Vaccination with DNA Encoding a Single-Chain TCR Fusion Protein Induces Anticonalotypic Immunity and Protects against T-Cell Lymphoma

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ABSTRACT

The clonotypic T-cell antigen receptor (TCR) provides unique Vα and Vβ sequences with potential as idiotypic targets for immunoregulation. For T-cell malignancies, vaccination with the TCR could induce therapeutic anti-idiotypic responses. To facilitate this approach, we have developed DNA vaccines that include the genes encoding TCR sequences from a T-cell lymphoma (TCL). To combine requirements for stable folding with a simple minimized single-chain construction, we used a three-domain VαVβββ sequence. To promote anti-TCR immunity, we fused a pathogen-derived sequence from tetanus toxin to the 3′-end of the single-chain TCR. The fusion gene vaccine induced anti-idiotypic antibodies and generated protection against the TCL. The critical requirement for the conformational integrity of the delivered TCR antigen was highlighted by the observation that DNA fusion vaccines containing either Vαββ or Vβββ sequences failed to generate antibodies reactive with the native TCR or provide protection. This is the first report of a DNA vaccine able to induce anti-idiotypic immunity against TCL, and it presents a simple strategy for selectively eliminating T-cell clones in vivo.

INTRODUCTION

The structural diversity of the TCRα and β chains is generated by somatic recombination of V(D)J gene segments (1). The unique combination of these genetic elements gives rise to Id determinants that are expressed clonotypically by the T cell. When expressed by malignant or autoreactive T-cell clones, these Id determinants can serve as targets for active immunotherapy. The Id immunoglobulin of malignant B cells provides a similar molecular target. Vaccination strategies against B-cell Id have provided encouraging results in both preclinical models and human trials (2–5). However, the difficulty of preparing individual Id proteins has driven the development of DNA vaccines that include the encoding V-region genes (6, 7). Although V-region sequences are weak tumor antigens, when fused to pathogen-derived sequences, they can elicit strong protective immunity. The greater difficulty of producing Id protein vaccines from the TCL is slow to investigation of this approach. However, recombinant TCR protein vaccines have been shown to prevent experimental autoimmune diseases and the development of a murine TCL (10–12). Here we extend the concept of DNA fusion gene vaccination to T-cell malignancies. We delineate two critical elements required for the vaccine, including a minimal VαVβββ sequence able to encode a scTCR with conformational integrity as predicted from crystallographic studies (13–15). Our data reveal that products of VαVβ or Vβββ DNA constructs have conformational deficits and fail to induce effective immunity. The second element necessary for a successful vaccine is fusion of the VαVβββ sequence to FrC, without which the scTCR fails to generate antibody or protective responses.

MATERIALS AND METHODS

Cell Lines. C6VL, a radiation-induced thymoma/lymphoma derived from the C57BL/Ka mouse strain (16), was kindly provided by Craig Okada (Stanford, CA). C6VL cells have a mature T-cell phenotype expressing TCRββ, CD3, CD4, and H-2b. The TCR α chain is composed of AV9*04, A56, and the β-chain of BV19*01, BD1*01, and BJ1–3*01. TCL-1 arose spontaneously in a C57BL/Ka mouse bred in house and served as a control in tumor challenge experiments. 293 cells were purchased from the European Collection of Animal Cell Cultures (Salisbury, United Kingdom).

Construction of DNA Vaccines. The TCR Vα and Vβ domains for C6VL were spliced together with a (Gly4Ser)7 peptide linker to generate the scTCR sequence VαVβ (Fig. 1a) by primer overlap extension, using the paired primers: VA leader, 5′-AACGTTAGCATGCTTCCGGACACTCTTCCCC-3′; JA linker, 5′-AGGACACATCTGCCGATCCGCGTCCAAGTGTGTAACACTCGAAC-3′; VB linker, 5′-GGCGGAGGGTGGCTTGCGGCGTG-3′; JB reverse, 5′-GCTAGTC-TACACAATGAGCCGGCTCTGGG-3′; and JB reverse, 5′-GCTAGTC-TACACAATGAGCCGGCTCTGGG-3′. To generate Vαβββ (Fig. 1a), the Vα domain was linked to the entire β-chain by extending the β-chain sequences to the residue right before the terminal cysteine, using the CB reverse primer, 5′-GCTAGTC-TACACAATGAGCCGGCTCTGGG-3′. For Vβββ, 5′-AAGCTTACGATGCTTCCGGACACTCTTCCCC-3′; CB reverse primer, 5′-GCTAGTC-TACACAATGAGCCGGCTCTGGG-3′. Plasmid DNA was purified for vaccination using a Qiagen Giga kit (Qiagen, Hilden, Germany). All constructs were sequenced and checked for expression in vitro using the TNT T7 Coupled Reticulocyte Lysate System (Promega, Southampton, United Kingdom).

Expression in Vitro. The ability of scTCR-FrC fusion constructs to synthesize and export protein was assessed after transient transfection into 293 cells by lipofection (Effectene; Qiagen). Supernatants were collected after transient transfection into 293 cells and assessed for protein expression by ELISA plates with a monoclonal anti-FrC antibody. To promote Id-TCR immunity, we transfected these cells with the expression vector pscDNA3 (Invitrogen, Leek, The Netherlands) as HindIII-NcoI fragments in frame and 5′ to the coding sequence for FrC (aa 865-1316 of tetanus toxin). Construction of pFrC with a BCL1 Vλ gene derived leader sequence has been described previously (8). Plasmid DNA was purified for vaccination using a Qiagen Giga kit (Qiagen, Hilden, Germany). All constructs were sequenced and checked for expression in vitro using the TNT T7 Coupled Reticulocyte Lysate System (Promega, Southampton, United Kingdom).

Production of Recombinant scTCR. Recombinant Vαβββ protein, biotinylated at the COOH terminus in vivo, was expressed in Drosophila melanogaster S2 cells. Two synthetic oligonucleotides encoding a biotin-acceptor sequence (17) were annealed and then ligated directly into pMT/Bip/V5-His (Invitrogen) as a NolI-Agel fragment, generating the construct pMT-BA. Vαββββ DNA was then cloned into pMT-BA as a SpeI-NolI fragment. A PCR product encompassing Escherichia coli biotin ligase was amplified from the plasmid pACYC184 (Avidity, Denver, CO) and subcloned into pMT/Bip/V5-His as an NolI-NolI fragment. The Vαββββ and biotin ligase constructs were cotransfected into S2 cells (Invitrogen) by calcium phosphate precipitation together with pCohHYGO (Invitrogen). Stable transfectants, derived by selection in Schneider Drosophila expression medium containing 300 μg/ml hygromycin-B, were expanded in protein-free medium (Insect Xpress; Bio-whittaker, Wokingham, United Kingdom), supplemented with 50 μg/ml-d-biotin.
RESULTS AND DISCUSSION

Construction of DNA Vaccines. To investigate DNA vaccination against T-cell malignancies, V-region sequences from the murine TCL cell line C6VL were assembled as scTCR by PCR and cloned in-frame and 5' to the coding sequence for FrC. The Va domain was spliced via a (Gly Ser) flexible linker to either the VB domain alone or to the entire β-chain (minus the transmembrane/cytoplasmic regions), generating the constructs VaVB-FrC and VaVβCβ-FrC, respectively (Fig. 1a). To assess whether Va was required to stabilize VB folding, a VβCβ-FrC fusion construct was also assembled (Fig. 1a).

Cβ Is Required to Stabilize Vβ Folding and Pairing with Va. We initially evaluated the structural integrity of the scTCR DNA vaccine products by transfecting the constructs into 293 cells. All supernatants were found to contain levels of FrC protein similar to those produced from a plasmid containing the FrC gene alone (Fig. 1b). Using a sandwich ELISA with an anti-FrC mAb as capturing antibody and the conformation-dependent, Vβ6-specific mAb RR4-7 for detection (18), we demonstrated clear reactivity with expressed VaVβCβ-FrC (Fig. 1b). There was also partial reactivity with VβCβ-FrC, indicating limited Vβ-folding in the absence of the Va domain. VaVβCβ-FrC protein failed to react with RR4–7 (Fig. 1b), suggesting inappropriate folding of this molecule. Reactivity with mAb 124–40, which recognizes a clonotypic determinant on the C6VL Va chain (16), could only be demonstrated to VaVβCβ-FrC (Fig. 1b). These findings confirm the premise that Cβ is required to stabilize Vβ-folding (19, 20).

VaVβCβ-FrC Induces Antibody Reactive with Native C6VL TCR. We next investigated whether the scTCR-FrC fusion constructs would induce antibody reactive with the C6VL lymphoma cells. Mice were vaccinated i.m. with 50 μg of plasmid DNA on days 0, 21, and 42. Serum samples were collected on day 56 and pooled and analyzed for antibody reactive against native C6VL cells by flow cytometry (Fig. 2). Although all FrC-containing constructs generated antibodies against FrC (Table 1), only those animals vaccinated with VaVβCβ-FrC were able to induce antibody reactive with C6VL cells (Fig. 2). An absolute requirement

### Table 1 Serum antibody responses induced by DNA vaccination

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Target antigen</th>
<th>rFrC</th>
<th>rVaVβCβ</th>
<th>C6VL cells</th>
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<tr>
<td>VaVβCβ-FrC</td>
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* Determined by ELISA.

† Serum samples were collected on day 56, after vaccination with the constructs on days 0, 21, and 42.
for the scTCR to maintain a native conformation was highlighted by the failure of \( \alpha \beta \)-FrC or \( \beta \beta \)-FrC to induce anti-C6VL antibodies. The essential role of the FrC sequence was shown by the failure of the \( \alpha \beta \)-FrC vaccine to induce anti-C6VL antibody (Fig. 2). Importantly, immune serum raised by vaccination with \( \alpha \beta \)-FrC was unable to stain peripheral lymphocytes from nonimmunized mice (data not shown), suggesting that the antibody response was directed against private Id determinants. Analysis of pooled sera for immunoglobulin subclasses showed comparable levels of IgG1 and IgG2a (0.8:1), consistent with a mixed T(H)1/T(H)2-dominated response.

The \( \alpha \beta \beta \)-FrC vaccine induced antibody recognizing C6VL cells in only 42% of mice (Table 1). In contrast, sera from all mice recognized recombinant \( \alpha \beta \beta \)-protein by ELISA. Surprisingly, however, immune sera from mice vaccinated with \( \alpha \beta \)-FrC or \( \beta \beta \)-FrC constructs, known to produce mainly misfolded protein, also recognized recombinant \( \alpha \beta \beta \)-protein (Table 1). The antibody response was specific for C6VL, with no reactivity against \( \alpha \beta \beta \)-protein prepared from an unrelated T-cell line (data not shown). This indicates that the recombinant \( \alpha \beta \beta \)-protein contains some misfolded molecules, possibly a common feature of recombinant proteins. It points to a requirement to confirm reactivity of apparent anti-Id antibodies with native cell-expressed Id determinants.

**\( \alpha \beta \beta \) Induces Protective Immunity against C6VL Lymphoma.** The relevance of conformational integrity for induction of protective immunity against C6VL lymphoma was then addressed. C6VL is a highly aggressive tumor, with as few as 50 cells killing 100% of recipient mice (data not shown). Vaccination with the DNA construct \( \alpha \beta \beta \)-FrC induced strong protection against challenge with C6VL (Fig. 3a), compared with nonvaccinated control mice and mice vaccinated with FrC alone (\( P < 0.001 \)). Vaccinations with \( \alpha \beta \beta \)-FrC, \( \beta \beta \)-FrC, or \( \alpha \beta \beta \)-alone were also completely ineffective. The protective response generated was specific for C6VL, with no protection induced against TCL-1, an unrelated T-cell tumor (data not shown). The pattern of protection has been confirmed in two subsequent experiments, with survival rates of mice vaccinated with \( \alpha \beta \beta \)-FrC ranging from 50 to 80%, whereas the other constructs were ineffective at prolonging survival.

**DNA Vaccination Does Not Perturb the Normal T-cell Repertoire.** One potential problem with this approach is that the DNA vaccine could raise an autoimmune response capable of eliminating normal T cells expressing the same \( \alpha \) or \( \beta \) framework determinants. To assess if vaccination with \( \alpha \beta \beta \)-FrC caused a change in TCR usage, peripheral lymphocytes from immunized mice were processed 3 weeks after the final vaccination and stained with RR4-7. Cells expressing V\( \beta6 \) were still detectable at levels comparable with those found in nonimmunized mice or mice vaccinated with FrC alone, indicating no autoimmune effect (data not shown).

**Tumor Protection Is Independent of CD8+ Cells.** Previous studies using a TCR protein vaccine against C6VL lymphoma had indicated a role for CD8+ T cells in mediating suppression of tumor growth (12, 21). We investigated this effector pathway by \textit{in vivo} depletion of CD8+ cells with mAb YTS 169.4.2.1. Flow cytometric analysis of peripheral lymphocytes 1 day before tumor challenge confirmed >98% depletion of CD8+ T cells (data not shown). However, depletion did not compromise protection against tumor (Fig. 3b). Thus, despite the known efficacy of DNA vaccines in inducing CD8+ T-cell responses, there was no evidence for involvement of this pathway with TCR as antigen. The critical requirement for native folding of the scTCR points to B cells being involved in the protective response. This was also suggested for TCR protein vaccines, which failed to induce immunity in B cell-deficient (J\( H^{-/-} \)) mice (12, 21). However, B cells could be involved in antigen presentation, as well as antibody production. In our vaccinated mice, there was no clear correlation between anti-C6VL antibody levels and survival, and some mice with no detectable anti-Id were protected. This raises the possibility of a role for anti-Id CD4+ T cells, known to be capable of mediating protection against B-cell malignancies (22, 23). Parallel depletion of CD4+ T lymphocytes could not be performed because of expression of CD4 by C6VL cells.

**Antibody Transfer Can Provide Protection.** The ability of immune serum to mediate tumor protection was assessed \textit{in vivo} by passive antibody transfer. The efficacy of antibody was revealed by the fact that 90% of naive recipients given purified IgG from mice immunized with \( \alpha \beta \beta \beta \)-FrC were protected against a subsequent lethal challenge with C6VL cells (Fig. 3c). Mice similarly treated with total IgG from mice immunized with an irrelevant fusion vaccine were
not protected (Fig. 3c). Therefore, it appears that antibody alone can mediate protection but that it may not be the only mechanism. Evidence from the use of recombinant scTCR as antigen to suppress autoimmune T lymphocytes has implicated CD4+ regulatory cells as effectors (11). Consistent with our findings, no antibody was produced by the VαVβ protein used in this study (11). Similarly, recombinant VαVβ scTCR protein from C6VL did not induce antibody or protective immunity (12), again supporting a requirement for conformational integrity. One interpretation is that anti-Id antibody is a highly effective mechanism for attacking T-cell clones and that recombinant protein may be an inefficient inducer because of inappropriate folding. For DNA vaccines where levels of protein expression are low, folding must be optimized, and the presence of the stabilizing Cβ domain is essential. There are likely to be additional cellular mechanisms capable of attacking T-cell targets, and these may be induced by less well-folded protein. However, our analysis reveals the power of anti-Id antibody and points to inclusion of this weapon in our attack against T-cell tumors.

These results reveal a strategy for selectively eliminating T-cell clones by DNA vaccination. A combination of optimally folded scTCR sequence with a pathogen-derived sequence induces antibody and generates protection against TCL. The range of application is wide but may be especially attractive for cutaneous TCLs, where clinical need and a feasible clinical setting are evident.

ACKNOWLEDGMENTS

We thank Kerry Cox, Sam Martin, and Lisa Davis for invaluable technical assistance.

REFERENCES

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