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CD4+ T cells inhibit the neu-specific CD8+ T-cell exhaustion during the priming phase of immune responses against breast cancer

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Abstract

Studies conducted in animal model of infectious diseases or H-Y antigen model suggest a crucial role for CD4⁺ T cells in providing help for CD8⁺ T-cell memory responses. This concept suggests that inclusion of T helper epitopes in vaccine formulation will result in improved CD8⁺ T-cell responses. Although this concept has been applied to cancer vaccine design, the role of CD4⁺ T cells in the memory differentiation of CD8⁺ T cells and retention of their anti-tumor function have never been tested in breast cancer model. Using the FVB mouse model of neu-positive breast carcinoma we report for the first time that helpless T cells showed cytostatic or tumor inhibitory effects during primary tumor challenge whereas, helped T cells showed cytotoxic effects and resulted in complete tumor rejection. Such differential effects, *in vivo*, were associated with higher frequency of CD8⁺PD-L1⁺ and CD8⁺PD-1⁺ T cells in animals harboring helpless T cells as well as higher titer of IL-2 in the sera of animals harboring helped T cells. However, depletion of CD4⁺ T cells did not alter the ability of neu-specific CD8⁺ T cells to differentiate into memory cells and to retain their effector function against the tumor during recall challenge. These results suggest the inhibitory role of CD4⁺ T cells on CD8⁺ T-cell exhaustion without substantial effects on the differentiation of memory T cells during priming phase of the immune responses against breast cancer.

Keywords

Breast cancer; HER-2/neu; Helpless CD8⁺ T cells; CD4⁺ helper T cells; Memory T cells

Introduction

The ability to elicit an immune response to primary tumors and sustain a memory response upon the tumor recurrence is the basis of vaccination against cancers. Given that breast tumors usually lack the expression of MHC class II, CD8⁺ T cells attracted more attention than CD4⁺ T cells for the induction of cell-mediated immune responses against cancers. However, it is critical for vaccine design to understand the roles that CD4⁺ T cells play in the induction of CD8⁺ T-cell responses as well as the generation and persistence of memory cytotoxic T-cell (CTL) responses against tumors. This understanding allows us to determine whether and how we should include T helper epitopes in vaccine formulation for the induction and/or persistence of anti-tumor CD8⁺ memory T cells, or to include CD4⁺ T cells in the adoptive CD8⁺ T-cell therapy of cancer. There are two hypotheses, *i.e.*, programming model [1, 2] and maintenance model [3] describing the role of CD4⁺ T-cell help in CD8⁺ T-cell responses. According to the programming model, CD4⁺ T cells are required during the primary response to provide help for CD8⁺ T cells via the CD40L–CD40 interaction inducing differentiation of CD8⁺ memory T cells [4]. Once memory T cells are programmed, recall responses could occur in the absence of CD4⁺ T cells. The maintenance model suggests that memory T cells are generated even in the absence of CD4⁺ T cells; however, persistence and effector function of CD8⁺ memory T cells during recall challenge would require help from CD4⁺ T cells otherwise helpless CD8⁺ T cells will undergo TRAIL-mediated apoptosis [5]. Thus far, numbers of helper factors have been identified including, most recently, IL-21 [6]. These models have been proposed based on observations derived during bacterial or viral infections or CTL responses to the male Ag H-Y. No study has addressed whether activation and persistence of CD8⁺ memory T-cell responses to tumors may follow similar trend. In fact, clinical experience suggests that the

combined induction of MHC class I and class II responses during anti-cancer vaccination may be deleterious for the induction of CD8⁺ T cells [7]. These observations raise the question of whether helpless CD8⁺ T cells may exhibit anti-tumor function and maintain functional memory against tumor cells during recall responses.

Here, we used FVB mouse model of HER-2/neu positive breast carcinoma in order to determine the contribution of helper CD4⁺ T cells in the generation, maintenance, and effector function of the neu-specific CD8⁺ effector and memory T cells to the primary or recall tumor challenge. We found that depletion of CD4⁺ T cells during priming or effector phase of the immune response did not alter the ability of neu-specific CD8⁺ T cells to differentiate into memory cells and to retain their anti-tumor effector function. We report that CD4⁺ T cells are mainly involved in inhibiting the tumor-induced CD8⁺ T-cell exhaustion that could affect their anti-tumor function only during priming phase of the immune responses.

Materials and methods

Mice

Wild-type FVB (Jackson Laboratories) female mice (6–8 weeks of age) were housed in aseptic condition and used throughout these studies. The studies have been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University.

Tumor cell line

Neu positive MMC cell line was established from a spontaneous tumor harvested from FVBN202 transgenic mice as previously described by our group [8, 9].

Depletion of lymphocytes in vivo

Depletion of CD4⁺ and/or CD8⁺ T cells were performed by i.p. injections of GK1.5 and/or 2.43, as previously described by our group [8, 9]. Briefly, the Abs were purified by ammonium sulfate precipitation from ascites of SCID mice injected i.p. with the hybridoma cells. The depletions were started on days –2 and –1 and animals were challenged with the tumor on day 0. Animals were then injected with the depleting Abs (200–250 Ig/mouse) once every 5 days until the completion of the trial. Depletion of the lymphocyte subsets was assessed on the day of challenge and bi-weekly thereafter by FACS analysis of peripheral blood. Depletion of B cells was performed by i.p. injection of LEAFTM purified anti-mouse B220 Ab (100 Ig/mouse; two sequential days prior to tumor challenge followed by once every 5 days) (Biolegend, San Diego, CA).

In vivo tumor challenge

Female mice were inoculated s.c. with MMC (5×10^6 cells/mouse unless otherwise stated). Animals were inspected twice every week for the development of tumors. Masses were measured with calipers along the two perpendicular diameters. Tumor volume was calculated by: V (volume) = L (length) $\times$ W (width)² $\div$ 2. Mice were killed before a tumor mass exceeded 2,000 mm³.

Flow cytometry

A three color staining flow cytometry analysis of the mammary tumor cells (10^6 cells/tube) was carried out as previously described by our group [8, 9]. Fc blocking was performed using anti-CD16/CD32 Ab (Biolegend). We used following antibodies: mouse anti-neu (Ab-4) (Calbiochem, San Diego, CA), isotype control Ig, PE-conjugated anti-mouse Ig

(Biollegend), FITC-conjugated Annexin V and propidium iodide (PI) (BD Pharmingen, San Diego, CA), PE/Cy5-CD8, FITC-CD44, PE-CD44, PE-CD69, FITC-CD62L, PE-PD-L1, PE-PD-1, isotype Ig (Biollegend). Cells were finally washed and analyzed at 50,000 counts with the Beckman Coulter EPICS XL within 30 min.

Microarray performance and statistical analysis

Total RNA from CD8+ T cells was extracted using Trizol reagent according to the manufacturer's instructions. Total RNA was amplified into anti-sense RNA (aRNA) as previously described [10, 11] and the quality of both, total RNA and secondarily amplified RNA was tested with the Agilent Bioanalyzer 2000 (Agilent Technologies, Palo Alto, CA). Confidence about array quality was based on the principle of reference concordance as previously described [12]. Mouse reference RNA was prepared by homogenization of the following mouse tissues (lung, heart, muscle, kidneys, and spleen) and RNA was pooled from four mice. Pooled reference and test aRNA was isolated and amplified in identical conditions during the same amplification/hybridization procedure to avoid possible inter-experimental biases. Both, reference and test aRNA was directly labeled using ULS aRNA Fluorescent labeling Kit (Kreatech, Netherlands) with Cy3 for reference and Cy5 for test samples.

Whole genome mouse 36 k oligo arrays were printed in the Infectious Disease and Immunogenetics Section of the Department of Transfusion Medicine (IDIS), Clinical Center, National Institute of Health, Bethesda using oligos purchased from Operon (Huntsville, AL). The Operon Array-Ready Oligo Set (AROS™) V 4.0 contains 35,852 longmer probes representing 25,000 genes and about 38,000 gene transcripts and also includes 380 controls. The design is based on the Ensembl Mouse Database release 26.33b. 1, Mouse Genome Sequencing Project, NCBI Ref-Seq, Riken full-length cDNA clone sequence, and other GenBank sequence. The microarray is composed of 48 blocks and one spot is printed per probe per slide. Hybridization was carried out in a water bath at 42°C for 18–24 h and the arrays were then washed and scanned on a Gene Pix 4000 scanner at variable PMT to obtain optimized signal intensities with minimum (<1% spots) intensity saturation.

Resulting data files were uploaded to the mAdb data-bank (<http://nciarray.nci.nih.gov>) and further analyzed using BRBArrayTools developed by the Biometric Research Branch, National Cancer Institute [13] (<http://linus.nci.nih.gov/BRB-ArrayTools.html>) and Cluster and Treeview software [14]. The global gene-expression profiling consisted of nine experimental samples. Gene ratios were average corrected across experimental samples and displayed according to uncentered correlation algorithm [15].

Statistical analysis

Gene expression profiles were compared statistically by un-paired Student's *t* test using the BRBArrayTools and Stanford Cluster program to identify differentially expressed genes among naive, helpless, and helped CD8+ T cells. The significance cut-off level of $P < 0.01$ was chosen as demanded by the statistical power of the comparison and sample number. Statistical significance and adjustments for multiple test comparisons were based on univariate and multivariate permutation test as previously described [16, 17]. Pathways analysis was done using Ingenuity pathway analysis software.

RT-PCR

RT reaction was performed as described by our group [8].

Cytotoxicity assay

Cytotoxicity assays were performed as previously described by our group [8]. Briefly, neu-specific effector lymphocytes derived from MMC-sensitized FVB mice [8] were cultured with MMC at 10:1 E:T ratios in complete medium (RPMI 1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FBS) and 20 U/ml recombinant IL-2 (Preprotech) in 6 well culture dishes. After 48 h, cells were harvested and stained for neu (anti-neu), Annexin V and PI according to the manufacturer's protocol (BD Pharmingen).

IFN-γ ELISA

Secretion of MMC-specific IFN-γ by T cells was detected by co-culture of lymphocytes (4×10^6 cells) with irradiated MMC (15,000 rads) at 10:1 E:T ratios in complete medium (RPMI1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin) for 24 h. Supernatants were then collected and subjected to IFN-γ ELISA assay using a Mouse IFN-γ ELISA Set (BD Pharmingen, San Diego, CA) according to manufacturer protocol. Results are reported as the mean values of triplicate ELISA wells.

Multiplex cytokine array

Sera of animals harboring helped T cells ($n = 3$) or helpless T cells ($n = 6$) were sent to Allied Biotech, Inc. for multiplex array analyses in a blinded fashion. The following cytokines/chemokines were included in the array: VEGF, GM-CSF, MCP-1, IFN-γ, TNF-α, IL-13, IL-5, IL-2, IL-4, IL-12p70, IL-12p40, IL-10, IL-1β, IL-6.

Telomere length analysis

Telomere length was determined using the terminal restriction fragment (TRF) length assay as before [18]. DNA was isolated from cells using genomic tips (Qiagen). Seven microgram of genomic DNA was digested with a cocktail of restriction enzymes (*AluI*, *HaeIII*, *HinfI*, *MspI*, and *RsaI*, New England Biolabs) and resolved on a 0.7% agarose gel. The G-rich telomeric probe [(TTAGGG)₄] was labeled with [γ-³²P]ATP (6000 Ci/mmol), followed by removal of unincorporated label (QIAquick nucleotide removal kit, Qiagen). The gel was dried and subjected to in-gel hybridization with the radiolabeled G-rich probe for 12–16 h, followed by washing and exposure to the PhosphorImager cassette (Molecular Dynamics).

Results

Helped and helpless CD8+ T cells exhibit differential gene expression profile

To determine whether provision of help by CD4+ T cells may significantly affect patterns of gene expression in CD8+ T cells we performed microarray analysis using neu-specific helped and helpless CD8+ T cells during primary tumor challenge as well as naïve CD8+ T cells. The CD8+ T cells were derived from FVB mice that are capable of rejecting mouse mammary carcinomas (MMC) in a neu-specific manner [8, 9]. Mice were inoculated with MMC to generate helped CD8+ T cells. A group of animals was depleted of CD4+ T cells prior to and during the tumor challenge in order to generate helpless CD8+ T cells. The efficiency of depletion was above 99% as determined by flow cytometry of blood cells as well as lack of the neu-specific IgG Ab responses in the sera of these animals (data not shown). Unchallenged FVB mice were served as source of naïve CD8+ T cells. Animals were killed 3–4 weeks after MMC challenge when those with helped T cells almost rejected MMC while those with helpless T cells only inhibited tumor growth [8, 9]. Splenocytes were then subjected to negative selection for the isolation of CD8+ T cells. Although only a fraction of T cells was tumor-specific, this fraction was expected to be substantial because of collecting T cells at the time of tumor challenge in vivo and housing the mice in aseptic condition. Purity of CD8+ T cells was above 97% (data not shown). RNA were extracted

and subjected to microarray analysis, using a 36 k whole genome mouse array. Class comparison between helped and naïve CD8⁺ T cells (cut-off P value of 0.01) revealed 261 genes that were differentially expressed. A heat map was generated using Treeview software that included (for visual comparison) also helpless CD8⁺ T cells [14]. Compared to naïve T cells, helped and helpless CD8⁺ T cells shared a similar gene expression profile upon tumor challenge (Fig. 1a). Thus, the transcriptional program that differentiates the naïve from the memory phenotype cannot differentiate the transcriptional phenotype resulting from the presence of help during memory T cell development. In second tier of analysis, we compared helpless with helped CD8⁺ T cells by applying a student t -test with a cut-off P value of 0.01. This analysis identified 433 transcripts differentiating the two groups (Fig. 1b). Treeview visualization of up- and down-regulated genes including also naïve CD8⁺ T cells identified transcripts particularly representative of helpless T cells since naïve and helped CD8⁺ T cells shared a similar gene expression profile with 168 up- and 265 down-regulated genes compared to helpless CD8⁺ T cells (Fig. 1b). Thus, lack of help following Ag challenge has a deep effect on the general profile of CD8⁺ T cells independent of previous Ag exposure. We then compared up and down-regulated genes from the two separate student t -tests (cut-off P value 0.01) using Boolean diagrams for visualization (Fig. 1c). Helpless and helped CD8⁺ T cells shared only 58 down regulated (Fig. 1c, top) and 36 up-regulated (Fig. 1c, bottom) genes when compared with naïve CD8⁺ T cells. Compared to naïve CD8⁺ T cells, helpless and helped T cells showed downregulation of 270 and 96 genes as well as upregulation of 499 and 71 genes, respectively (Fig. 1c). Ingenuity pathway analysis (IPA, Fig. 1d) revealed that the transcripts differentiating helped and helpless T cells were almost exclusively related to response to stress and immune challenge, and were proportionally down-regulated in helped T cells compared to helpless T cells. These data propose a prolonged status of hyper-stimulation of helpless CD8⁺ T cells during MMC challenge.

Higher viability of helped T cells vs. helpless T cells despite comparable levels of TRAIL and c-FLIP

Since it was reported that helpless T cells undergo apoptosis because of the expression of TRAIL, we wanted to see whether similar mechanism may exist during anti-tumor immune responses. As shown in Fig. 2a, we found slightly higher viability (Annexin V-/PI-) of the neu-specific helped T cells compared to the helpless T cells prior to (average 84.35% vs. 66.3%; $P = 0.046$) or after stimulation with the neu positive MMC tumor cells in vitro (average 86.4% vs. 71.57%; $P = 0.042$). Viability of helpless or helped T cells did not diminish following stimulation with the nominal MMC tumor cells in vitro (Fig. 2a, helpless: average 66.3% vs. 71.57%; helped: average 84.35% vs. 86.4%). Both helpless and helped T cells showed comparable levels of apoptosis-inducing TRAIL, its receptor DR5 as well as c-FLIP (Fig. 2b).

Helpless CD8⁺ effector T cells tend to be exhausted during primary tumor challenge

Since FVB mice harboring the neu-specific helpless T cells showed tumor inhibitory effects but did not completely reject MMC tumors within 3–4 weeks, we hypothesized that the lack of CD4⁺ T cells during activation of CD8⁺ T cells may accelerate exhaustion of helpless effector T cells in response to the tumor challenge. Ingenuity pathway analysis of microarray data (Fig. 1d) also suggested a prolonged status of hyper-stimulation of helpless CD8⁺ T cells during MMC challenge that could facilitate T cell exhaustion [19, 20]. To test this, we looked at the known markers of T-cell exhaustion, i.e., PD-L1 and PD-1 expression during primary tumor challenge. As shown in Fig. 3a, helpless T cells had higher expression of PD-L1 than helped T cells, prior to the neu-specific stimulation with MMC in vitro (average 61% vs. 22%; $P = 0.04$). Whereas helpless T cells did not markedly increase PD-L1 expression upon in vitro stimulation with MMC (average 61% vs. 81%; $P = 0.13$),

helped T cells increased PD-L1 expression in the presence of MMC (average 22% vs. 66%; $P = 0.026$) to the levels comparable to that in helpless T cells (average 66% vs. 81%; $P = 0.50$). Compared to helped T cells, helpless T cells also showed higher expression of PD-1 prior to in vitro stimulation (average 4.14% vs. 0.45%; $P = 0.030$), though there were only helped T cells that increased expression of PD-1 upon in vitro stimulation with MMC (average 0.45% vs. 2.25%; $P = 0.032$). Then, we asked the question why increased expression of PDL-1/PD-1 by helped T cells because of MMC stimulation did not result in T-cell exhaustion in animals harboring helped T cells. We hypothesized that animals harboring helped T cells might have higher levels of IL-2 that could overcome PD-L1/PD-1-mediated T-cell exhaustion in vivo. It has been shown in several models that IL-2 can overcome PDL-1/PD-1-induced T-cell exhaustion [21]. To test this, we collected sera from animals harboring helped or helpless T cells and performed multiplex cytokine array analysis for the detection of 14 different cytokines, including IL-2. As shown in Fig. 3b, animals harboring helped T cells had higher titer of IL-2 in their sera compared to those with helpless T cells ($P = 0.038$). We also looked at another known marker of T cells activation, CD44, before and after stimulation with MMC in vitro. As shown in Fig. 3c, helpless T cells (gated CD8^{high} fraction) had higher expression of CD44 than helped T cells prior to stimulation with MMC (average 61% vs. 36.8%; $P = 0.047$). Similar to the expression of PD-L1, CD44 expression was increased only in helped T cells following stimulation with MMC in vitro (average 36.8% vs. 51.8%; $P = 0.028$). Given that in vitro stimulation of helpless T cells with MMC did not increase CD44 expression (average 61% vs. 48.7%; $P = 0.11$), we sought to determine whether helpless T cells were unable to undergo further activation in vitro. Therefore, we looked at the expression of an early activation marker CD69 prior to and after MMC stimulation (Fig. 3d). Helpless T cells markedly increased expression of CD69 on effector T cells (gated CD8+CD44^{high}) following stimulation with MMC (average 8.5 vs. 22.3; $P = 0.015$). Similar results were obtained from helped T cells (average 16.7% vs. 34.7%; $P = 0.002$). However, capacity of helped T cells for activation was greater than helpless T cells because they showed higher expression of CD69 prior to (average 16.7% vs. 8.5%; $P = 0.04$) and after stimulation with MMC in vitro (average 34.7% vs. 22.3%; $P = 0.019$). The neuspecific helpless T cells secreted moderately lower IFN- γ ($P = 0.058$) in the neu-specific fashion when stimulated with irradiated MMC in vitro (Fig. 3e).

Helpless CD8+ T cells exhibit anti-tumor effector function in vivo and in vitro

In order to determine whether presence of the exhausted effector phenotypes in helpless T cells may inhibit their anti-tumor function, we performed cytotoxic assays in vivo and in vitro. FVB mice harboring helped T cells received anti-B220 Ab injections for the depletion of B cells in order to eliminate contribution of the neu-specific Ab responses in tumor rejection. To established helpless CD8+ T cells, another group of mice were injected with anti-CD4 Ab. Animals were then challenged with neu positive MMC. As expected, control FVB mice that were depleted of CD4/CD8 T cells showed aggressive tumor growth within 3–4 weeks following tumor challenge (Fig. S1). As shown in Fig. 4a, FVB mice harboring helped T cells rejected the tumor within 3 weeks whereas mice harboring helpless T cells showed only tumor inhibitory effects and tumors remained plateau (<500 mm³) by the time tumor size reached 2000 mm³ in control mice (Fig. S1). However, both helped and helpless T cells showed comparable cytotoxic effects on MMC in vitro such that viable tumors (annexin v-/PI-) were reduced from an average 79% to 38% ($P = 0.012$) and 40% ($P = 0.017$) (Fig. 4b) after 48 h culture with helpless or helped T cells (E:T, 10:1), respectively. Phenotype analysis of the T cells (Fig. 4c) showed that both helpless and helped T cells had comparable percent of CD44+CD62L^{low} effector/memory (EM: 7.8% vs. 6.7%) and CD44+CD62L^{high} central memory (CM: 29% vs. 31%) phenotypes whereas helpless T cells had higher proportion of CD44+CD62L-effector and lower proportion of CD44-CD62L+

naïve phenotypes compared to helped T cells (E: 36% vs. 15%, $P = 0.0019$; N: 23.6% vs. 42%, $P = 0.00011$). These data suggested that depletion of CD4⁺ T cells did not alter differentiation of CD8⁺ T cells toward memory phenotypes. In order to determine whether presence of helpless memory T cells may have tumor inhibitory function during recall tumor challenge we inoculated CD4-depleted mice with lower dose of MMC (2×10^6 instead of 5×10^6 cells/mouse) during primary challenge so that animals can reject low dose tumor. Depletion of CD4⁺ T cells was maintained during the recall challenge. Four weeks after the primary challenge, tumors were not measurable, and two weeks after the tumor rejection animals were re-challenged with MMC (5×10^6 cells/mouse) on the contralateral side. As shown in Fig. 4d, animals managed to reject recall tumor challenge such that tumors were not measurable 3–4 weeks after the recall challenge. To determine whether neu-specific helpless memory T cells may be maintained in vivo, telomere length analyses of helpless and helped CD8⁺CD44⁺CD62L⁺ memory T cells were performed. As shown in Fig. 4e, both helpless and helped memory CD8⁺ T cells exhibited substantial telomere shortening as compared to naïve T cells. Although global telomere lengths appeared relatively constant after normal exposure times, significant erosion was detected within the population of helpless and helped T cells after over-exposure. These data suggest that a subset of telomeres is shortening as a result of increased proliferation, which could ultimately lead to premature aging phenotypes, as suggested previously [22].

Discussion

Here, we used the FVB mouse model of HER-2/neu positive breast carcinoma and showed for the first time that generation, maintenance and effector function of the neuspecific CD8⁺ memory T cells occurred in the absence of CD4⁺ T cells. Whereas the neu-specific helped CD8⁺ T cells were more efficient against the tumor than helpless CD8⁺ T cells, during priming phase of the immune responses, no substantial differences in their anti-tumor efficacy was observed during recall tumor challenge. In this breast tumor model, CD4⁺ T cells inhibited exhaustion of CD8⁺ T cells during primary tumor challenge but they had no effect on the generation and effector function of CD8⁺ memory T cells during recall challenge.

Although several studies have been conducted on understanding the role of CD4⁺ helper T cells in supporting the activation and effector function of CD8⁺ memory T cells, none of these studies was performed in cancer model. We used FVB mice that are capable of rejecting MMC in neu-specific manner because of the expression of high affinity T cell receptor for the neu antigen [8, 9]. These mice were housed in aseptic condition and T cells were collected at the time of tumor challenge in order to increase yield of the neu-specific T cell fraction. Microarray analysis of helpless and helped T cells showed differential expression of hundreds of genes. These CD8⁺ T cells were clustered differently with helped T cells being similar to naïve T cells. This suggests that helped T cells underwent substantial contraction during late stage of tumor rejection (3–4 weeks following primary tumor challenge) while helpless T cells were still active during the plateau phase of tumor burden. Ingenuity pathway analysis showed an increased expression of stress-related genes in helpless T cells (Fig. 1d), suggesting a prolonged status of hyper-stimulation of helpless CD8⁺ T cells during MMC challenge. To test this, we looked at known markers of T cell exhaustion, i.e., PD-L1/PD-1 as well as a marker of T cell activation, CD44. We found a greater proportion of exhausted helpless effector T cells (increased PD-L1, PD-1, and CD44 expression) compared to helped T cells. Given that in vitro stimulation of helpless T cells with MMC did not increase CD44 expression, we sought to determine whether helpless T cells were unable to undergo further activation in vitro. Therefore, we looked at the expression of early activation marker, CD69, and determined that while helpless T cells increased expression of CD69 upon in vitro stimulation, their capacity for activation was

lower than helped T cells because they showed lower expression of CD69 prior to and after stimulation with MMC in vitro. These data were consistent with a moderately lower production of the neu-specific IFN- γ by helpless T cells. Greater expression of PD-L1+ and PD-1+ in helpless T cells compared to helped T cells may account for failure of these T cells in complete rejection of the primary tumor challenge. Lowering the dose of tumor challenge, however, resulted in nearly complete rejection of the tumors by helpless T cells. These suggest that helpless T cells were less efficient, but not defective, than helped T cells during priming phase of the immune responses. It has been reported that antigenic stimulation of T cells results in T-cell exhaustion [19, 20]. Detection of higher levels of apoptosis in helpless than helped T cells (Fig. 2) was consistent with higher proportion of PD-L1+ helpless T cells. If CD8+ T-cell turnover is doubled in the absence of CD4+ T cells, it may have significant biological implications. We also showed that in vitro stimulation of helped T cells increased expression of PD-L1 and PD-1, however, helpless T cells were more prone to exhaustion because they had higher expression of these molecules during tumor challenge and prior to in vitro stimulation with the tumor. Higher proportion of CD69+ population in helped T cells compared to helpless T cells, both prior to and after the in vitro stimulation with MMC, also suggested that helpless T cells to be more prone to the exhaustion. In addition, higher levels of IL-2 in the sera of animals harboring helped T cells could overcome the activation-induced expression of PD-L1 in helped T cells in vivo.

Compared to helpless T cells, helped T cells showed greater tumor inhibitory effects during primary responses in vivo. Whereas helped T cells showed cytotoxic effects and induced complete rejection of primary tumor challenge, helpless T cells showed cytostatic effects and only inhibited tumor growth. Despite failure of helpless T cells in complete rejection of primary tumor challenge, their ability to inhibit neu-positive MMC in vitro was comparable to that of helped T cells. These suggest that helpless T cells are not defective in anti-tumor immune responses, but because of being more prone to exhaustion during activation they are less efficient than helped T cells to provide complete protection against primary tumors. Such tendency of helpless T cells to the exhaustion was also supported by the data obtained from microarray analysis. Ingenuity pathway analysis revealed that the transcripts differentiating helped and helpless T cells were almost exclusively related to response to stress and immune challenge, and were proportionally down-regulated in helped T cells compared to helpless T cells. In addition, helpless T cells had significantly greater proportion of CD44+CD62L⁻ T_E and smaller proportion of CD44-CD62L+ T_N phenotypes compared to helped T cells. Such phenotype distribution, again, suggest that helpless T cells have more supply of naive T cells for in vivo activation and provision of full protection against the tumor. Very recently, it was reported that naive T cells are more potent than central memory T cells in differentiating into the effector cells and protecting the host against tumor challenge [23, 24]. Importantly, both helpless and helped CD8+ T cells showed comparable proportion of CD44+CD62L^{low} T_{EM} and CD44+CD62L^{high} T_{CM} phenotypes. These results do not suggest a crucial role for CD4+ T cells in the generation of CD8+ memory T cells. Interestingly, helpless memory T cells protected the mice against recall tumor challenge. This suggests that helpless memory T cells did not need CD4+ T cells for their effector function during recall responses. Telomere analysis of memory fractions also showed a comparable longevity of helped and helpless memory T cells. It was reported that telomere length of lymphocytes correlates with their anti-tumor efficacy in cancer patients receiving adoptive T cell therapy [25, 26].

In cases where adoptive CD8+ memory T-cell immunotherapy (AIT) of human cancers have shown promising results [27, 28], our findings suggest that such AIT followed by the depletion of CD4+ T cells in vivo could result in an increased homeostatic proliferation of the adoptively transferred CD8+ T cells and an improved objective response. The observed phenomenon should also be tested in other tumor models to determine whether this is a

general phenomenon during anti-tumor CD8+ T cell responses or it is specific to the neu-targeted T cell responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Gene expression profile of naïve, helped and helpless CD8+ T cells. Negatively selected CD8+ T cells were obtained from splenocytes of tumor bearing and naïve animals ($n = 3$). **a** Student's t test comparing naïve and helped CD8+ T cells (cut-off P value 0.01) reveals 261 differentially expressed genes. Gene expression values from helpless CD8+ T cells were included in the visualization but genes were selected based on the outcome of the t test. **b** Class comparison between helped and helpless CD8+ T cells (cut-off P value of 0.01) revealed 433 differentially expressed genes (permutation P value 0.1). For visualization, a heat map was generated using Treeview software that included also naïve CD8+ T cells. Cluster analysis uncovers the similarity between helped and naïve CD8+ T cells which is also indicated in the Boolean diagrams in **c** because of the smaller number of differentially expressed genes. **c** Boolean comparison of up- and down-regulated genes based on the outcome of two separate student's t tests comparing helped vs. naïve and helpless vs. naïve CD8+ T cells (cut-off P values 0.01). Overlapping up- or down-regulated genes from both t tests are displayed in regions AB. **d** Ingenuity pathway analysis based on 433 differentially expressed genes between helped and helpless T cells (cut-off P value of 0.01). Out of 71 significantly involved canonical pathways 11 with predominant relationship to immune mechanisms are shown. Red and green bar sections refer to up- or down-regulation of genes in the respective pathways in helped when compared to helpless T cells

Fig. 2.

Apoptosis in helpless and helped T cells. **a** FVB female mice ($n = 4$) harboring helpless or helped CD8⁺ T cells were killed 3 weeks after MMC challenge and their splenocytes subjected to flow cytometry analysis before or 24 h after in vitro stimulation with irradiated MMC (15000 rad) in E:T ratio of 10:1. Cells were stained with PE/Cy5-anti-mouse CD8, FITC-Annexin V and PI. Gated CD8⁺ cells in the lymphocyte region were analyzed to determine viability (Annexin V-/PI-) of T cells. Data are representative of four experiments. **b** CD8⁺ T cells were also isolated by EasySep negative selection kit (Stem Cell Technologies), and subjected to RT-PCR analyses for the detection of TRAIL, DR5, c-FLIP. GAPDH was used as internal control. Data are representative of three experiments

Fig. 3.

Helpless CD8⁺ T cells are more exhausted than helped T cells. FVB female mice ($n = 4$) harboring helpless or helped CD8⁺ T cells were killed 3 weeks after MMC challenge. Splenocytes were subjected to flow cytometry analyses 24 h after stimulation with irradiated MMC (15000 rad; E:T, 10:1) or medium alone in vitro. Gated CD8^{high} cells were analyzed for the expression of PD-L1 or PD-1 (**a**). Detection of IL-2 in the sera of animals harboring helped ($n = 3$) or helpless ($n = 6$) T cells (**b**). Gated CD8^{high} cells were analyzed for the expression of CD44 (**c**). Gated CD8⁺ CD44^{high} were also analyzed for the expression of CD69 (**d**). Supernatants were collected and subjected to IFN- γ ELISA (**e**). Representative data from four experiments are shown

Fig. 4.

Anti-tumor efficacy and phenotype analysis of helpless and helped T cells. **a** FVB mice ($n = 3$) were challenged with MMC (5×10^6 cells/mouse) after injections with anti-CD4 Ab for CD4 depletion (helpless) or injections with control Ig (helped). **b** Viability of MMC tumor cells were determined by Annexin V/PI staining after 48 h culture with T cells derived from animals harboring helped or helpless T cells. **c** Splenocytes of FVB mice ($n = 4$) harboring helpless or helped T cells and carrying MMC tumor cells for 3 weeks were stained with Abs to CD8, CD44, and CD62L. Gated CD8⁺ cells of the lymphocyte region were then analyzed for the detection of effector (E), effector/memory (EM), central memory (CM), and naïve (N) CD8⁺ T cells. Representative dot plot and average of each phenotype from 4 mice per group are presented. **d** FVB mice ($n = 3$) harboring helpless T cells were challenged with low dose MMC (2×10^6 cells/mouse) and two weeks after tumor rejection animals were re-challenged with MMC (5×10^6 cells/mouse) on the contra-lateral side. **e** Gated CD8⁺CD44⁺CD62L⁺ splenocytes of FVB mice harboring helped or helpless T cells and bearing MMC tumor cells for 3 weeks as well as naïve tumor-free mice ($n = 4$) were sorted and telomere lengths were determined. DNA was extracted and tested for telomere length using the TRF protocol as before [18]. The left panel was exposed for 24 h, while the right panel was exposed for 48 h. White bars indicate the lower limit (shortening) of telomere signal for each sample. H1299, which is a human lung adenocarcinoma cell line with longer than average human telomere length, served as a control for hybridization. Fragment sizes (in kb) are indicated on either side of the representative gel