCH class II. On top of that, the number of PADRE-specific CD4 T cells increasing continuously in the course of the immunization might compete with OVA-specific CD4 T cells for access to APCs. This phenomenon of competition of T cells at high frequencies for peptide–MHC complexes has been described for CD8 T cells (49–51) and as well for CD4 T cells (52), the latter affecting memory CD4 T cell generation (53).

A striking observation was the deleterious effect of PADRE on the OVA-specific CD8 T cell response and in consequence on diabetes induction in RIP OVA$^{\text{LOW}}$ mice in the presence of GM-CSF/IM. This phenomenon of cross-immunodominance has not been observed before, to our knowledge. The probable mechanisms of help provided by PADRE or other Th epitopes is the local provision of cytokines, which activate surrounding CD8 T cells and CD40-CD40L licensing of APCs to activate CD8 T cells. PADRE boosted the OVA-specific CD8 T cell response in the absence of GM-CSF/IM in both mouse strains and hence was able to provide help and should compensate for the lack of the OVA-specific CD4 T cell response. However, the help provided by PADRE-specific CD4 T cells might be superseded by their possible competition with OVA-specific CD8 T cells at very high cell frequencies in their surge for APC access, because the activation of both CD4 and CD8 T cells by the same APC at the same time has been suggested (54) and was observed in situ (55). Indeed, in the context of low-dose virus infection, inhibition of CD8 T cell responses by CD4 T cells has been reported (56). Another explanation for the failure of PADRE-specific CD4 T cells to induce autoimmune in the RIP OVA$^{\text{LOW}}$ model is their lack of recruitment to the target organ because PADRE is not expressed in the pancreas of RIP OVA$^{\text{LOW}}$ mice. The requirement for Ag-specific CD4 T cells in the physical proximity of the CTLs may be important in the effector phase as well as their role in the priming and expansion phases.

Four immunizations with OVA/GM-CSF/IM increased survival rate after tumor challenge and delayed tumor growth, accompanied by diabetes development. Furthermore, OVA/GM-CSF/IM-immunized mice that did not survive the tumor challenge had a slightly lower diabetes incidence than surviving mice. This indicates a possible coincidence of tumor protection with autoimmunity development. Immunization of tolerant animal models without supplying non-physiological high numbers of antigenic-specific T cells by adoptive transfer has previously been reported to result in parallel tumor protection and autoimmunity: immunotherapy of RIP-GP mice or SM-LacZ mice controlled tumor growth but was accompanied by fatal autoimmune diseases (57). Similarly, a recombinant Listeria monocytogenes vaccine accompanied with IM enhanced antimelanoma effects but also induced depigmentation of the skin, or vitiligo (58). In the clinic, successful melanoma immunotherapy was linked with development of vitiligo (59), indicating that autoreactive tumor-specific T cells are present in humans and have not been deleted in the process of central tolerance. A parallel situation was observed in RIP OVA$^{\text{LOW}}$ mice. As GM-CSF and IM enhanced DNA immunization breaks tolerance in the RIP OVA$^{\text{LOW}}$ model provides tumor protection, this approach has a good prospect of positive results in clinical cancer immunotherapy. The Th epitope PADRE has the potential to further enhance immunity but it has to be applied with care, especially as the mechanisms of the induced immunodominance effects have not been investigated experimentally yet.

**Acknowledgments**

We thank K. Benhassan-Chahour, O. Vidalin, and O. Barroso-Herrera for sharing unpublished data for discussion.

**Disclosures**

N.S. and J.T. are former employees of GlaxoSmithKline. N.S. was funded by a Marie Curie Fellowship while at GlaxoSmithKline. L.C., S.B., and J.C. are current employees of GlaxoSmithKline.

**References**


Supplemental data

Table 1: Statistics on SIINFEKL-H-2K\(^b\) tetramer response in RIP OVA\(^{LOW}\) mice (corresponding to figure 5A and 5C). RIP OVA\(^{LOW}\) mice were immunised four times with the PADRE containing plasmid pOVA-P (4x OVA-P), once with OVA-P and than three times with OVA (1x OVA-P, 3x OVA), twice with OVA-P and than twice with OVA (2x OVA-P, 2x OVA) or four times with OVA (4x OVA), each group with or without GM-CSF/IM. Blood samples were taken throughout the study at indicated time points, stained with SIINFEKL-H-2K\(^b\) tetramer and anti-CD8 mAb and analysed by flow cytometry gating on CD8 T cells. Statistical significance was assessed by a two-way ANOVA with Tukey-Kramer post test with ns = p>0.05; * = 0.05≥p≥0.01; ** = 0.01≥p≥0.001; *** = p<0.001.

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Table 2: Statistics on urine glucose level in RIP OVA\textsuperscript{LOW} mice (corresponding to figure 5B and 5D). RIP OVA\textsuperscript{LOW} mice were immunised four times with the PADRE containing plasmid pOVA-P (4x OVA-P), once with OVA-P and than three times with OVA (1x OVA-P, 3x OVA), twice with OVA-P and than twice with OVA (2x OVA-P, 2x OVA) or four times with OVA (4x OVA), each group with or without GM-CSF/IM. Mice were monitored throughout the study at indicated time points by measurement of the urine glucose level. Statistical significance was assessed by a two-way ANOVA with Tukey-Kramer post test with ns = p>0.05; * = 0.05≥p≥0.01; ** = 0.01≥p≥0.001; *** = p<0.001.

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