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Dendritic Cell Targeting of Survivin Protein in a Xenogeneic Form Elicits Strong CD4⁺ T Cell Immunity to Mouse Survivin¹

Anna Charalambous, Margarita Oks, Godwin Nchinda, Sayuri Yamazaki, and Ralph M. Steinman²

To determine whether strong CD4⁺ T cell immunity could be induced to a nonmutated self protein that is important for tumorigenesis, we selectively targeted the xenogeneic form of survivin, a survival protein overexpressed in tumors, to maturing dendritic cells in lymphoid tissues. Dendritic cell targeting via the DEC205 receptor in the presence of anti-CD40 and poly(I:C) as maturation stimuli, induced strong human and mouse survivin-specific CD4⁺ T cell responses, as determined by IFN-γ, TNF-α, and IL-2 production, as well as the development of lytic MHC class II-restricted T cells and memory. Immunity was enhanced further by depletion of CD25⁺Foxp3⁺ cells before vaccination. Anti-DEC205-human survivin was superior in inducing CD4⁺ T cell responses relative to other approaches involving survivin plasmid DNA or survivin peptides with adjuvants. However, we were unable to induce CD8⁺ T cell immunity to survivin by two doses of DEC205-targeted survivin or the other strategies. Therefore, significant CD4⁺ T cell immunity to a self protein that is overexpressed in most human cancers can be induced by DEC205 targeting of the Ag in its xenogeneic form to maturing DCs. The Journal of Immunology, 2006, 177: 8410–8421.

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with an unfavorable course of disease, including accelerated progression, higher rates of recurrences, increased resistance to therapy, and shortened survival (26, 27). Down-regulation of survivin expression in various tumor cell lines, induced by antisense compounds, reduces tumor growth potential and promotes tumor cell apoptosis (28, 29). Furthermore, injection of human MCF-7 breast cancer xenografts with replication-deficient adenoviruses encoding a phosphorylation-defective survivin Thr53→Ala (pAd-T34A) dominant-negative mutant, was shown to inhibit growth of established tumors and trigger tumor cell apoptosis in vivo (30).

We postulated that the relatively low levels of survivin expression in the thymus and periphery might limit the extent of immunological tolerance in the CD4+ T cell compartment. We evaluated different approaches for inducing CD4+ T cell immunity to this protein comparing human or xenogenic survivin (Ivsurvivin) to the mouse protein (which are 85% homologous) in BALB/c mice. We will report that one specific strategy, the incorporation of Ivsurvivin into the H chain of anti-DEC205 mAb, allowed for the induction of strong CD4+ T cell responses that cross-reacted with mouse survivin (msurvivin). DEC205 is a receptor for Ag uptake and processing that is expressed at high levels on a subset of DCs in lymphoid tissues. DEC205/CD205 targeting has been shown previously to be highly immunogenic for foreign microbial proteins (31–33). Now we find that targeting of a self-Ag via DEC205 elicits a CD4+ T cell response that involves high frequencies of IFN-γ, IL-2, and TNF-α producing cells that also include cytolytic and memory functions. As we shall discuss, this is the first description of strong CD4+ T cell immunity to a shared nonmutated self-Ag that is overexpressed in many tumors.

Materials and Methods

Mice

Female BALB/c mice from Taconic Farms were maintained under specific pathogen-free conditions and used at 7–8 wk of age under the guidelines of our Institutional Animal Care and Use Committee.

Reagents

Hamster mAb for CD25 (PC61) was a gift from S. Sakaguchi (Kyoto University, Kyoto, Japan). We purchased anti-CD16/32, PE-conjugated anti-CD8a and I-A^d and allophycocyanin-CD11c from BD Biosciences or eBioscience; goat polyclonal anti-hsurvivin Ab from R&D Systems; biotin, PE, FITC, PerCP, or allophycocyanin-anti-CD25 (7D4,PC61) from BD Pharmingen; anti-mouse PE-Foxp3 (FJK-16s) staining kit from eBioscience; goat polyclonal anti-hsurvivin Ab from R&D Systems; and a biotinylated rabbit anti-goat IgG (Southern Biotechnology Associate), as a conjugated secondary Ab. Genetic engineering of survivin into the H chain of the anti-DEC205 mAb was prepared by cloning the pCMV3-FLAG, using XbaI and RsrII restriction enzyme sites. Vaccine DNA was eluted into saline at 1 µg/µl; endotoxin in purified DNA was <5 EU/ml. One or two doses of Ivsurvivin DNA were administered i.m. with or without electroporation (four pulses of 1-ms duration and field strength of 200 V cm−1). BM-derived DCs (BMDCs) were prepared with GM-CSF (35). In brief, BM cells were grown in RPMI 1640 medium containing 5% FCS and the supernatant (5% v/v) from J558L cells transduced with murine GM-CSF (provided by A. Lanzavecchia, Bellinzona, Switzerland). On day 5, the Ivsurvivin peptide library, consisting of 36 overlapping 15-mer peptides, was added in some wells at 2 µg/µl per ml. Six hours later, LPS (Sigma-Aldrich) was added at 50 ng/ml for 16 h. On day 6, cells were collected and washed with PBS. Mice were immunized once s.c. and boosted twice at 2-wk intervals, with 3 × 107 peptide-pulsed or pulsated BMDCs per mouse, each time.

Ag targetting and maturation of DCs in vivo

Overlapping 15-mer peptides, staggered by four amino acids and spanning the entire sequence of survivin and msurvivin, were synthesized by the Proteomics Resource Center of The Rockefeller University. The 36-member Ivsurvivin and msurvivin libraries were divided into four pools of nine peptides, respectively, for use as a source of Ag in immune assays for survivin-specific immunity. The respective survivin peptide pools span from 1 to 45 aa (pool 1), 35 to 88 aa (pool 2), 68 to 108 aa (pool 3), 98 to 135 aa (pool 4) of the msurvivin protein. Also, two previously identified (37) msurvivin MHC-I-restricted 9-mer peptides (msurvivin (66–74) and (98–106)) were synthesized. The respective Ivsurvivin peptide pools span from 1 to 45 aa (pool 1), 35 to 88 aa (pool 2), 68 to 108 aa (pool 3), 98 to 135 aa (pool 4) of the Ivsurvivin protein. DNA for hsurvivin or msurvivin was administered s.c. at a concentration of 50 µg per mouse with CFA or with incomplete Freund’s adjuvant. We used a FACSCalibur with data analysis in FlowJo (Tree Star). All reagents were prepared by transient transfection (calcium-phosphate) in 293T cells in serum-free DMEM supplemented with Nutridoma SP (Roche), purified on protein G-Sepharose (Amersham Pharmacia Biotech), and characterized by SDS-PAGE and Western blotting (HRP-conjugated sheep anti-mouse IgG (Amersham Biosciences), or HRP-conjugated rabbit anti-goat Igs (Southern Biotechnology Associate), as a secondary to goat anti-hsurvivin (R&D Systems). Unconjugated anti-DEC205 mAb was expressed by stably transfected Chinese hamster ovary (CHO) cells was likewise purified. All reagents were verified to be endotoxin free using the Limulus Amebocyte Lysate QCL-1000 from Cambrex. The MHC-II-restricted immunodominant hsurvivin peptide epitope (13–27 aa), identified here, was synthesized by the Proteomics Resource Center of The Rockefeller University. For vaccination, the peptide was administered s.c. at a concentration of 50 µg per mouse with CFA or with anti-CD40 (25 µg) plus poly(I:C) (50 µg). Plasmid DNA hsurvivin vaccine was prepared by cloning the hsurvivin gene into the commercial plasmid pCMV3-FLAG, using EcoRI and XbaI restriction enzyme sites. Vaccine

Flowcytometry for hsurvivin- and msurvivin-responsive T cells

Spleen cells were stimulated with pools of peptides (2 µg/µl) or medium alone in the presence of costimulatory anti-CD28 (2 µg/ml; clone 37.51) for 6 h, adding 10 µg/ml brefeldin A (Sigma-Aldrich) for the last 4 h to accumulate intracellular cytokines. In some experiments, we used previously described MHC-I binding nonamer peptides (above) to look for CD8+ T cell responses. Cells were washed, incubated for 15 min at 4°C with CD16/32 mAb to block FcγR, and stained with FITC- or PE-conjugated anti-CD3 (145–2C11) and PerCP-conjugated anti-CD4 (RM4-5) for 20 min at 4°C. The cells were permeabilized (Cytofix/Cytperm Plus; BD Biosciences) and stained with allophycocyanin-anti-IFN-γ (XMG 1.2), PE-anti-IL-2 (JESG-5H4), and PE-anti-TNF-α (MP6-XT22) mAbs for 15 min at room temperature (BD Pharmingen). We used a FACSCalibur with data analysis in FlowJo (Tree Star). All plots were gated on CD3+ cells. To assess proliferation of immune T cells in response to Ag, bulk spleen cells were labeled with CFSE

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In vivo depletion of CD25⁺ T lymphocytes

Three days before vaccination with anti-DEC205-survivin plus anti-CD40 plus poly(I:C), mice were injected with either 100 or 300 μg of purified anti-CD25 IL-2Rα mAb, a rat IgG1 Ab produced by hybridoma PC61 (38). The depletion of regulatory T cells was monitored at various time points following PC61 injection, by staining splenocytes with anti-CD25 (7D4 clone) and anti-Foxp3 Abs, with 300 μg being sufficient to deplete most CD4⁺ CD25⁺ Foxp3⁺ T cells.

In vivo cytotoxicity assay

Splenocytes from naive mice were separated and pulsed with hsurvivin or msurvivin peptide 4 (13–27 aa) (2 mM, 1 h at 37°C, 5% CO₂) (39). Peptide-pulsed splenocytes were labeled with a high dose of CFSE (5 μM), while unpulsed splenocytes were labeled with a low dose of CFSE (0.5 μM). The CFSEbright and CFSElow-labeled populations were then combined at a 1:1 ratio, and 1.5 × 10⁶ total cells were injected i.v. into mice. Twelve hours later, the ratio of CFSElow to CFSEbright cells was determined in spleen by flow cytometry. To verify that the targets for killing were MHC-II positive, we separately analyzed the CFSE-labeled cells according to labeling with I-A^d-specific mAb (AF6-120.1; BD Pharmingen). The percentage of specific killing was calculated as follows: (1 – (ratio immune/ratio naive)) × 100. Ratio equals the numbers of events of hsurvivin or msurvivin peptide-coated targets, divided by the number of events of reference targets.

FIGURE 2. DC maturation requirements for primary response to anti-DEC205-hsurvivin. A, BALB/c mice were injected i.p. with PBS, 25 μg of 1C10 mAb plus 50 μg poly(I:C), 15 μg of anti-DEC205-hsurvivin with either 25 μg of 1C10 mAb or 50 μg of poly(I:C), or the combination of 1C10 mAb and poly(I:C). Twenty-one days later, the percentage of IFN-γ⁺ CD4⁺ cells was assessed in CD3⁺ splenic T cells, using hsurvivin and msurvivin peptide libraries as recall Ag. The averages and SDs for three similar experiments are shown.

FIGURE 3. Priming of survivin-specific CD4⁺ T cells with anti-DEC205-hsurvivin, but not anti-DEC205-msurvivin, in the presence of anti-CD40 and poly(I:C). A, BALB/c mice were injected i.p. with PBS, maturation stimulus alone (25 μg of 1C10 mAb and 50 μg of poly(I:C)), 15 μg of anti-DEC205-hsurvivin and maturation stimulus, 15 μg of anti-DEC205-hsurvivin and maturation stimulus or the III/10 control Ig-survivin mAbs. Twenty-one days later, the % of IFN-γ⁺ CD4⁺ cells and IL-2⁺ CD4⁺ cells were assessed in CD3⁺ splenic T cells, using hsurvivin and msurvivin peptide libraries (survivin mix) as recall Ag. B, As in A, but the averages and SDs for three similar experiments with two mice pooled in each experiment. C, Fifteen micrograms of anti-DEC-hsurvivin Ab in combination with 25 μg of anti-CD40 and 50 μg of poly(I:C) was administered i.p. to BALB/c mice. After 21 days, IFN-γ production was monitored by ICS, following in vitro restimulation of bulk splenocytes with single peptides from the reactive hsurvivin and msurvivin peptide pool 1 (2 μg/ml). The bold underlined amino acids differ in hsurvivin and msurvivin.

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<th>Mouse Survivin Peptide</th>
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Results

Anti-DEC205 hsurvivin fusion Ab induces a high frequency of CD4+ T cells reactive with both hsurvivin and msurvivin

To assess the capacity of DCs in lymphoid tissues to initiate an immune response to the self protein, survivin, we prepared fusion mAbs of anti-DEC205-hsurvivin and anti-DEC205-msurvivin (as well as control Ig fusion mAbs; Fig. 1) and injected these into naïve BALB/c mice along with anti-CD40 and poly(I:C) to mature the DCs. The combination of anti-CD40 and poly(I:C) resulted in a much higher primary immune response than either agent by itself (Fig. 2). Twenty-one days later, we isolated bulk splenocytes and restimulated the cells with hsurvivin or msurvivin peptide libraries, each library consisting of 36 overlapping 15-mer peptides, or with either of two msurvivin MHC-I-restricted 9-mer peptides (msurvivin; 66–74) (GWEPDDNPIL) and msurvivin (85–93) (AFLTVKKQM). Immunity was monitored by intracellular cytokine staining (ICS) for IFN-γ and IL-2 production, because the responses were sufficiently large to do so. A primary cytokine-producing immune response was observed for CD4+ T cells, but not CD8+ T cells, upon restimulation with the hsurvivin and msurvivin peptide libraries (Fig. 3A), and in BALB/c, but not C57BL/6 mice (data not shown). The use of the previously identified msurvivin, MHC-I-restricted, 9-mer peptides for restimulation did not induce any detectable immune responses (CD4 or CD8) (data not shown). Also, we could not elicit IFN-γ secretion with three different H-2D mouse tumor cells (A20 B cell lymphoma, CT26 colon carcinoma, and methA fibrosarcoma), each of which expressed survivin mRNA by RT-PCR but low amounts of protein (data not shown). Only anti-DEC205-hsurvivin and not anti-DEC205-msurvivin, in combination with the DC maturation stimuli, was able to initiate the production of survivin-specific CD4+ T cells, but importantly, both hsurvivin and msurvivin peptide libraries were able to recall the immune T cells primed with hsurvivin (Fig. 3). In the first three experiments of this type, the average frequency of msurvivin-reactive IFN-γ and IL-2-producing cells was 0.36 and 0.45% of the CD4+ T cells in spleen, respectively (Fig. 3B). When we tested four pools, each comprised of nine overlapping 15-mer peptides, the major epitope for MHC-II presentation in BALB/c mice was in pool 1 of both hsurvivin and msurvivin libraries. When we tested the individual 15-mers in pool 1, the dominant mimotope for in vivo-processed survivin was between 13 and 27 aa (Fig. 3C). The sequences of hsurvivin (13–27) (FLKDHRSFTKNWPFF) and msurvivin (13–27) (YLNKRATFKNWPF) differ in the NH2-terminal amino acids. We conclude that high frequencies of self-reactive CD4+ T cells specific for a tumorigenic protein can be elicited in mice with xenogeneic survivin delivered within anti-DEC205 mAb.

Comparison of DEC-205 targeting to other methods of survivin immunization

We compared the response to DC-targeted hsurvivin with other modes of immunization. When we injected 10 μg of the MHC-II-restricted immunodominant peptide of hsurvivin13–27 with anti-CD40 and poly(I:C) maturation, or the combination of hsurvivin13–27 with unconjugated anti-DEC205 mAb, the immune responses were weak, in contrast with DC-targeted hsurvivin protein with the same maturation stimulus (Fig. 4A). A single dose of hsurvivin13–27 likewise elicited only weak immunity by the ICS and ELISPOT assays when we used CFA (Fig. 4A). Immunization of mice with the MHC-I-restricted peptide epitopes, msurvivin66–74 or msurvivin85–93, with various different adjuvants (CFA, poly(I:C), and anti-CD40), did not elicit survivin-specific CD8+ T cell immunity (data not shown). We then tested xenogeneic plasmid DNA vaccination. We performed the DNA immunization experiments with or without electroporation at the time of injection, because recent evidence suggests that electroporation improves the effectiveness of DNA vaccination by increasing DNA uptake and protein expression in various tissues in vivo (40, 41). The immune response (CD4+ or CD8+) to one or two injections of 30 μg of a DNA plasmid encoding the hsurvivin gene was either weak or undetectable in an IFN-γ ICS assay, even when followed by electroporation (four pulses of 1-ms duration and field strengths of 200 V cm−1). IFN-γ production was monitored as in A, but the averages and SD are for three similar experiments with two mice pooled in each experiment.

Comparison of DEC205 targeting to other methods of survivin immunization. A, BALB/c mice were injected with either 10 μg of empty anti-DEC205 mAb, 10 μg of anti-DEC205-hsurvivin, 10 μg of hsurvivin peptide (13–27 aa) alone or in combination with 10 μg of empty anti-DEC205 mAb, all in the presence of anti-CD40 and poly(I:C). BALB/c mice were also injected with CFA alone or in combination with MHC-II-restricted hsurvivin peptide (13–27 aa). Fifteen days later, IFN-γ production was monitored. Numbers are the percentage of CD4+ cells producing IFN-γ upon in vitro restimulation with the hsurvivin and msurvivin peptide 4 (13–27 aa). Data represent averages and SD for two similar experiments. B, BALB/c mice were injected with PBS, anti-DEC205-hsurvivin, or control Ig-hsurvivin (10 μg) in the presence of anti-CD40 and poly(I:C), DNA encoding the hsurvivin gene (30 μg) i.m. without or with electroporation (four pulses of 1-ms duration and field strengths of 200 V cm−1). IFN-γ production was monitored as in A, but the averages and SD are for three similar experiments with two mice pooled in each experiment.
Responses to anti-DEC205-hsurvivin cross-react efficiently with msurvivin but not other mouse self-Ags

Central tolerance mechanisms reduce the number of self-reactive cells, especially those with a higher affinity for Ag recognition (42). To evaluate the efficiency of anti-DEC205-hsurvivin immunization, as well as the functional affinity of the immune T cells, we injected naive BALB/c mice with three different doses (30, 10, and 3 \( \mu \)g) of either anti-DEC205-hsurvivin or control Ig-hsurvivin in the presence of anti-CD40 and poly(I:C). Twenty-one days later, the percentage of IFN-\( \gamma \)-CD4\(^+\) cells and IL-2-CD4\(^+\) cells was assessed in CD3\(^+\)-splenic T cells, using hsurvivin and msurvivin peptide libraries (2 \( \mu \)M). As in A, but the splenocytes were restimulated in vitro with different concentrations of hsurvivin and msurvivin peptide libraries ranging from 2 to 0.02 \( \mu \)M. One of two similar experiments.

Long-term memory after primary immunization with anti-DEC205-hsurvivin

To begin to assess the generation of memory, we tested whether immunized mice would develop secondary responses of a greater magnitude than the primary response. We immunized naive BALB/c mice with either PBS or anti-DEC205-hsurvivin (5 \( \mu \)g) with anti-CD40 and poly(I:C). Sixty days later, each group of mice was boosted with PBS, anti-DEC205-hsurvivin (10 \( \mu \)g) alone, or anti-DEC205-hsurvivin (10 \( \mu \)g) with either anti-CD40 or poly(I:C), or the combination of anti-CD40 and poly(I:C), as maturation stimuli. Seven days later, we isolated bulk splenocytes and performed both ICS for IFN-\( \gamma \) production, in a rapid restimulation assay for effector memory cells (Fig. 6A) and proliferation by CFSE dilution as well as CD62L expression, during 4 days of response to Ag in culture (Fig. 6B). As shown in Fig. 6A, persisting effector cells were no longer detected in the nonboosted groups (see PBS column), but a higher percentage of survivin-specific T cells, relative to the primary response, was noted in the boosted

![FIGURE 5.](http://www.jimmunol.org/)
mice (see arrows). When we assessed memory with a CFSE dilution assay, we again found that boosting with anti-DEC205-hsurvivin, even without full DC maturation, expanded the frequency of proliferating cells, which were both CD62Llow (Fig. 6B; see first arrow from left) and capable of producing IFN-γ (data not shown).

Although DC maturation stimuli were necessary during the primary immunization, some degree of boosting was evident in the absence of these maturation stimuli (Fig. 6; e.g., black arrowheads in A). These data indicate that DC-based immunization with anti-DEC205-hsurvivin induces memory cells that have reactivity to msurvivin.

**CD25+ T cell depletion amplifies the CD4+ T cell response to anti-DEC205-hsurvivin**

Because immune responses to self and foreign Ags can be negatively regulated by CD25+ suppressor T cells (43, 44), we examined whether depletion with an anti-CD25 mAb (clone PC61) can increase effector CD4+ T cell responses. We injected naive BALB/c mice with either PBS or 300 μg of PC61, and 3 days later, we immunized each group with PBS, anti-CD40 and poly(I:C), anti-DEC205-hsurvivin (15 μg per mouse) in the presence of anti-CD40 and poly(I:C), or control III/10-hsurvivin in the presence of anti-CD40 and poly(I:C) (Fig. 7). We verified at the time of immunization and 21 days later that CD4+CD25+Foxp3+ T cells were depleted by PC61 (data not shown). We found that the depletion of CD4+CD25+ regulatory T cells with 300 μg of PC61 amplified the survivin-specific CD4+ T cell response, induced by immunization with anti-DEC205-hsurvivin in the presence of anti-CD40 and poly(I:C) (Fig. 7). A dose of 100 μg of PC61 did not amplify immunity but also only reduced the number CD4+CD25+ T cells by ~50% (data not shown). We also observed sizable increases in immunity to both hsurvivin and msurvivin when we measured T cell proliferation in a CFSE dilution assay (see arrows in Fig. 7C) and increased IFN-γ and IL-2 production in an ICS assay (Fig. 7, A and B). However, the enhanced survivin-specific CD4+ T cell response was only seen upon immunization with anti-DEC205-hsurvivin and not anti-DEC205-msurvivin (data not shown), and PC61 treatment was not capable of allowing for the development of a CD8+ T cell response following immunization with anti-DEC205-hsurvivin. These data indicate that CD4+ T cell immune responses to a self tumor Ag can be enhanced by treatment of mice with PC61 mAb at a dose sufficient to deplete CD4+CD25+Foxp3+ suppressor T cells for prolonged periods.

**Immunization with anti-DEC205-hsurvivin induces cytolitic CD4+ T cells**

To determine whether the immunization protocol in Fig. 7 could expand cells with in vivo cytolytic function, we immunized control mice or mice depleted of regulatory T cells with PC61 with anti-DEC205-hsurvivin and anti-CD40 and poly(I:C), or with control Ig-hsurvivin. Thirty-five days postimmunization, we injected the mice with 15 x 10^6 BALB/c spleen cells of two types: one pulsed with peptide 4 from the hsurvivin or msurvivin peptide pool 1 and labeled with CFSE^{high} (5 μM) and the other un pulsed but CFSE^{low}-labeled (concentration of 0.5 μM). Effective and specific CTLs were observed in the lymph nodes (data not shown) and spleens of mice immunized with anti-DEC205-hsurvivin in the presence of anti-CD40 and poly(I:C), with greater responses upon depletion of CD4+CD25+ regulatory T cells (see arrows in Fig. 8, A, top row, and C, left). After 12 h in vivo, ~50% of the CFSE^{high}-labeled, MHC-II^{high} donor cells pulsed with hsurvivin peptide were lost, compared with the reference CFSE^{low} labeled, unpulsed...
donor population, in the immunized mice depleted of CD4+CD25+ regulatory T cells. The percentage loss of CFSEhigh-labeled, MHC-IIhigh donor cells pulsed with hsurvivin peptide was reduced to 38% in immunized mice not depleted of CD4+CD25+ regulatory T cells, (see arrows in Fig. 8, A, second row, and C, left). CFSEhigh-labeled, MHC-IIhigh donor cells pulsed with msurvivin peptide showed a smaller ~20% level of killing (see arrows in Fig. 8, B and C, left). To establish the importance of MHC-II presentation, we verified that peptide-pulsed MHC-II low donor cells in the same recipient mouse were not eliminated (right half of Fig. 8). Therefore, the CD4+ T cells that are actively immunized to msurvivin are capable of some cytolytic activity.

**Immunity to anti-DEC205-hsurvivin includes a high frequency of TNF-α-producing CD4+ T cells**

Several effector mechanisms have been demonstrated in the lysis of target cells in vitro by CD4+ T cells, including TNF-α (45), Fas ligand (FasL) (46), and perforin (47). To implicate a mechanism for CD4-directed killing in vivo, following immunization with anti-DEC205-hsurvivin, in the presence of anti-CD40 and poly(I:C), after depletion of CD4+CD25+ regulatory T cells (using PC61), we evaluated production of FasL, perforin, and TNF-α by hsurvivin and msurvivin peptide-responsive IFN-γ producers in an ICS assay. Neither FasL nor perforin could be detected (data not shown). However, the ICS data show that CD4+ T cells, from mice immunized with anti-DEC205-hsurvivin with anti-CD40 and poly(I:C), following depletion of CD4+CD25+ regulatory T cells, produce TNF-α, in response to in vitro stimulation of hsurvivin and msurvivin peptide libraries (Fig. 9).

**Discussion**

**Classes of tumor Ags**

Mutated self-Ags that are associated with tumor development can serve as rejection Ags in mice (45, 48, 49). These mutant self-Ags can contribute to tumorigenesis, but different mutations are typically...
found in different examples of a particular type of tumor (50). In contrast, many of the alterations that are shared among tumors and are likely to drive different components of carcinogenesis represent normal, nonmutated self-Ags (51–55). Immune responses to these nonmutated self-Ags in tumors are typically weak, primarily due to central and peripheral mechanisms for self tolerance. Nonetheless, the thymus allows for the formation of low-affinity T cells specific to self-Ags (56, 57). The purpose of this paper was to use as Ag a nonmutated self protein important in tumorigenesis (in our case, survivin) and identify mechanisms required to elicit strong immunity, particularly a CD4^+ helper T cell response. By strong, we mean T cell responses that are 0.1–1.0% of total CD4^+ T cells, involve the expression of IFN-γ, IL-2, and TNF-α, and are associated with T cell memory, including proliferation.

**A new strategy for enhancing CD4^+ T cell immunity to tumors**

Cancer immunology and immunotherapy has focused on CD8^+ CTL, due to their ability to recognize and lyse MHC-I^+ tumor cells. However, CD4^+ T cells play a crucial role in immunity to tumors as well. They provide help to CD8^+ T cells, produce cytokines that are important for immune response, and can directly lyse tumor cells. In this section, we describe a new strategy for enhancing CD4^+ T cell immunity to tumors using survivin as an Ag.

**FIGURE 8.** Immunization with anti-DEC205-hsurvivin and adjuvants induces long-lived, survivin-specific, MHC-II-restricted killing. BALB/c mice were injected i.v. with either PBS or 300 μg of PC61. Three days later, each group was immunized with PBS, anti-CD40 (25 μg) and poly (I:C) (50 μg), anti-DEC205-hsurvivin, or control III/10 Ig-hsurvivin (15 μg per mouse) in the presence of anti-CD40 and poly(I:C). Thirty-five days later, the immunized mice were injected i.v. with 7 × 10^6 each of syngeneic splenocytes pulsed with hsurvivin (A) or msurvivin (B) peptide 4 (CFSE^+^) and unpulsed (CFSE^-^), to detect active killer cells in the lymph nodes and spleen (see arrows). Histograms are gated on MHC-II^high^ cells (top and bottom left panels), or MHC-II^low^ cells (top and bottom right panels). C, Averages and SD for two experiments for percentage lysis of hsurvivin or msurvivin peptide-pulsed targets as in A and B, for MHC-II^high^ cells (left) and MHC-II^low^ cells (right).

**FIGURE 9.** Survivin-specific CD4^+ T cells produce TNF-α. BALB/c mice were injected i.v. with 300 μg of PC61. Three days later, each group was immunized with PBS, anti-CD40 and poly(I:C), anti-DEC205-hsurvivin, or control III/10-Ig hsurvivin (15 μg per mouse) in the presence of anti-CD40 and poly(I:C). Twenty-one days later, the percentages of TNF-α^+^ CD4^+^ T cells relative to isotype control Ab were assessed in gated CD3^+^ splenic T cells using hsurvivin and msurvivin peptide libraries.
cells (4, 58). Various strategies have been developed to induce CD8\(^+\) T cell responses to tumor Ags, including adoptive transfer of tumor Ag-specific CD8\(^+\) T cells (5), engineering of MHC-I binding tumor peptides into viral (59) or bacterial (60) expression vectors, transfection of DCs with defined RNA for tumor Ags or bulk RNA from tumors (61, 62), and loading of DCs with MHC-I binding tumor peptides or tumor cell lysates (63, 64) and naked plasmid DNA (65, 66). To date, trials in cancer patients have demonstrated that these approaches are able to induce some Ag-specific CD8\(^+\) T cell responses, including IFN-\(\gamma\) production (63, 67). However, the CD8\(^+\) T cell responses have typically been short lived (68), small in magnitude relative to antiviral responses, and typically not associated with objective tumor regression in the patients with advanced cancer that have been tested (69–71).

To improve on the induction of tumor immunity to tumors, one possibility is to consider a need for CD4\(^+\) T cell help to sustain memory CD8\(^+\) T cells. “Helped” CD8\(^+\) T cells are able, upon encounter with their cognate tumor Ag, to proliferate and acquire cytolytic capabilities (72). Thus, the induction and efficacy of a vaccine-induced antitumor response, even when aimed at solid tumors that themselves lack MHC-II (6), in many cases requires the cooperation of CD4\(^+\) Th cells (73).

In this study, we evaluated the hypothesis that the direct targeting of a xenogeneic form of a self-Ag, survivin, to maturing DCs in lymphoid tissues via the DEC205 receptor might be able to overcome barriers to eliciting immunity to conserved self proteins important in tumorigenesis. Previously, it was shown that xenogeneic forms of an Ag were better able to induce CD8\(^+\) T cell responses to the corresponding self protein (65). To target hsurvivin to mouse DCs in situ, we used a strategy developed previously with foreign model (31, 34) and microbial (32, 33) Ags. In this approach, antigenic peptides or proteins were engineered as fusion proteins in the H chain of anti-DEC205/CD205 mAbs. Ag-based targeting then enhanced Ag presentation in vivo to CD4\(^+\) and CD8\(^+\) T cells at least 100-fold relative to nontargeted Ag. We found that DC targeting with anti-DEC205-hsurvivin fusion mAb could overcome tolerance to this overexpressed self/tumor Ag and induce survivin-specific CD4\(^+\) T cell immunity of improved quantity and quality relative to prior reports with other shared, nonmutated tumor Ag vaccines.

The basis for the improved immunity via anti-DEC205 targeting relative to other standard approaches could be severalfold: 1) Ab targeting ensures that large numbers of DCs systemically are presenting Ag (31), whereas other approaches typically load small numbers of DCs in draining lymph nodes; 2) there is evidence that DEC205 is a specialized endocytic receptor, able to recycle through MHC-II compartments and markedly enhance presentation on MHC-II (74); and 3) DEC205 is expressed on a subset of DCs that may be more effective in inducing Th1 T cell immunity (75). Resolution of these possibilities might benefit from targeting approaches that examine other DC subsets and other receptors on the DEC205\(^-\) DC subset.

**Evidence for improved CD4\(^+\) T cell immunity to survivin following targeted delivery to maturing DCs in vivo**

We obtained several lines of evidence for improved CD4\(^+\) T cell immunity to survivin following targeted delivery to maturing DCs in vivo. First, the CD4\(^+\) T cell response to survivin was sizable relative to prior studies on this limb of cell-mediated immunity. Following a single immunization of naive BALB/c mice with anti-DEC205-hsurvivin, but not anti-DEC205-msurvivin, in the presence of anti-CD40 and poly(I:C) as DC maturation stimuli, 0.36 ± 0.05% of the CD4\(^+\) T cells made IFN-\(\gamma\) and 0.45 ± 0.05% made IL-2 in response to msurvivin, while the response to hsurvivin was 2- to 3-fold greater. Only 3–10 \(\mu\)g of the anti-DEC205-hsurvivin (which corresponded to 0.3–1.0 \(\mu\)g of hsurvivin protein) could generate these responses. With an IFN-\(\gamma\) ELISPOT assay, the same immunization induced ~300 spots per 3 \(\times\) 10\(^5\) CD4\(^+\) T cells upon in vitro hsurvivin peptide restimulation vs 10 spots per 3 \(\times\) 10\(^5\) CD4\(^+\) T cells in the absence of in vitro peptide restimulation (data not shown).

Although we were unable to induce survivin-specific, CD4\(^+\) T cells by injecting DCs loaded with survivin peptide, others have succeeded in inducing some IFN-\(\gamma\) producing cells by an ELISPOT assay, following multiple doses of DCs pulsed with a melanoma peptide in humans with cancer (76). Soares et al. (53) also immunized mice with three injections of human MUC-1 peptide-pulsed DCs at 3-wk intervals. The CD4\(^+\) MUC-1 T cell response was ~160 ELISPOTs in immune lymph nodes with a background of 120 spots. A current limitation to the use of ex vivo-derived DCs as adjuvants is that only a few percent of the injected cells arrive in the lymph nodes, whereas DEC-targeting loads large numbers of DCs that express DEC and systemically (31).

Other groups have used MHC-II-restricted peptide epitopes of self/tumor Ags (e.g., hTERT, wild-type p53, carcinoembryonic Ag (CEA)) in their soluble form. In these studies, immunization required high doses of Ag and two or three injections to overcome tolerance and induce a CD4\(^+\) T cell response (45, 77). For example, HLA-DR4-transgenic mice had to be immunized twice with 100 \(\mu\)g per injection of an MHC-II-restricted peptide from human telomerase reverse transcriptase (htERT), emulsified in CFA, to detect hTERT-specific CD4\(^+\) T cell responses (77). After two immunizations, the hTERT-specific CD4\(^+\) T cell responses were 140 spots per 10\(^6\) bulk splenocytes upon in vitro htERT peptide restimulation in an IFN-\(\gamma\) ELISPOT assay vs 10 spots per 10\(^6\) bulk splenocytes in the absence of in vitro peptide restimulation. Similarly, high doses (50 \(\mu\)g per mouse) of wild-type and mutant mouse p53 peptides in CFA were necessary for the induction of p53-specific CD4\(^+\) T cell responses in naive mice (45).

A second consequence of DEC205 targeting was that the immune T cells had a relatively good functional affinity. We have used mimotope peptides derived from an overlapping library of 15-mers spanning the full length of hsurvivin or msurvivin rather than naturally processed peptides, but even so, relatively low doses of the mimotope peptides (0.02 \(\mu\)g/ml for the human peptide and 0.2 \(\mu\)g/ml for the mouse peptide) were required to activate the population of primed T cells in the immune assays. Previous studies have used 10- to 500-fold higher peptide concentrations for in vitro restimulation of bulk splenocytes (54, 55, 77). For example, to elicit the CD4\(^+\) T cells primed in vivo with htERT peptide emulsified in CFA (in HLA-DR4-transgenic mice), bulk splenocytes were restimulated in vitro with 20 \(\mu\)g/ml htERT peptide (77). Similarly, to elicit T cells primed and boosted in vivo with recombinant vaccinia-CEA, the CD3\(^+\) T cells were restimulated in vitro with 50 \(\mu\)g/ml human CEA (54, 55).

A third consequence, which has not been reported with other strategies to immunize helper T cells, was that the CD4\(^+\) T cell response was long lasting, and upon boosting, effector memory was noted. The use of a maturation stimulus during the primary immunization was necessary to induce long-term effector memory CD4\(^+\) T cells (CD62L\(^{$low}$)) that could be boosted with anti-DEC205-hsurvivin. However, memory T cells with a central memory phenotype (CD44\(^{high}\)CD62L\(^{$low}$)) were not detected. A similar result was reported recently by Benigni et al. (78). They found that injection of LACK-expressing tumor cells into 16.2 mice (LACK is a protein Ag from *Leishmania major*) and 16.2 mice express a
TCR Tg β-chain derived from a LACK-specific hybridoma) induces the generation of CD4 effector T cells (CD4L2.1high and capable of IFN-γ production) in tumor-draining lymph nodes (78). The authors also report that LACK-specific CD4+ T cells of a central memory phenotype (IL-2+ LACK-specific CD4highCD62Lhigh) could be found within the peripheral lymphoid tissues of mice, only if LACK-expressing tumors (the only cell type in the mouse expressing the Ag) were surgically resected. This result is in accordance with infectious disease models, which have shown that complete Ag clearance favors the development and survival of central T cell memory (79, 80). The absence of central memory CD4+ T cells in our system may thus be explained by the fact that survivin is a normal self-Ag and is thus able to stimulate the memory cells induced by DEC205 targeting. Alternatively, DEC205 targeting may induce predominantly effector-type memory cells.

Fourth, we noted that the CD4+ T cell-dependent immune response could be further amplified if CD4+CD25+ regulatory T cells were first depleted with PC61 anti-CD25 mAb before immunization. Sakaguchi and coworkers (38) have emphasized the amplifying effect of CD25 depletion in most experimental mouse tumors. Furthermore, Schreiber et al. (81) have reported a selective accumulation of CD4+CD25+ T cells inside tumors, especially at late stages of tumor progression, while depletion of these T cells during the effector phase successfully enhanced antitumor immunity. Previous studies demonstrating enhanced immunotherapy following depletion of CD4+CD25+ regulatory T cells with PC61 mAb have used either viral tumor Ags or MHC-I-restricted tumor/self-Ags (82–84). Casares et al. (82) studied an MHC-II-restricted epitope derived from the sequence of murine leukemia virus gp70 envelope protein, which is a tumor rejection Ag expressed by CT26 but not an authentic self-Ag. Jones and Gallimore (83) demonstrated that treatment of mice with anti-CD25 mAb facilitated long-term CD4+ T cell-mediated tumor immunity, as indicated by the fact that these cells were able to reject B16F10 tumor cells when injected into B6.RAG2−/− mice in the absence of CD8+ T cells. When specific melanocyte differentiation Ags were tested, all the mice that had rejected melanoma 8 mo previously and that exhibited signs of depigmentation were also protected against infection with recombinant vaccinia vector-expressing tyrosinase, but not Trp2, gp100, MART-1, or Trp1 (83). Other studies have concentrated on demonstrating enhanced CD8+ T cell responses, upon depletion of CD4+CD25+ regulatory T cells, to MHC-I-restricted tumor/self-Ags, such as gp100 (84), Trp2 (85), and Her2 Neu (86) in various tumor models. Our findings represent the first time that depletion of regulatory T cells has been shown to augment CD4+ T cell reactivity to a shared nonmutated tumor/self-Ag.

Fifth, we were also able to elicit survivin-specific, cytolytic CD4+ T cells. The survivin-dependent CTLs acted on MHC-IIhigh targets in vivo, and we were able to identify high frequencies of TNF-α-producing CD4+ T cells as well. Several effector mechanisms have been demonstrated for the lysis of target cells in vitro by CD4+ T cells, including TNF-α (87), FasL (46), and perforin (47). Neither FasL nor perforin could be detected on our survivin-responsive T cells (data not shown). Therefore TNF-α is a possible mediator of the lysis we observed.

**Inability to observe CD8+ T cell responses to survivin following DEC-205 targeting**

Despite the capacity of anti-DEC205-targeting to induce survivin-specific, CD4+ T cell immunity, we were unable to detect CD8+ T cell immunity even when we used previously described MHC-I binding survivin peptides for BALB/c mice (37). Previous studies have used survivin as a potential cancer vaccination target and have shown the induction of survivin-specific CD8+ T cell responses, leading to protection from a survivin-expressing tumor challenge. For example, vaccination of BALB/c mice with 5 × 10⁵ DCs transfected with murine survivin RNA (three s.c. injections) leads to long-term resistance to challenge by A20 lymphoma (88). Two MHC-I-restricted peptide epitopes derived from the murine survivin protein were identified, and BALB/c mice treated with syngeneic DCs pulsed with the two survivin epitopes were able to reject an otherwise lethal tumor inoculation of the A20 B cell lymphoma (37). In addition, a DNA vaccine encoding murine survivin and the secretory chemokine CCL21, orally delivered by doubly attenuated Salmonella typhimurium (dam− and AraA−) to secondary lymphoid organs, elicited marked activation of DCs and an effective CD8+ T cell immune response. This resulted in eradication or suppression of pulmonary metastases of non-small cell lung carcinoma in both prophylactic and therapeutic settings in C57BL/6J mice (89).

In contrast, we have been unable to induce a CD8+ T cell response to these previously defined survivin peptides by immunizing with peptides in CFA (both 15-mer peptide mixtures or two nonmeric MHC-I binding peptides), or peptides pulsed onto DCs, or plasmid DNA encoding survivin under the control of a CMV promoter, or DCs loaded with dying A20 lymphoma cells. We think that two factors may have limited the induction of CD8+ T cell immunity. The repertoire for this self protein may be too small to allow for the detection of immunity with only one or two immunizations. Secondly, in the case of the anti-DEC205 targeting strategy, the current mAb may be suboptimal for eliciting CD8+ T cells, although clearly some CD8+ T cell immunity is observed with anti-DEC targeting of foreign proteins like OVA, HIV gag, and malaria circumsporozoite protein (31–33).

**Inability to observe protection against A20 lymphoma growth following DEC-205 targeting**

We also were unable to document protection of immunized mice to an in vivo tumor challenge by A20 lymphoma cells expressing msurvivin mRNA (data not shown). This result is in contrast to our earlier data with anti-DEC205-OVA conjugates, which when administered in conjunction with anti-CD40, proved to be a superior means to protect mice against an OVA-expressing tumor challenge (31). One possible explanation for this discrepancy is that survivin is a self-Ag that has presumably induced some tolerance at thymic and/or peripheral levels, in contrast to the foreign Ag, OVA. Furthermore, the MHC-I-restricted peptide epitope of the OVA protein (SINFEKL) may be easier to process or following processing, it binds more stably to the MHC-I molecule, leading to enhanced CD8+ T cell activation, compared with candidate CD8+ epitopes in the survivin protein. We also note that expression of msurvivin in mouse tumor cell lines like A20 lymphoma is quite low, using immunoblotting or FACS with goat anti-survivin polyclonal Ab (data not shown). This may have reduced the chance for recognition by T cells immunized by anti-DEC-survivin Abs. We are currently testing other immunization strategies to determine whether CD8+ T cell responses to survivin can be induced in the BALB/c mice used in our experiments.

In summary, targeting via the DEC205 receptor on maturing DCs within lymphoid tissues allows CD4+ T cell immunity to be generated to a shared nonmutated self-Ag that is overexpressed in human cancers (19–21). Therefore, the CD4+ helper T cell repertoire need not be blind to hyperexpressed self-Ags that drive the pathogenesis of cancer.
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