Differential Suppression of Tumor-Specific CD8+ T Cells by Regulatory T Cells

Edward James, Alex Yeh, Cathy King, Firouzeh Korangy, Ian Bailey, Denise S. Boulanger, Benoît J. Van den Eynde, Nicholas Murray and Tim J. Elliott

*J Immunol* 2010; 185:5048-5055; Prepublished online 4 October 2010; doi: 10.4049/jimmunol.1000134

http://www.jimmunol.org/content/185/9/5048

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2010/10/04/jimmunol.1000134.DC1

**References**

This article cites 42 articles, 21 of which you can access for free at:

http://www.jimmunol.org/content/185/9/5048.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Differential Suppression of Tumor-Specific CD8+ T Cells by Regulatory T Cells

Edward James,* Alex Yeh,*† Cathy King,* Firouzeh Korangy,† Ian Bailey,* Denise S. Boulanger,* Benoît J. Van den Eynde,‡ Nicholas Murray,* and Tim J. Elliott*

In the CT26 BALB/c murine model of colorectal carcinoma, depletion of regulatory T cells (Tregs) prior to tumor inoculation results in protective immunity to both CT26 and other BALB/c-derived tumors of diverse histological origin. In this paper, we show that cross-protection can be conferred by adoptively transferred CD8+ CTLs. Other schedules for inducing immunity to CT26 have been described, but they do not lead to cross-protection. We show that Treg ablation facilitates the development of new CTL specificities that are normally cryptic, and have mapped the root epitope of one of these responses. This work has allowed us to demonstrate how the specificity of CTL responses to tumor Ags can be controlled via differential suppression of CTL specificities by Tregs, and how this can result in very different physiological outcomes. The Journal of Immunology, 2010, 185: 5048–5055.

Studies in mouse models of cancer have shown that the acquired immune system can prevent both onset and progression of tumors and that Ag-specific T cells may confer lifelong protection against malignancy (1). Descriptive studies in human cancers, particularly melanoma, ovarian cancer, and colonic cancer, have strongly suggested that the presence of tumor-infiltrating lymphocytes is associated with better survival outcomes (2–4). Tumors, however, possess many strategies for evading the immune system. One critical pathway is the induction of tumor Ag-specific immune tolerance mediated through the generation of T regulatory cells (Treg), and several lines of evidence support the immunosuppressive role of Tregs in influencing cancer prognosis (5, 6). There is an increase in the number of CD4+CD25+ Tregs in the peripheral blood of cancer patients in parallel with advancing stage of disease, and this plays a major part in the failure of many immunotherapies against tumors. Patients with early non-small cell lung and breast cancer with higher proportions of tumor Tregs relative to tumor-infiltrating lymphocytes had a significantly higher risk of recurrence, and in melanoma this predicted reduced patient survival (7–9). In ovarian cancer, the absolute numbers of Tregs in tumor tissue was shown to be a significant, independent predictor of mortality even after controlling for stage, surgical debulking, and other factors known to affect survival (5). Patients with high numbers of Tregs in ovarian tumor tissue were observed to have a mortality of 100% at 5 y, compared with 50% in the low-Treg group. In addition, Tregs capable of inhibiting tumor-associated Ag-specific immune responses were increased in patients with colorectal cancer (10). These data indicate an important role of Tregs in modulating the antitumor immune response, and the induction of Tregs may present a significant barrier to the successful application of anticancer immunotherapy.

In mouse models, depletion of Tregs generally results in better immune protection against tumors (11–14), and we have shown that mice depleted of Tregs can reject the colorectal tumor line CT26, which is not normally immunogenic. The rejection of the primary tumor was accompanied by development of protective immunity, because mice vaccinated with CT26 were able to reject the same tumor when rechallenged 50–74 d later. Both CD4+ and CD8+ T cells could mediate immunity to the second CT26 challenge. In addition, mice rendered CT26 immune following anti-CD25 mAb treatment were able to reject other tumor lines of diverse histological origin (12).

Our results indicate, therefore, that depletion of Tregs uncovers cryptic responses to Ags that are shared among different tumor cell lines. CT26-specific T cell responses can be elicited by different forms of vaccination in the presence of regulatory cells, but in these cases T cell responses are highly focused on a single tumor-specific epitope, AH1 (corresponding to amino acids 423–431 of the endogenous murine leukemia virus [MuLV], gp90 gene), presented by MHC class I H-2Ld (15).

Taken together, these data suggest that immune responses to some Ags are more tightly regulated than others. This may be a consequence of the types of Ags that have the ability to activate Tregs. We therefore undertook to identify the Ag or Ags recognized by cross-protective CD8+ CTLs and to determine the immunological correlates of cross-protection.

Materials and Methods

Mice, Abs, and in vivo depletion

BALB/c mice were bred locally under specific pathogen-free conditions in Southampton, U.K. Female or male mice (6–8 wk old) were used in all experiments. During experimental procedures, mice were housed in conventional facilities. Hybridomas secreting CD25 (PC61, rat IgG1)-specific

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10/$16.00
mAb have been described previously (16, 17) and were grown in culture and mAb purified by precipitation in saturated ammonium sulfate. For depletion, mice received 1 mg mAb PC61 injected i.p. in volumes of 100–200 µl, twice with a 1-d interval. The Ab was administered 3 and 1 d prior to the injection of tumor cells.

Tumor cells
Cultures of the cell lines, CT26, CT26/GM-CSF, P815, MCFL, Renca, and A20 (American Type Culture Collection, Manassas, VA), were maintained in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FCS (Globepharm, Surrey, UK), 2 mM L-glutamine, penicillin/streptomycin (Sigma-Aldrich), 50 µM 2-ME, 1 mM sodium pyruvate (Life Technologies-BRL, Rockville, MD), and 1mM HEPES (PAA Laboratories, Dortmund, MA). CT26/GM-CSF was derived as described (18). BCL1 was passaged in vivo only (19). MCFL was a generous gift from Dr. Mario Colombo (Istituto Nazionale Tumori, Milan, Italy). In all experiments, mice were injected s.c. with 10^5 tumor cells in PBS. All these cell lines were tested and found to be mycoplasma free.

Isolation and adoptive transfer of lymphocyte subsets
Single-cell suspensions were prepared from the spleens of donor mice. The cells were labeled with MACS Abs for CD4^+ and CD8^+ T cells and purified using MACS V+ separation columns according to the manufacturer’s instructions (Miltenyi Biotec, Bisley, U.K.). Following elution of the purified cells off the columns, the purity of the cells (>90%) was confirmed by FACS analysis. Cells were resuspended in PBS at a concentration of 5 × 10^6/ml. Mice were injected i.v. with 200 µl cell suspension.

Cell lines and hybridomas
Generation of CTL lines, clones, β-galactosidase (lacZ)-inducible T cell hybrids has been described (20, 21). Briefly, spleen cells from CT26-immune Treg-depleted BALB/c mice were removed of CD4^+ T cells, as described above, and stimulated in vitro every 12–14 d with irradiated CT26 cells and 20 IU/ml IL-2. CTL clones were generated using limiting dilution of the parental CTL lines. The lacZ-inducible T cell hybridoma CCD2Z was generated by fusing the CTL clone CT26-specific B6.H2^b restricted T cell clone 2 (CCD2) with the BWZ.36/CD8α fusion partner, as described (21).

T cell activation assays
LacZ-inducible T cell hybrids were cocultured with APCs that either were transfected with Ag cDNAs or were pulsed with exogenous peptides. The lacZ activity was measured as described previously (22). CTL lysis assays were performed with 51Cr-labeled tumor cells. WEHI killing was carried out using supernatant from CTL lines/clones cultured in the presence of tumor cells, and cocultured overnight. Supernatant from the overnight cultures was incubated for 1 h at 4˚C with anti–H-2Dd (34-5-8S), -Ld (B22.249) or Kd (B7) Abs, washed with PBS and extracted with 800 µl chilled 10-KDa filter (Millipore, Billerica, MA). The cDNA library was screened in pools of 50–100 CFUs by transfection with recombinant plasmids, as described previously (23, 24). A cDNA library using mRNA from CT26 cells was cloned into the EcoRI site of the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA). The cDNA library was screened in 96-well plates by forming bacteria with recombinant plasmids, as described previously (23, 24).

Statistical analysis
Analyses were performed using the Prism software (GraphPad, San Diego, CA). The p values were calculated using either the two-tailed unpaired t test or the paired t test.

Expression constructs and peptides
All plasmid constructs were in the expression vector pcDNA3. Nested deletions were generated by PCR of the P20E8A1 cDNA with a vector-specific forward primer and a P20E8A1-specific reverse primer (C2’-GGTTCTAGAGTGGTTCTAGAATTCTGAGACACCCGAG-3’); C3 (5’-GACTCTAGAATTCTGGCTGCGCAGCTG-3’), C7 (5’-GACTCTAGAATTCTGGCTGCGCAGCTG-3’), C8 (5’-GACTCTAGAATTCTGGCTGCGCAGCTG-3’), C9 (5’-GACTCTAGAATTCTGGCTGCGCAGCTG-3’), C10 (5’-GACTCTAGAATTCTGGCTGCGCAGCTG-3’) and C12 (5’-GACTCTAGAATTCTGGCTGCGCAGCTG-3’) with KOD polymerase (Merck, Darmstadt, Germany). PCR fragments were digested with EcoRI and XbaI and cloned into the corresponding sites of pcDNA3. The synthetic peptides GSA9 (GGPESPYCFA), GS10 (GGPESPYCFS), GS11 (GGPESPYCFS), GS12 (GGPESPYCFS), GFC13 (GGPESPYCFSWGC), GSF11 (GGPESPYCFS), GSF11 (GGPESPYCFS), AH1 (SPSVYVYHQF) (15), QL9 (QLSPFFPD) (25), and YF9 (YGPSPYRFF) (26) were purified by HPLC and confirmed by mass spectrometry (GL Biochem, Shanghai, China).

Extraction and HPLC analysis of endogenously processed peptides
Total acid-soluble peptide pools from tumor cells CT26, CT26/GM-CSF, A20, and MCFL were extracted as described (27). Briefly, 10^5 CT26 and CT26/GM-CSF, 4 × 10^5 MCFL, 10^5 A20, 10^5 Renca, and 10^8 P815 cells were washed with PBS and extracted with 800 µl 10% boiling formic acid in the presence of a 10-µM “martyr” peptide ASNENMETM for 10 min. Cellular debris was removed by centrifugation and fractionated by HPLC after filtration through a 10-KDa filter (Millipore, Billerica, MA). Reverse-phase C18 column (Grace-Vydac, Hesperia, CA, 2 × 25 mm, 5 µm, 300 Å) was run in 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). The gradient was from 17 to 45% (solvent B) at a rate of 1%/min. Flow rate was maintained at 0.25 mll/min, and 150-µl fractions were collected and dried in a vacuum centrifuge. Mock injections with sample buffer alone were performed prior to each extracted sample, using the same column and identical run conditions. The collected fractions were assayed in the same experiment, using the same APCs and T cells, in parallel with fractions from the cell extracts and synthetic peptide standards.

RMA-S stabilization
RMA-S-D^3-L^3 cells were cultured overnight at 26˚C and then for 60 min in the presence of a saturating concentration of peptide (100 µM). The cells were washed twice with PBS, then cultured for the indicated times at 37˚C. Cells were taken at the indicated times and stained with either anti-D^3 Ab (34-5-8S) or anti-L^3 Ab (30-5-7S) mAbs conjugated with PE (BD Biosciences, San Jose, CA). The cells were washed twice and analyzed by FACS (BD Bioscience). Flow cytometry analysis was performed using FlowJo software (Tree Star, Ashland, OR).

CD8 T cell responses
Spleen cells from CT26-challenged mice (Treg depleted or replete) were harvested between days 12 and 14. The CD8^+ T cell responses to the CT26 Ags GSW11 and AH1 were measured by assay of inducible IFN-γ by those cells in response to incubation with peptide or CT26, CD8^+ T cells, APCs, and peptides/tumors were cultured together for 4 h in the presence of a 10^-5 Ab before being stained for surface CD8 and intracellular IFN-γ (BD Biosciences). Cells were then analyzed on a FACS (BD Biosciences) and data analyzed with FlowJo software (Tree Star). Numbers are reported as above the background response of T cells alone. For in vitro restimulation, 5 × 10^6 spleen cells were cultured with 20 IU/ml IL-2 (PeproTech, Rocky Hill, NJ) and 1 µM peptide (AH1 or GSW11). After 7 d, the cells were harvested and assayed for AH1- and GSW11-specific CD8 T cell responses, as described above. For the second round of restimulation, cultures were harvested and live cells purified using Ficoll-Paque (GE Healthcare, Chalfont, U.K.). A total of 1 × 10^6 purified cells were incubated with 5 × 10^6 irradiated (10 Gy) BALB/c spleen cells, 20 IU/ml IL-2, and 1 µM AH1 or GSW11 peptide. As for the first round of stimulation, cells were harvested at day 7 and analyzed for AH1- and GSW11-specific CD8 T cells.

Cytotoxic T cell lysis
CT26 cells were stained with PKH26 (Sigma-Aldrich) according to the manufacturer’s instructions and incubated with AH1-specific CTLs at 37˚C for 2 h; then they were stained for 30 min on ice with 1 µM YoPro-1 (Molecular Probes, Eugene, OR) and analyzed by flow cytometry. Specific lysis of the PKH^+ target cells was calculated as 100% [YoPro^+ × YoPro^+ × E:T = 0]/[100 - % YoPro^+ × E:T = 0]. As negative controls, we included AH1-specific CTLs with BALB/c splenocytes.

Downloaded from http://www.jimmunol.org/ by guest on July 26, 2017
Results

Derivation of cross-reactive CD8\(^+\) T cells in mice primed to CT26 in the absence of Tregs

We have previously shown that both CD4\(^+\) and CD8\(^+\) effector T cells generated to the transplantable colon carcinoma CT26 in the absence of Tregs can mediate cross-protection and possibly act synergistically (12). Because, in the presence of Tregs, other forms of vaccination to CT26 using whole cells (either irradiated or transfected with GM-CSF) result in a protective CTL response that is highly focused on a single epitope (L\(^d\)-restricted AH1) and are not cross protective, our results suggested that depletion of Tregs uncovers responses to cryptic Ags that are shared among different tumor cell lines. We therefore sought to identify the source Ag(s) recognized by cross-protective CD8\(^+\) T cells, to improve our understanding of their apparent suppression by Tregs. To this end, we set out to establish CD8\(^+\) T cell lines that were representative of the cross-protective response in terms of their pattern of tumor recognition. T cell lines were generated from pooled purified CD8\(^+\) splenocytes from three mice immunized with live CT26 after Treg ablation. The splenocytes were shown to confer cross-protection to tumors of diverse origin when transplanted into SCID mice with a high potency (Fig. 1A). Several lines were generated in this way and found to contain predominantly H-2D\(^d\), and H-2K\(^d\)-restricted reactivities (data not shown). H-2L\(^d\)-restricted responses, which would include responses to the previously described immunodominant epitope from CT26, AH1 (SPSYVYHQF) derived from an endogenous ecotropic leukemia virus, were rarely seen, although in one case, the H-2L\(^d\)-restricted AH1 reactivity, which we detected with fluorescent tetramers, appeared after months in culture and came to dominate after a period of 6 mo in continuous culture (data not shown). The anti-AH1 response was, however, the dominant reactivity when splenocytes from CT26 tumor-bearing, Treg-replete mice were cultured in the same way (data not shown). Thus, the CD8\(^+\) responses elicited to CT26 in the absence of Tregs appeared qualitatively different from those previously described, even at this low level of resolution. One T cell line, CCD, which contained reactivities that were inhibited by anti–H-2K\(^d\) and anti–H-2-D\(^d\) but not anti–H-2-L\(^d\) Abs, was selected for its ability to recognize CT26, A20, and Renca, but not the mastocytoma cell line P815, in both IFN-\(\gamma\) secretion and killing assays (Fig. 1B), therefore displaying a pattern of reactivity that closely matched the bulk culture of CT26-stimulated splenocytes for Treg-depleted mice. We generated CTL clones from this line, of which one, designated CCD2, was prosecuted in detail. Clonality was confirmed with a panel of anti-TcRV Abs and \(\beta\)-Abs (Vo.8.3\(\beta\)-3 and continuously monitored by nested-set PCR). CCD2 reacted strongly against CT26, A20, and BCL-1; however, the response to Renca was significantly lower (Fig. 1A). Several lines were generated in this way and found to contain predominantly H-2D\(^d\), and H-2K\(^d\)-restricted reactivities (data not shown). H-2L\(^d\)-restricted responses, which would include responses to the previously described immunodominant epitope from CT26, AH1 (SPSYVYHQF) derived from an endogenous ecotropic leukemia virus, were rarely seen, although in one case, the H-2L\(^d\)-restricted AH1 reactivity, which we detected with fluorescent tetramers, appeared after months in culture and came to dominate after a period of 6 mo in continuous culture (data not shown). The anti-AH1 response was, however, the dominant reactivity when splenocytes from CT26 tumor-bearing, Treg-replete mice were cultured in the same way (data not shown). Thus, the CD8\(^+\) responses elicited to CT26 in the absence of Tregs appeared qualitatively different from those previously described, even at this low level of resolution. One T cell line, CCD, which contained reactivities that were inhibited by anti–H-2K\(^d\) and anti–H-2-D\(^d\) but not anti–H-2-L\(^d\) Abs, was selected for its ability to recognize CT26, A20, and Renca, but not the mastocytoma cell line P815, in both IFN-\(\gamma\) secretion and killing assays (Fig. 1B), therefore displaying a pattern of reactivity that closely matched the bulk culture of CT26-stimulated splenocytes for Treg-depleted mice. We generated CTL clones from this line, of which one, designated CCD2, was prosecuted in detail. Clonality was confirmed with a panel of anti-TcRV Abs and \(\beta\)-Abs (Vo.8.3\(\beta\)-3 and continuously monitored by nested-set PCR). CCD2 reacted strongly against CT26, A20, and BCL-1; however, the response to Renca was significantly lower (Fig. 1C; \(p = 0.001\)). Further characterization showed the response to CT26 was inhibited by anti-D\(^d\) and D\(^d\)/K\(^d\) but not anti-L\(^d\) or isotype control Abs (Fig. 1D). Thus the CCD2 CTL displayed an H-2D\(^d\)-restricted pattern of cross-reactivity that was representative of the cross-protective CD8\(^+\) T cell response in Treg-depleted, CT26-immunized mice.

Identification of a cross-reactive Ag by expression cloning

To identify the Ag recognized by the cross-reactive CTL CCD2, we employed an expression cloning technique (20). A cDNA library was prepared from CT26, composed of inserts with a size range of 0.4–5 kb cloned nondirectionally into pcDNA3 and organized into pools of 100 clones. The CCD2 T cell clone was used as a probe for expression of the cognate ligand in \(D\(^d\)-expressing\) COS-7 cells that were transiently transfected with pools of a plasmid cDNA expression library prepared from CT26. Representative data from one of twenty 96-well plates show two cDNA pools with above background killing of WEHI cells (Fig. 2A). Indeed, several stimulatory plasmid clones were found after fractionating the mixture into individual colonies (Fig. 2B). One of these plasmids, P20E8A1, was further tested for its ability to stimulate CCD2 in an Ag-specific and MHC-restricted manner. COS-7 cells transfected with P20E8A1 generated the T cell epitope in a dose-dependent manner—only when P20E8A1 cDNA was cotransfected with cDNA encoding the MHC molecule \(D\(^d\), but not \(K\(^d\) (Fig. 2C, 2D). In addition, the response to P20E8A1 was inhibited by anti-D\(^d\) Abs, as seen previously with the CCD2 anti-CT26 response. To facilitate the subsequent identification of the immunostimulatory epitope, we generated a lacZ-inducible hybridoma, CCD2Z, from fusion of the CCD2 T cell and the BWZ.36/CD8\(^+\) fusion partner (21), which displayed...
exactly the same recognition properties (Supplemental Fig. 1A).

In addition, CCD2Z was stimulated by most tumor cell lines, apart from P815, which also did not stimulate CCD2 (Supplemental Fig. 1B). The cross-reactive epitope is encoded by the endogenous ecotropic murine leukemia virus (MuLV).

Sequencing of the P20E8A1 cDNA insert revealed 99% sequence identity with the final 600 bp of pol and entire env (gp90) genes of an endogenous ecotropic MuLV, env-1 (Fig. 3A). The minimal sequence required to confer antigenic activity within P20E8A1 was determined by testing the functional activity of 39 deletion constructs. As determined by the stimulation of CCD2Z, the antigenic peptide was encoded within the gp90 protein, because the C3 truncation encoding the 39 600 bp of pol failed to stimulate (Fig. 3A, 3C). Interestingly, the L3'-restricted AH1 epitope is encoded within the same MuLV gene, gp90, (800 bp downstream of GSW11) in reading frame +1. Further truncations indicated that the epitope was encoded within or overlapped the 51 nucleotides represented by the C7 and C8 constructs (Fig. 3B, 3C). There was no contiguous sequence in any of the three reading frames within this region that coded for the optimal H-2Dd binding motif of XGPXXXXX[IL,F], although one sequence contained the N-terminal XGP motif without a typical hydrophobic C terminus. We therefore synthesized the 13-mer GGPFYCASWGC (GFC13), as well as sequen- tial C-terminal truncations, down to the 9-mer GGPFYCA (GSW9), and measured their ability to stimulate CCD2Z when added to P815. All peptides assayed showed a degree of stimulation, but one peptide, GSW11 (Dd) restricted synthetic peptides titrated on P815 cells. The peptide identities are shown below the graph. Dd and L3'-MHC binding by GSW11 (Dd) or AH1 (SPSYVYHQF; L3') was measured by RMA-S stabilization assay with Dd or L3' mAb. YF9 (YGPSLYRF) and QL9 (QLSPFPFDL) served as Dd and L3' binding controls, respectively. Data are representative of three independent experiments.

The cross-reactive epitope is encoded by the endogenous ecotropic marine leukemia virus.

FIGURE 2. Expression cloning and mapping of a cross-protective tumor Ag. A, Primary screen of a CT26 cDNA plasmid library in which the cDNA pools were transiently transfected into recipient COS cells along with Dd MHC class I. The WEHI killing assay was used to assess CCD2 stimulation. Dashed line represents background WEHI killing. B, A secondary screen of individual colonies from the cDNA pool P20E8 transfected into recipient cells was performed as above. The full-length clone, P20E8A1, was selected for further study. Dashed line represents background WEHI killing. C, Recipient COS cells were transfected with 1 µg of the P20E8A1 plasmid DNA or vector alone and cDNA encoding either the Dd or the Kd MHC I molecule. In addition, incubation with an α-Dd blocking Ab was also used. Stimulation of CCD2 was assessed by WEHI killing. D, COS cells were transfected as above and assayed for stimulation of CCD2Z measured by the LacZ response. Data are representative of two or three independent experiments.

FIGURE 3. Genetic mapping of a cross-protective Ag and its character- ization. A, Schematic of the alignment of a P20E8A1 cDNA insert with the endogenous ecotropic MuLV. Nested deletions C2, C3, C7, and C8 of the P20E8A1 cDNA were generated with P20E8A1-specific primers C2, C3, C7, and C8, respectively. Response of CCD2Z to truncated P20E8A1 constructs is indicated. B, The minimal region encoding the cross-protective Ag between the C7- and C8-truncated P20E8A1. The peptide epitope is highlighted in the gray box. C, CCD2Z responses to COS cells transfected with C2, C3, C7, and C8 reveal the minimal region encoding the cross-protective Ag. D, CCD2Z responses to candidate Dd restricted synthetic peptides titrated on P815 cells.
with peptide at saturating concentrations overnight at 26°C. Cells were washed of unbound peptide and incubated at 37°C. Peptide binding was measured as the rate at which MHC expression was lost at the cell surface: a commonly used surrogate measurement of peptide off-rate. As a further comparison, binding of the previously characterized AH1 Ag was assessed using Ld-expressing RMA-S cells. GSW11 was found to have a very rapid half-life for stabilization of Dd (20 min) and was faster than a second known Dd epitope, YGPSLYRRF (YPF9) (26), which had a half-life of 50 min (Fig. 3E). AH1 binding to Ld had a slower half-life still of 60 min, yet this was significantly faster than that of the control high-affinity peptide QLSPFPFDL (QL9), derived from P1 mastocytoma cells (25), which had a half-life of >120 min. Surprisingly, when we made C-terminal variants of GSW11 to include either the “classical” anchor variants I or F, neither of these, GSI11 or GSF11, showed improved affinity for Dd (half-life = 20 min; Supplemental Fig. 2). This finding indicated that the unstable nature of the interaction with Dd was not due to the C-terminal tryptophan residue.

GSW11 is generated in multiple tumor cell lines

We next wanted to confirm that GSW11 was endogenously generated in tumors recognized by the cross-protective CTL response. To achieve this, we generated peptide extracts from live tumor cells, fractionated them by reverse-phase HPLC, and assayed for the presence of GSW11 in the fractionated mixture by its ability to stimulate CCD2Z. Calibration with synthetic peptides indicated that differential identification of GSA9 and GSW11 peptides was possible (Fig. 4A). The profile obtained from CT26 peptide extracts revealed one significant peak that coeluted with synthetic GSW11 (Fig. 4B). A minor peak eluting ahead of GSW11 was shown to be an oxidized adduct (or dimer) that probably formed during the extraction process, because treatment of peptide extracts before and following fractionation with DTT, or treatment of cells with methyl methanethiosulfonate (which binds to free sulfhydryl groups on cysteine side chains and prevents interaction with other sulfhydryl groups) prior to extraction, resulted in loss of this peak and recovery of a single peak corresponding to the synthetic GSW11 (Supplemental Fig. 3). Extraction of peptides using a freeze/thaw method also led to the disappearance of the additional minor peak. We were able to determine the relative quantity of GSW11 generated by different tumor cell lines by comparing the minor peak. We were able to determine the relative quantity of GSW11 generated in the different tumor cell lines did not correlate to the level Dd cell surface expression (data not shown).

Anti-GSW11 and anti-AH1 CD8+ T cells are differentially suppressed by Treg

Having identified GSW11 as the source epitope of a cryptic response to CT26, we wanted to determine whether GSW11-specific CD8+ T cells are important in the cross-protective responses observed in the absence of Tregs; to achieve this, we investigated CD8+ responses in BALB/c mice inoculated with CT26 in the presence and absence of Tregs. Spleens and draining lymph nodes were assayed for GSW11- and AH1-specific CD8+ T cells, using intracellular cytokine staining after 12 d. This schedule allowed us to measure T cell responses in Treg-replete, tumor-bearing mice—even though the majority of these would eventually succumb to tumor growth. In the presence of Tregs, we were able to detect GSW11-specific T cells in only 4 of 11 mice investigated, with only ~0.04% of CD8+ T cells being GSW11 specific in the four mice that responded. These were not considered genuine anti-GSW11 responses because they failed to expand when stimulated in vitro (Supplemental Fig. 5). By contrast, all mice mounted a strong response to AH1, with 0.2–1% of CD8+ T cells being AH1 specific (Fig. 5A). This AH1-specific response is nevertheless insufficient to reject the tumor. A similar response is seen in mice vaccinated with irradiated CT26/GM-CSF. However, a different response profile was observed in Treg-depleted mice. In this case, all mice contained GSW11-specific CD8+ T cells, with an average of ~1.5%, representing an ~300-fold increase in numbers compared with Treg-replete mice. We also observed a 2- to 3-fold elevation of the anti-AH1 response in Treg-depleted mice (Fig. 5A). Overall, GSW11-specific T cells were seen to be codominant with AH1-specific T cells (~1.5% versus ~1.3%). A similar response pattern was seen in CT26/GM-CSF primed in Treg-depleted mice. Codominance between AH1- and GSW11-specific T cells was also seen at an earlier time point, day 8; however, the responses were much lower (data not shown). Because Treg-depleted mice reject tumor, the correlation of protection could be either the appearance of new specificities (exemplified by GSW11), broadening of the response to allow interclonal synergy, or enhancement of the immunodominant AH1 response. The last option would seem unlikely, given
results pooled from three separate experiments.

T cell responses of individual Treg-depleted mice challenged with CT26 target cells was found to be the same (Fig. 6A, data not shown). The ability of these AH1 CTL lines to kill CT26 tumor cells from both Treg-depleted and -replete CT26-challenged mice. The level of IFN-γ production in response to peptide stimulation and their ability to kill CT26 tumor cells from both Treg-depleted and -replete splenocytes was found to be the same following stimulation with either AH1 or GSW11 peptides. B, Anti-AH1 and -GSW11 CD8 T cell responses of individual Treg-depleted mice challenged with CT26 are indicated by joining lines. Each data point is from one mouse, with results pooled from three separate experiments.

To investigate this further, we analyzed AH1-specific CD8 T cells for their level of IFN-γ production in response to peptide stimulation and their ability to kill CT26 tumor cells from both Treg-depleted and -replete CT26-challenged mice. The level of IFN-γ produced from individual CD8 T cells derived from Treg-depleted or -replete splenocytes was found to be the same following stimulation with AH1 peptide (Fig. 6A, 6B). Owing to the lower quantity of AH1-specific CD8 T cells in Treg-replete mice, we were unable to purify enough to ascertain their ex vivo CTL killing capacity. To address this, we assessed the CTL cytotoxicity of splenocyte cultures from Treg-depleted and -replete CT26-challenged mice stimulated in vitro for 7 d in the presence of AH1. These cultures showed expansion of AH1-specific CD8 T cells (Supplemental Fig. 5) and did not contain any detectable GSW11-specific CD8 T cells (data not shown). The ability of these AH1 CTL lines to kill CT26 target cells was found to be the same (Fig. 6C). These results support the interpretation that the protection observed in the absence of Tregs is due to a broadening of the immune response rather than the acquisition of a more potent effector function. Interestingly, the codominance of AH1 and GSW11 responses seen in these ex vivo experiments was rarely recapitulated in short-term CT26-stimulated T cell cultures from Treg-depleted CT26-immunized mice. In this instance, the AH1 response was not easily detected, with GSW11 and other non-Ld-restricted responses predominating. A clue to why this might be the case came from closer examination of each response in individual mice in which there was a suggestion that high responders for GSW11 tended to be lower responders for AH1 and vice versa (Fig. 5B). This finding suggests a dynamic relationship between competing reactivities as they are established within the responding individual and may represent the process that is targeted by Tregs, resulting in differential suppression of the two responses.

FIGURE 5. GSW11-specific CD8 T cells are observed only in Treg-depleted mice. A, Treg-depleted or -replete BALB/c mice were challenged with CT26. After 12 d, CD8 T cell responses to AH1 and GSW11 were measured with intracellular IFN-γ staining following in vitro stimulation with either AH1 or GSW11 peptides. B, Anti-AH1 and -GSW11 CD8 T cell responses of individual Treg-depleted mice challenged with CT26 are indicated by joining lines. Each data point is from one mouse, with results pooled from three separate experiments.

Discussion

We have used an expression cloning technique to reveal the molecular identity of what we believe is a novel murine cross-protective tumor Ag. We have previously shown that removal of Tregs through Ab depletion induced a robust protective immunity upon subsequent CT26 tumor challenge. More importantly, the antitumor immunity induced was found to be cross protective to challenge with histologically distinct tumors (12). In this paper, we describe the characterization of one of the tumor Ags responsible for the cross-protective immunity, GGPESPYCASW (GSW11), derived from the gp90 gene of an endogenous ecotropic MuLV, env-1; this has provided valuable insights into the role of Treg suppression in the generation of a protective antitumor response.

FIGURE 6. AH1-specific CD8 T cells are functionally identical when primed in the presence or absence of Tregs. A, At 12 d after CT26 challenge, AH1-specific CD8 T cell responses from Treg-replete (left) or -depleted (right) BALB/c mice were measured with intracellular IFN-γ staining. The mean fluorescence intensity of IFN-γ–producing AH1-specific CD8 T cells is indicated on the dot plots. B, The comparative mean fluorescence intensity of AH1-specific CD8 T cells from CT26-challenged Treg-depleted or -replete BALB/c mice was assessed. Results are the mean ± SEM of six mice from two separate experiments. NS, p = 0.22. C, Following in vitro stimulation with AH1 pulsed irradiated BALB/c splenocytes, specific lysis of CT26 cells by AH1-specific CD8 T cells was measured. Data are representative of two independent experiments.
GSW11 may be a factor, although this is likely to be a minor consideration because there are multiple D\(^d\)-restricted peptides that are longer than the typical 9-mers suggested (29–31). A further anchor residue is suggested at position 5 (28); manipulation of this residue may enhance its binding to D\(^d\). CD8\(^+\) T cell responses to GSW11 are absent in naive mice following CT26 challenge; GSW11-specific responses are observed only when Tregs are depleted prior to challenge. This finding is in contrast to AH1-specific T cell responses, which are present in naive and Treg-depleted mice. The appearance of GSW11-specific T cells indicates a broadening of the antitumor response in the absence of Tregs. Indeed, in the absence of Tregs, the anti-CT26 response consists of at least an additional two Ags, apparent from CTL clones generated from spleens of Treg-depleted CT26-challenged mice; in addition to anti-GSW11–specific T cells, further D\(^d\), and K\(^d\)-restricted CD8\(^+\) T cell responses are observed (data not shown). These responses are likely to account for the inability of CCD2 T cells to be stimulated by Renca (Fig. 1C). The anti-GSW11 response is, therefore, a surrogate marker for the induction of a broader antitumor response in Treg-depleted mice, which is not solely restricted to anti-AH1 T cells and is ultimately protective. This broadening of the T cell response has also been confirmed from spectrotyping studies (32). Depletion of Tregs in mice has been shown to result in enhancement of T cell responses directed to many pathogen- and tumor-derived epitopes (11–13, 33). These studies showed stronger responses and, in most cases, a better outcome against pathogens or tumors. However, none of these investigations distinguished between a quantitative and a qualitative mechanism for improved immunity. In the majority of these studies, peptide-specific T cell responses were not measured, and in those that did, no new T cell reactivities were observed. In fact, examination of antiviral CD8\(^+\) T cell responses directed to influenza A-, vaccinia virus-, or SV40-transformed cells by Haer- yfar et al. (33) identified that the greatest Treg suppression was observed to the most immunodominant CD8\(^+\) T cells, which in some instances altered the immunodominance of the response. Our study is, therefore, the first to our knowledge to isolate the source epitope of a cryptic CTL response that is uncovered by Treg ablation.

AH1-specific CD8\(^+\) T cells are immunodominant in naive mice challenged with CT26, with no other CD8\(^+\) T cell response detectable (Fig. 4; Ref. 15). CT26-challenged Treg-depleted mice show a codominant phenotype, with anti-GSW11 T cells being equivalent to those directed against AH1. However, upon closer examination, individual mice have a tendency toward clonal immunodominance of either GSW11 (4 of 10) or AH1 (3 of 10), with 3 of 10 mice maintaining the codominant phenotype, suggesting that competition between GSW11- and AH1-specific CD8\(^+\) T cell responses is at least 50 times higher in the absence of Tregs than in their presence. We do not know the precise mechanism underlying this competition for dominance, but we note that in medium-term cultures of splenocytes from Treg-depleted CT26-immune mice, the GSW11 response dominates, even though long-term cultures permit outgrowth of the AH1 response (data not shown). Interestingly, we have found that AH1-specific lines grown from these cultures following peptide stimulation expand more slowly than similarly derived GSW11-specific lines, which may account for the dominance observed in vitro. The presence of codominance between AH1- and GSW11-specific responses is an unexpected finding. In the vast majority of cases, Ags with low MHC binding induce only subdominant T cell responses and are outcompeted by those responses directed against Ags with high MHC binding (34).

GSW11 is generated in all tumor cell lines assayed (Fig. 3). However, the generation of a cross-protective response is not reciprocal; challenge with A20 in the absence of Tregs does not lead to cross-protection against CT26 (Supplemental Fig. 6). One explanation is the finding that the amount of GSW11 generated by tumor cell lines differs significantly. CT26 generates >50 times more GSW11 than A20, resulting in lower cell surface expression of GSW11/D\(^d\). This is also reflected in the ability of the tumor cell lines to stimulate CCD2Z, which is independent of costimulatory molecules and thus responds in a way directly linked to the number of GSW11/D\(^d\) complexes present (Supplemental Fig. 1B). The lower level of cell surface GSW11/D\(^d\) complexes may prevent induction of GSW11-specific T cells and thus failure to induce a cross-protective response.

Despite its generation of GSW11, the ability to generate anti-GSW11 CD8\(^+\) T cells following CT26 challenge is observed only in Treg-depleted mice. AH1-specific T cells are observed in all mice; therefore, differential suppression of GSW11 and AH1 T cell responses is apparent in Treg-replete mice. An explanation for this differential suppression of T cell responses may be linked to the low affinity GSW11 has for binding to D\(^d\). GSW11/D\(^d\) complexes are very short lived, as determined by MHC binding and from the observation that we have been unable to generate GSW11/D\(^d\) tetramers; the interaction between GSW11 and D\(^d\) is not strong enough to promote their formation. As these complexes have a short half-life at the cell surface, GSW11-specific CD8\(^+\) T cells might be expected to require a high-affinity interaction between the TCR and GSW11/D\(^d\) to reach an activation threshold. Differences in T cell avidity have been shown to correlate with differential ability to secrete cytokines, such as IL-2, and may correlate with their dependence on them (36–38). We have observed this difference in GSW11- and AH1-specific T cell lines. GSW11-specific CTLs are more dependent on IL-2 for their activation and growth in culture than are AH1-specific lines, even though they expand more rapidly (data not shown). Because GSW11-specific T cells are suppressed by Tregs, the IL-2 dependence of these CTLs may provide a mechanism of differential suppression. Tregs have been shown to facilitate suppression of CTL responsiveness through acting as an IL-2 “sink,” thus inhibiting their proliferation (39–41). It is likely, therefore, that in situ Tregs could lower local IL-2 concentrations below a critical threshold required for the activation of some T cell clones, such as GSW11-specific ones, but not others, thereby resulting in differential suppression of T cell responses within “reach” of Tregs. It will be interesting to identify whether the Tregs are tumor specific.

The env-1 retrovirus is not active in the neonatal or juvenile thymus but is reactivated in 80% of mice at 6 mo of age (42). Similarly, human endogenous retroviruses (HERV) are present within the human genome and reactivate late in life and are often expressed ectopically in human tumors (43). It is likely that T cell responses to HERV are present in the same way as we have described in this paper, perhaps with the development of suppressive immunity accompanying endogenous expression. Therefore, understanding how to break these immunosuppressive networks may offer a therapeutic avenue for HERV-expressing tumors.

**Acknowledgments**

We thank Nasia Kontouli for invaluable assistance and technical support.

**Disclosures**

The authors have no financial conflicts of interest.
References


Supplementary figure 1

The CCD2Z response to CT26 is D^d restricted. A, CT26 cells were incubated with anti-L^d, K^d or D^d antibodies and a dilution series established. The ability of antibody bound CT26 to stimulate CCD2Z was measured. B, Various tumour cells were incubated with CCD2Z and their ability to stimulate was measured. Data is representative of 3 independent experiments.

Supplementary figure 2

Anchor residue substitutions have no effect on MHC binding. D^d MHC binding of GSW11, GSF11 or GSI11 was measured by RMA/S stabilisation assay with D^d mAb. Data is representative of 2 independent experiments.
Disruption of sulfhydryl groups prevents addition activity peaks. Peptide extracts from CT26 were treated with DTT (A) or MMTS (B) and fractionated by HPLC. Fractions were measured for CCD2Z stimulatory activity using P815 cells as APCs. Data is representative of two independent experiments.
GSW11 peptide is not detectable in all tumour cell lines. Peptide extracts from A) P815 and B) RENCA (closed circles) were fractionated by RP-HPLC. Mock runs (open circles) and synthetic GSW11 (open triangles) were added as controls. Fractions were assayed for CCD2Z stimulatory activity using P815 as APCs. Data is representative of 2 independent experiments.
GSW11-specific CD8+ T cells from CT26 challenged fail to expand following in vitro stimulation. Spleen cells from CT26 or PC61 treated CT26 challenged mice assayed for the presence of AH1 and GSW11-specific CD8+ T cells. In addition, spleen cells from these mice were re-stimulated in vitro with irradiated Balb/c spleen cells with either 1μM AH1 or GSW11 peptide and assayed for AH1 and GSW11-specific CD8+ T cells 7 days later. A further round of re-stimulation was performed at day 14 and the presence of AH1 and GSW11-specific CD8+ T cells was assessed 7 days later. Data is representative of 3 independent experiments.
Anti-A20 immunity induced in the absence of Tregs is not cross-protective. A, Treg depleted (PC61; n=10) or Treg replete (IgG1; n=10) Balb/c mice were challenged with A20 and tumour growth measured. B, Treg depleted A20-immune mice were rechallenged after 50 days with either A20 (open circles; n=5) or CT26 (closed circles; n=5) and survival measured. Data is representative of 2 independent experiments.