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Modification of One Epitope-Flanking Amino Acid Allows for the Induction of Friend Retrovirus-Specific CD8$^+$ T Cells by Adenovirus-Based Immunization

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While Friend retrovirus-infected mice readily mount a vigorous CD8$^+$ T cell response to the leader-gag-derived peptide GagL$_{85–93}$, no GagL$_{85–93}$-specific T cells were detectable in mice immunized against Friend virus (FV) with viral vectors or DNA vaccines. By exchanging one epitope-flanking amino acid or using a scaffold protein we were able to demonstrate for the first time the induction of GagL$_{85–93}$-specific CD8$^+$ T cells by genetic vaccination and show their high protective effect against FV challenge infection.

The Gag polyprotein p65 of Friend murine leukemia virus (F-MuLV) consists of the structural proteins p15, p12, p30, and p10, which are the main components of the viral capsid. Usage of an alternative start codon upstream of the *gag* open reading frame adds a leader region to the Gag polyprotein, resulting in the glycosylated protein gp85^{gag} (12). The leader region of gp85^{gag} contains an immunodominant CD8$^+$ T cell epitope of Friend virus (FV) (9), and in acutely FV-infected mice up to 15% of the activated CD8$^+$ T cells are GagL$_{85–93}$ specific (26). Despite the immunodominance of this epitope in natural FV infection, no induction of GagL$_{85–93}$-specific CD8$^+$ T cells could be detected after immunization of mice with adenovirus (Ad)-based vectors encoding leader-gag (3). Similarly, in mice genetically immunized by plasmid DNA encoding leader-gag, no GagL$_{85–93}$-specific CD8$^+$ T cells could be detected before FV challenge (11), and in vaccination studies with vaccinia virus-based vectors encoding different parts of p65^{gag} or gp85^{gag}, no difference in protection was seen whether or not the leader region was included in the vaccine (19). Thus, although the FV model has been used extensively for the development of new concepts for vaccination against retroviruses, none of the genetic vaccines utilized in the past were able to induce CD8$^+$ T cell responses against the immunodominant GagL$_{85–93}$ epitope of FV. Therefore, the goal of this study was to develop a genetic vaccine capable of inducing GagL$_{85–93}$-specific immunity.

Two factors critically influence if a peptide sequence can be presented as an epitope on a major histocompatibility complex (MHC): the ability to bind to a certain MHC allele and the efficiency of proteasomal degradation resulting in that peptide. Interestingly, neither property is predicted for the immunodominant GagL$_{85–93}$ epitope in H-2D^b mice using the prediction tools NetMHC (17) and NetChop (20). As the tyrosine flanking the GagL$_{85–93}$ epitope C terminally (C + 1) in the native sequence has been described to be unfavorable for proteasomal degradation (16), inefficiency of processing might be an explanation for the lack of CD8$^+$ T cell immunity after genetic immunization. To overcome this problem, we exchanged the C+1 tyrosine with lysine, since for the resulting protein, leader-gag$_{C1K}$, proteasomal cleavage after Leu$_{93}$ is predicted (20).

As an alternative approach, we constructed an adenoviral vector encoding a fusion protein of the murine cellular protein thioredoxin (Txn) and the GagL$_{85–93}$ peptide, TxnGagL. Thioredoxin is a cellular protein involved in redox processes, and, while being a nonantigenic self-protein, it has been ascribed immunostimulatory and chemotactic properties, making it an attractive protein scaffold for immunization (5, 6).

Adenoviral vectors encoding leader-gag$_{C1K}$ or TxnGagL (Fig. 1A) were constructed as described before (14); transgene expression and sequence identity were verified (data not shown). To analyze whether expression of the new transgenes leads to processing of the GagL$_{85–93}$ epitope, an *in vitro* proliferation assay was performed using GagL$_{85–93}$-specific CD8$^+$ T cells from T cell receptor-transgenic mice (22) and vector-transduced bone marrow-derived dendritic cells (DC). While no proliferation of the CD8$^+$ T cells was detectable when DC were transduced with Ad5.Gag (3), a vector encoding the native leader-gag sequence (Fig. 1A), proliferation was observed after transduction of DC with either Ad5.Gag$_{C1K}$ or Ad5.TxnGagL (Fig. 1D to F), indicating efficient processing of the GagL$_{85–93}$ epitope from the engineered transgenes.

To analyze the induction of CD8$^+$ T cell responses by the new vaccine vectors, highly FV-susceptible CB6F1 hybrid mice (H-2$^{b/d}$, FV2$^{r/s}$, Rfv3$^{r/s}$) were immunized twice in a 3-week interval with 10^9 particles of the adenoviral vectors using a heterologous prime-boost combination of Ad5-based vectors and fiber-chimeric Ad5F35 (21) vectors as described before (3). Immune responses were analyzed 2 weeks after the second immunization using MHC class I (MHC-I) tetramers containing a modified GagL$_{85–93}$ epitope peptide (25) for staining of spleen or blood cells. As reported before (3), immunization with Ad.Gag did not lead to the induction of GagL$_{85–93}$-specific CD8$^+$ T cells, yet when mice were immunized with Ad.Gag$_{C1K}$ or Ad.TxnGagL, GagL$_{85–93}$-specific CD8$^+$ T cells were readily detectable by tetramer staining in most mice (Fig. 2). Especially high levels of GagL$_{85–93}$-specific CD8$^+$ T cells were found in mice immunized with Ad.TxnGagL; the levels

Received 25 June 2012 **Accepted** 20 August 2012
Published ahead of print 29 August 2012
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doi:10.1128/JVI.01607-12

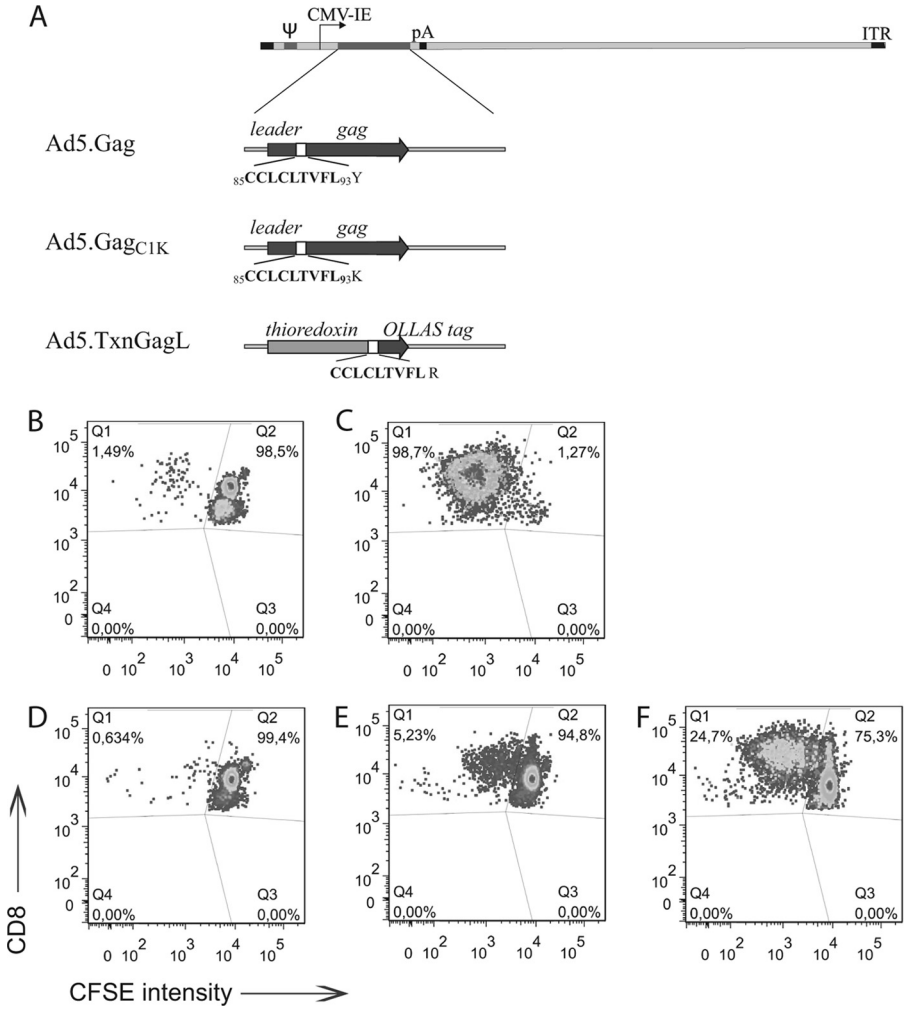

FIG 1 Vector design and *in vitro* processing of the Ad-encoded GagL epitope. (A) Schematic presentation of the employed adenoviral vectors. The location of the GagL$_{85–93}$ epitope CCLCLTVFL is shown, and the C+1 flanking amino acids in the native and modified sequences are indicated. ITR, inverted terminal repeat; Ψ, packaging signal; CMV-IE, cytomegalovirus immediate early promoter; pA, polyadenylation signal. (B to F) For the analysis of antigen processing, an *in vitro* proliferation assay was performed using carboxyfluorescein succinimidyl ester (CFSE)-stained, naive CD8$^+$ T cell receptor (TCR)-transgenic T cells specific for the GagL$_{85–93}$ epitope and bone marrow-derived dendritic cells (DC) transduced with the adenoviral construct Ad5.Gag (D), Ad5.Gag$_{C1K}$ (E), or Ad5.TxnGagL (F) at a multiplicity of infection of 1,000. Nontransduced DC served as the negative control (B), and DC loaded with a modified GagL$_{85–93}$ peptide (where the cysteine residues are replaced by α-aminobutyric acid) served as the positive control (C). CFSE intensity of CD8$^+$ T cells was analyzed after 3 days of coincubation with the respective DC. Plots are representative for three independent experiments.

were significantly higher than those in mice immunized with Ad.Gag$_{C1K}$ ($P < 0.05$).

To possibly augment vaccine-induced CD8$^+$ T cell responses, we coapplied vectors encoding interleukin-12 (IL-12), IL-15, IL-18, IL-21, or granulocyte-macrophage colony-stimulating factor (GM-CSF) together with Ad.Gag$_{C1K}$, since these cytokines have been previously reported to enhance CD8$^+$ T cell responses to peptide or DNA vaccines (1, 2, 7). However, no significant differences in levels of GagL$_{85–93}$-specific CD8$^+$ T cells compared to those resulting from immunization with Ad.Gag$_{C1K}$ alone were found (data not shown).

To analyze protection conferred by the new vectors, mice were challenged with 5,000 spleen focus-forming units of FV 3 weeks after boost immunization. Only mice that had received Ad.Gag$_{C1K}$ or Ad.TxnGagL were strongly protected, with no detectable viral loads in the plasma on day 10 (Fig. 3A) and very low viral loads in the spleen on day 21 (Fig. 3B) after FV challenge. Thus, although a few mice immunized with Ad.Gag$_{C1K}$ exhibited very low frequencies of MHC-I tetramer-positive (Tet I$^+$) CD8$^+$ T cells, they were still protected from high-dose FV challenge, suggesting that also these mice had vaccine-primed CD8$^+$ T cells.

The strong protection after vaccination with the new vectors emphasizes the importance of CD8$^+$ T cells for protection against retroviral infection. The CD8$^+$ T cell response induced by the TxnGagL construct is especially high and similar in strength to the CD8$^+$ T cell response seen in acutely FV infected mice (26). Also in this construct, the epitope-flanking amino acid is modified compared to the native sequence (C + 1: R), evidently allowing for efficient processing. Thioredoxin has been ascribed immunostimulatory properties (5, 6), and bacterial Txn has been used before as a peptide carrier for antibody induction (8, 24); the high frequency of GagL$_{85–93}$-specific CD8$^+$ T cells in Ad.TxnGagL-im-

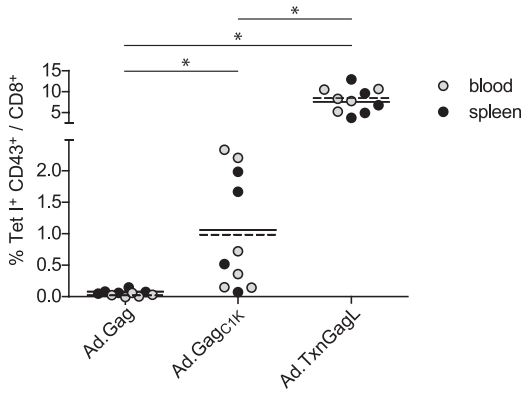

FIG 2 Vaccine-induced CD8$^+$ T cell response. CB6F1 mice were immunized twice in a 3-week interval using 10^9 viral particles (vp) of adenoviral vectors carrying the indicated transgenes; Ad5-based vectors were used for prime immunization, and Ad5F35 vectors were used for boost immunization. Two weeks after the second immunization, the induction of MHC-I tetramer (Tet I)-positive CD8$^+$ T cells was analyzed either in spleen cells or blood cells. Shown are percentages of Tet I$^+$ CD43$^+$ cells. Lines indicate mean values for spleen (solid lines) and blood cells (dashed lines), respectively. Statistically significant differences are indicated by asterisks ($P < 0.05$; analysis of variance [ANOVA] on ranks, Student-Newman-Keuls test). Data for blood cells were combined from two independent experiments; data for spleen cells were obtained from a single additional experiment. Spleen cells and blood cells were collected from different mice immunized in two independent experiments.

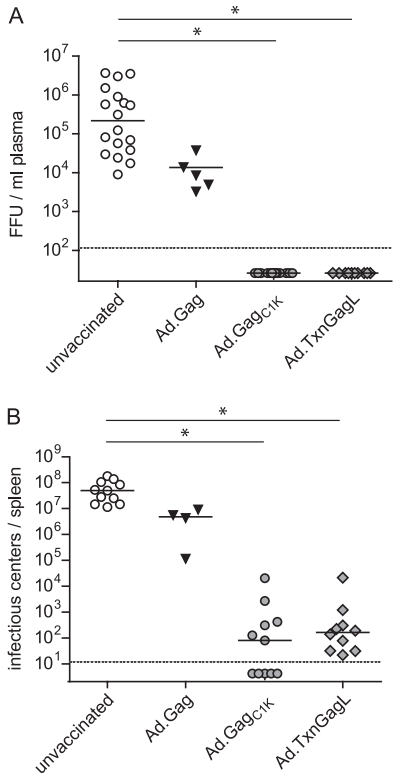

FIG 3 Protection from high-dose FV challenge infection. CB6F1 mice were immunized twice in a 3-week interval using 10^9 vp of adenoviral vectors carrying the indicated transgenes; Ad5-based vectors were used for prime immunization, and Ad5F35 vectors were used for boost immunization. Three weeks after boost, mice were challenged with 5,000 spleen focus-forming units (SFFU) of FV complex. Viral load in plasma (A) was analyzed 10 days after FV challenge; viral load in spleen (B) was analyzed on day 21 after FV challenge. Solid lines indicate median values. Statistically significant differences are indicated by asterisks ($P < 0.05$; ANOVA on ranks, Dunn's test). Dashed lines indicate the detection limit. Data were acquired in three independent experiments.

munized mice indicates that its immunogenicity may be beneficial for T cell induction as well.

The reasons for the apparent discrepancy in processing of the leader-gag protein expressed from F-MuLV or a genetic vaccine are not completely understood. As this finding might have possible implications for the use of adenoviral vectors for vaccine development in general, it shall be thoroughly investigated. Possible mechanisms are an involvement of other F-MuLV proteins in processing and differences in the induction of the immunoproteasome, although it is known that nonreplicating adenoviral vectors are very immunogenic and induce immunoproteasome formation (13, 18). More likely, it may be competition between the GagL$_{85–93}$ epitope and adenovirus-derived epitopes that hampers GagL$_{85–93}$-specific T cell induction. The GagL$_{85–93}$ epitope is the immunodominant epitope of FV, where CD8$^+$ T cell responses to other epitopes are very weak or undetectable. In the context of an adenoviral vector, however, the GagL$_{85–93}$ epitope has to compete with strong epitopes from adenoviral proteins. While the unfavorable C + 1 amino acid may not play a significant role in the barely competitive environment of FV, it may be a strong disadvantage when the epitope is competing with adenoviral epitopes, and this may explain the subdominance of the GagL$_{85-93}$ epitope. Enhancing processing of GagL$_{85-93}$ through the amino acid substitution in the Gag$_{C1K}$ construct apparently improves its rank in the immunodominance hierarchy. Similar suppression of immune responses to transgene epitopes by adenovirus epitopes has recently been described (15). Epitope competition is also a possible explanation for the considerably higher levels of GagL$_{85-93}$-specific CD8$^+$ T cells induced by the TxnGagL vaccine. The TxnGagL fusion protein is likely secreted from vector-transduced cells, as has been reported for Txn before (23). Secreted protein could then be taken up by nontransduced antigen-presenting cells, and the GagL$_{85-93}$ epitope would be processed and presented in the absence of adenovirus-derived epitopes.

The ability to induce CD8$^+$ T cell responses by genetic vaccination is an important prerequisite to further advance immunization strategies in the FV model. It is known that for complete protection from retrovirus infection, complex immune responses are necessary, comprising CD4$^+$ and CD8$^+$ T cells as well as neutralizing antibodies (10). In the past we reported on a new adenovirus-based vector type that displays the encoded antigen on the capsid and thereby induces strong CD4$^+$ T cell and antibody responses (4). The combination of this vector with the cytotoxic T lymphocyte (CTL)-inducing vectors described here shall be evaluated to further improve vaccination efficiency, with sterile immunity against a retrovirus infection mediated by a genetic vaccine probably being within reach.

ACKNOWLEDGMENTS

This work was supported by a grant to P.G. from the internal research promotion program IFORES of the Medical Faculty of the University Duisburg-Essen, by a grant from the Mercator Research Center Ruhr to U.D., and by grants from the Deutsche Forschungsgemeinschaft (DFG grant TRR60 to U.D. and WI 1329/6-1 to U.D. and W.B.).

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