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Award Number: W81XWH-08-1-0733

TITLE: The Role of Myeloid-Derived Suppressor Cells in the Immunotherapy of HER2/neu-Positive Breast Carcinomas

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REPORT DATE: October 2009

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

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REPORT DOCUMENTATION PAGE

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OMB No. 0704-0188

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1. REPORT DATE *(DD-MM-YYYY)*
01-10-2009

2. REPORT TYPE
Annual Summary

3. DATES COVERED *(From - To)*
1 Oct. 2008-30 Sep 2009

4. TITLE AND SUBTITLE
The Role of Myeloid-Derived Suppressor Cells in the Immunotherapy of HER2/neu-Positive Breast Carcinomas

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0733

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Johanna K. Morales, Maciej Kmieciak, Masoud H. Manjili

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Virginia Commonwealth University

Richmond, VA 23219

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research
And Materiel Command
Fort Detrick, Maryland
21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We have found myeloid-derived suppressor cells (MDSC) to be the major inhibitors of adoptively transferred anti-HER2/neu T cells *in vivo.* Depletion of these cells results in tumor inhibition coupled with increased anti-HER2/neu antibody titers. Significantly, we have found that the combination of the chemotherapeutic drug gemcitabine, which selectively inhibits MDSC, with adoptive immunotherapy results in complete tumor rejection, elevated antibody titers, and immunological memory capable of rejecting further tumor challenge. Additionally, the generation of *de novo* MDSC from the bone marrow is dependent on GM-CSF, which is frequently used as an adjuvant in the immunotherapy setting. Based on these findings, we propose that the use of GM-CSF with immunotherapies be re-evaluated and suggest that immunotherapy should be administered in conjunction with gemcitabine for the inhibition of MDSC.

15. SUBJECT TERMS
Breast Cancer, HER2/neu, myeloid-derived suppressor cells, gemcitabine

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU		**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U		24	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction:

This study aimed to investigate the accumulation of myeloid-derived suppressor cells (MDSC) in hosts bearing HER2/neu positive breast carcinomas. Since MDSC have been found to be highly suppressive of anti-tumor immune functions, it is important to determine specific factors that cause the accumulation of these cells in tumor-bearing individuals. Furthermore, we aimed to develop means of inhibiting or otherwise subverting these cells *in vivo* as a first step to developing clinically relevant immunotherapies in tumor-bearing hosts. Given the widespread observation of MDSC accumulation in tumor-bearing individuals and the fact that 30% of breast cancers overexpress HER2/neu, the identification of soluble factors contributing to MDSC accumulation and the development of therapies that will circumvent this problem could have widespread therapeutic effects (1).

Body:

Determine the effect that MMC-derived Supernatants have on the viability and phenotype of sorted CD11b+Gr1-, CD11b-Gr1+, and CD11b+Gr1+ cells from the bone marrow of FVBN202 mice.

We confirmed that MMC-derived supernatants cause the generation of CD11b+Gr1+ cells through the intradermal injection of concentration MMC supernatants (Fig. 1). Through the use of cell sorting, we have determined that GM-CSF is the main tumor-derived soluble factor that supports the maintenance of sorted CD11b+Gr1+ cells *in vitro*. Importantly, we have also determined that GM-CSF causes the generation of *de novo* MDSC from sorted CD11b-Gr1- cells (Fig. 2). Other tested cytokines/chemokines (G-CSF, M-CSF, VEGF, MCP-1) did not support the generation or maintenance of MDSC. Technical limitations prevented us from obtaining adequate numbers of sorted CD11b-Gr1+ and CD11b+Gr1- cells. We also found that GM-CSF, but not G-CSF, M-CSF, VEGF, or MCP-1, augmented the viability of both pre-existing MDSC (sorted cells that were CD11b+Gr1+) and of newly-derived MDSC (sorted cells that were CD11b-Gr1-) generated using GM-CSF (Fig. 3). In order to eliminate unnecessary variables, RPMI with and without the recombinant cytokines/chemokines were used in cultures instead of MMC-conditioned medium with blocking antibodies, since conditioned medium may contain other un-identified factors.

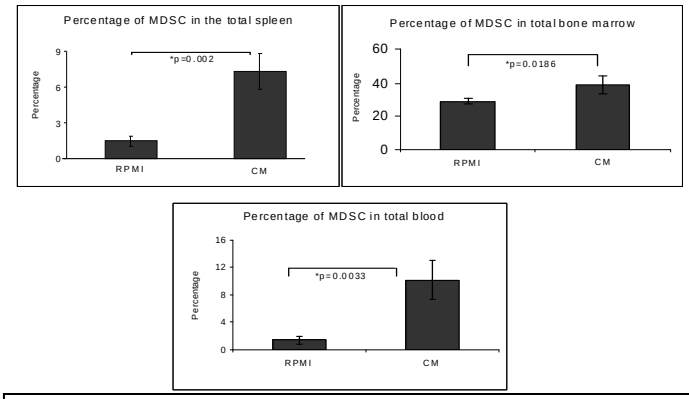

Figure 1: MMC-derived soluble factors cause an increase in MDSC in the spleen, bone marrow, and blood of FVB mice. FVB mice received 3 consecutive daily injection of either RPMI (containing 5% FBS) or with RPMI that had been conditioned with $10X10^6$ MMC's (in 10mL) for 24 hours. Media was removed and concentrated by centrifugation with a VivaSpin tube with a 10,000 kDa cutoff to a volume of 600uL. MMC conditioned medium (CM) was used to inject 3 FVB mice (200uL per mouse). Bars are the average percentage of total CD11b+Gr1+ cells as assessed by flow cytometry of the spleen, bone marrow, or blood +/- SD.

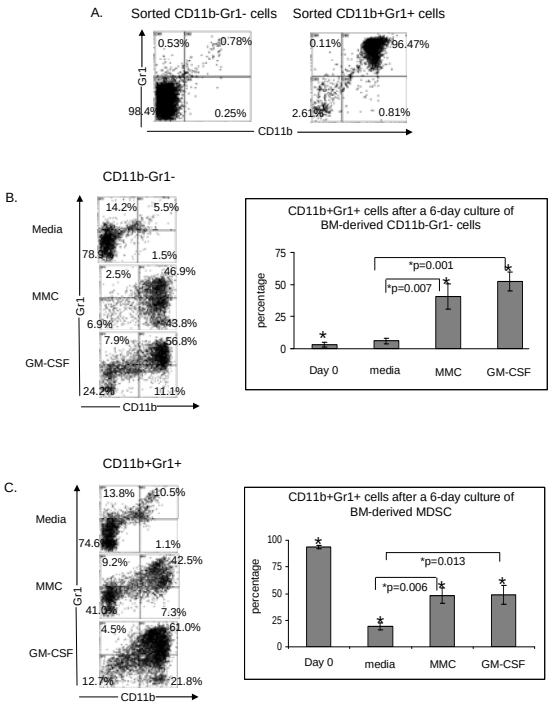

Figure 2: MMC-derived soluble factors and GM-CSF can generate MDSC from CD11b-Gr1- progenitor cells, and can maintain existing MDSC. Bone marrow cells from naïve FVBN202 mice were stained and sorted into 2 populations: CD11b+Gr1+ and CD11b-Gr1- cells. (A) Flow cytometry plots of the purity of CD11b+Gr1+ and CD11b-Gr1- populations after sorting. Data are representative of duplicate experiments. (B) Representative flow cytometry plots showing the percentage of CD11b+Gr1+ cells 6 days after culture of sorted CD11b-Gr1- cells (left) and averages of 3-4 experiments (right). (C) Representative flow cytometry plots showing the percentage of CD11b+Gr1+ cells remaining on day 6 after culture of sorted CD11b+Gr1+ cells (left) and averaged data from 3-4 experiments (right).

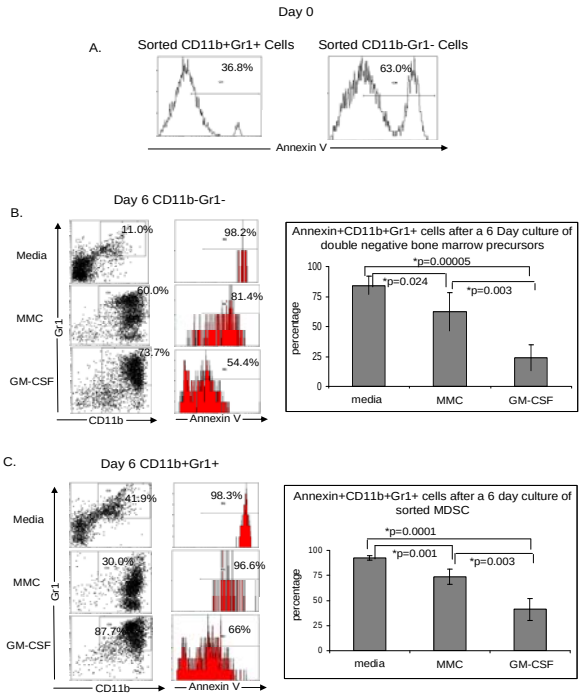

Figure 3: MMC-derived soluble factors and GM-CSF can protect newly-derived, and existing MDSC from apoptosis.
Sorted populations of CD11b+Gr1+ or CD11b-Gr1- cells were cultured for 6 days with media, MMC, or GM-CSF and stained on day 6 for CD11b, Gr1, and Annexin V. (A) Representative flow cytometry plots of duplicate experiments showing the expression of Annexin V on sorted CD11b+Gr1+ cells (left) or sorted CD11b-Gr1- cells (right) just after sorting. (B) Representative flow cytometry plots of CD11b+Gr1+ cells after 6 days of culture of sorted CD11b-Gr1- cells (boxed region) that are Annexin V positive. Bar graph shows the average percentage of CD11b+Gr1+AnnexinV+ cells from 3-4 experiments. (C) Representative flow cytometry plots of CD11b+Gr1+ after 6 days of culture of sorted CD11b+Gr1+ cells (boxed region) that are Annexin V positive. Bar graph depicts the averages of 3-4 experiments.

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To further characterize MDSC generated from GM-CSF and their suppressive functions, we stained for 2 different epitopes of the Gr1 molecule, designated as Ly6G and Ly6C. We found that that CD11b+Gr1+ cells from FVBN202 transgenic mice bearing HER2/neu positive tumors consisted of CD11b+Ly6G-Ly6C+ suppressive and CD11b+Ly6G+Ly6C+ non-suppressive subsets. Importantly, GM-CSF specifically supported the generation of the CD11b+Ly6G-Ly6C+ suppressive subset that inhibited proliferation as well as anti-tumor function of neu-specific T cells (Morales et al, manuscript submitted).

Prove that Gr1+ cells are a major obstacle to effective adoptive immunotherapy (AIT) in the FVBN202 transgenic mouse model of breast carcinomas

We have shown in Morales et al that the combination of AIT along with the depletion of MDSC (through the administration of anti-Gr1 antibodies) results in effective tumor inhibition (2; attached as Appendix 1). We have also shown that the expansion of effector T cells using a regimen of "alternating gamma chain cytokines", which includes a one time pulse of IL-2 on day 2 of cultures otherwise expanded solely in the presence of IL-7 and IL-15, results in greater expansion and a more highly cytotoxic population *in vitro*. MDSC depletion, however, is still critical, as transfer of these cells alone does not result in tumor regression (2). In addition, AIT using CD69+ versus CD69- cells showed no difference in tumor volumes, indicating that T cell migration and activation state are not as vital as MDSC depletion (Fig. 4).Paramount to our studies was the goal of combining AIT with the inhibition or depletion of MDSC *in vivo* in a clinically relevant manner. Due to some level of toxicity

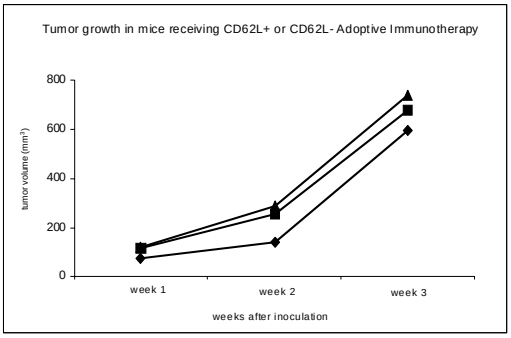

Figure 4: Adoptive transfer of CD62L- T cells does not prevent T cell suppression by MDSC (A) Tumor growth chart of FVBN202 recipient mice that were pretreated with Cyclophosphamide and then received either no treatment (squares), adoptive transfer of CD62L+ T cells expanded in alternating cytokines (triangles) or CD62L- T cells expanded in alternating cytokines (diamonds).

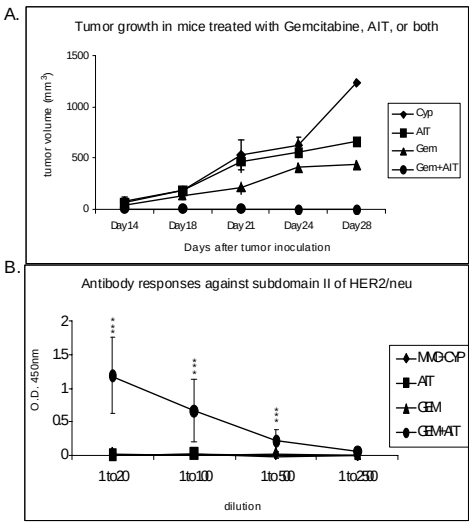

Figure 5: Gemcitabine combined with AIT causes complete tumor regression, along with strong antibody responses against HER2/neu (A) FVBN202 mice were treated with Cyclophosphamide (100mg/kg), challenged with MMC tumors 24 hours later, and administered $70X10^6$ adoptively transferred T cells 24 hours after that. Control mice were not given either AIT or Gemcitabine (Cyp, diamonds). Mice given Gemcitabine were given 1.5mg i.p. on days 6, 11, and 16. Mice give Gemcitabine alone (Gem) are indicated by triangles, mice given AIT alone by squares, and mice given both Gem and AIT (Gem+AIT) by circles. Tumor growth was measure with a digital caliper. Data represent averages of 3-5 mice per group +/- SEM. (B) Serum was collected on day 21 after tumor inoculation and ELISAs run against subdomain II of the ECD of HER2/neu. Data are averages of 2-4 mice per group, each sample run in duplicate +/- SEM with *** indicating that the Gem+AIT is significant as compared to each of the other 3 treatment groups.

resulting from high doses of anti-Gr1 depleting antibody, we sought the use of the chemotherapeutic drug, Gemcitabine, recently reported for its selective inhibition of MDSC (3,4). Although neither the use of Gemcitabine alone nor AIT alone resulted in complete tumor regression, the combination of these two therapies resulted in complete tumor rejection in 100% of mice (n=5, Fig. 5a). Importantly, these otherwise tolerant FVBN202 mice showed large antibody titers against the rat neu antigen (Fig. 5b). Immunological memory was also confirmed *in vivo* through the rejection of subsequent neu+ tumor challenges, increasingly robust anti-neu antibody responses, and high levels of IFN-γ secretion after restimulation of cells *in vitro* (Figs. 6 and 7). These data support that hypothesis that the presence of elevated MDSC in tumor bearing individuals is a major cause of reduced AIT efficacy and suggests that AIT should be combined with the chemotherapeutic agent Gemcitabine in order to achieve maximum effects.

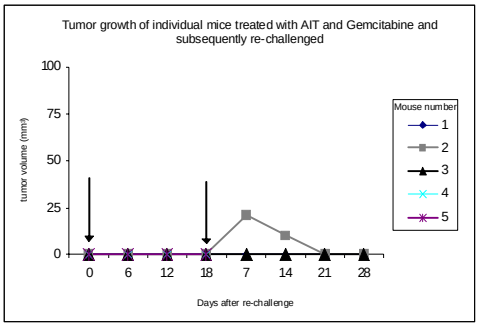

Figure 6: Mice treated with Gemcitabine and AIT can reject further MMC challenge Five mice that previously were treated with Gemcitabine and AIT and completely rejected MMC tumors, were re-challenged 74 days after the original challenge on the contralateral side with 3X106 MMC (Day 0). Re-challenged mice were treated with 1.5mg of Gemcitabine on days 6 and 11. Tumors did not grow in any of the five mice, therefore, 18 days after the first re-challenge, 3 of the five mice were re-challenged again the contra-lateral side with $3X10^6$ MMC (Day 18) and were not treated further with Gemcitabine. Tumor growth was measured again starting 7 days after the second challenge. Black arrows indicate days of re-challenge with MMC.

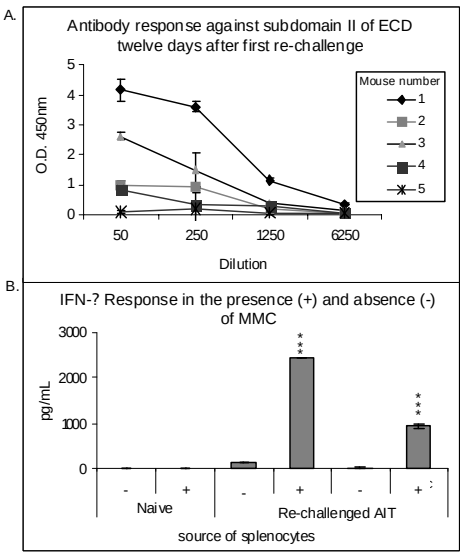

Figure 7: Mice receiving AIT and Gemcitabine treatment mount an immune response against subsequent MMC challenge (A) Serum was taken 12 days after FVBN202 mice that had received AIT and Gemcitabine had rejected a second challenge with MMC. ELISA was run against subdomain II of the HER2/neu ECD and read at 450nm. Data are averages of each sample run in duplicate +/- SD (B) Two of the 5 mice from above were killed and their splenocytes were cultured in duplicate either alone (-) or at a 10:1 E:T ratio with MMC cells (+). FVBN202 naïve splenocytes were used as a control. Supernatants were collected after 24 hours and used in an ELISA for the detection of IFN-ɣ. Data are averages of duplicate samples +/- SD. Triple asterisks (***) indicate that after subtracting the baseline value of IFN-ɣ secretion by splenocytes alone, the resultant value achieved a p-value <0.001 as compared to MMC alone, media alone, and naïve+MMC alone.

Key Research Accomplishments:

- Identification of MDSC as the major suppressive cell type of adoptive immunotherapy treatments
- Identification of GM-CSF as the key tumor-derived cytokine driving both the generation and maintenance of MDSC
- Optimization of cytokine expansion protocol for the generation of greater numbers of more highly cytotoxic, and more viable, donor T cells.
- Determination that the use of Gemcitabine in conjunction with AIT results in complete tumor rejection.
- Combinational therapy using Gemcitabine and AIT also results in immunological memory, evident by increasingly high antibody titers, large amounts of IFN-g secretion upon restimulation, and the rejection of subsequent challenges with neu+ tumors.

Reportable Outcomes

- Manuscripts:
    - **Morales, J. K.**, M. Kmieciak, L. Graham, M. Feldmesser, H. D. Bear, and M. H. Manjili. (2009). Adoptive transfer of HER2/neu-specific T cells expanded with alternating gamma chain cytokines mediate tumor regression when combined with the depletion of myeloid-derived suppressor cells. *Cancer Immunol. Immunother. 58: 941-953*
    - Le, H. K., L. Graham, E. Cha, **J. K. Morales**, M. H. Manjili, and H. D. Bear. (2009). Gemcitabine directly inhibits myeloid derived suppressor cells in BALB/c mice bearing 4T1 mammary carcinoma and augments expansion of T cells from tumor-bearing mice. *Int. Immunopharmacol. 9: 900-909.*
    - **Morales, J. K.**, M. Kmieciak, H.D. Bear, M.H. Manjili. GM-CSF is one of the main tumor-derived soluble factors involved in the differentiation of CD11b-Gr1- bone marrow progenitor cells into myeloid-derived suppressor cells. Manuscript Submitted.
- Poster Presentations:
    - **Morales, J.K**., Kmieciak, M., Graham, L. Feldmesser, M., Bear, H.D. Manjili, M.H. Adoptive Transfer of HER2/neu Specific T Cells Expanded with Alternating Gamma Chain Cytokines Mediates Tumor Regression when Combined with the Depletion of Myeloid-Derived Suppressor Cells. Cancer Immunotherapy: Realizing the Promise, National Institutes of Health 2008.
    - **Morales, J.K**., Kmieciak, M., Manjili, M.H. Adoptive Immunotherapy Targeting HER2/neu Positive Breast Carcinomas Requires the Depletion of Myeloid-Derived Suppressor Cells. Massey Cancer Center Retreat, Virginia Commonwealth University 2008.
    - **Morales, J.K**., Kmieciak, M., Manjili, M.H. Tumor-Derived Soluble Factors Cause the Differentiation and Maintenance of Myeloid-Derived Suppressor Cells. Watt's Day, Virginia Commonwealth University 2008.

- Degrees Obtained:
  - Doctor of Philosophy in Microbiology and Immunology from the Medical College of Virginia Campus of Virginia Commonwealth University, May 2009.
- Employment Received:
  - Postdoctoral Fellow position with Dr. John Ryan at Virginia Commonwealth University beginning April 2009.

Conclusions:

This work has identified MDSC as the single major inhibitor of effective adoptive immunotherapy treatments against HER2/neu+ breast carcinomas. We have found that the depletion of these cells results in the success of adoptive immunotherapies directed against the rat neu antigen. Importantly, we have found that the use of the drug Gemcitabine in combination with adoptively transferred T cells results in complete tumor rejection and long-lasting immunological memory. These findings are of great significance given that Gemcitabine is already approved for clinical use in humans and that adoptive immunotherapy is also being used in clinical trials. The identification of the effectiveness of this combinatorial therapy could have very positive implications for patients who are refractory to standard chemotherapeutic treatments and given the fact that adoptive transfers in humans have met with limited success. Furthermore, the identification of GM-CSF as the main factor driving the derivation of new MDSC from the bone marrow strongly suggests that the role of GM-CSF as an immune adjuvant should be re-evaluated. Clinically, the administration of GM-CSF to patients who already have GM-CSF-secreting tumors may elevate serum levels of GM-CSF sufficiently so as to cause the generation of MDSC and therefore dampen any potentially positive effects of the immune-based therapy. Taken together, we believe these data to be extremely important in its implications of the potential negative effects of the use of GM-CSF in immune-based therapies while highlighting the benefits of Gemcitabine.

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2. Morales, J. K., M. Kmieciak, L. Graham, M. Feldmesser, H. D. Bear, and M. H. Manjili. 2009. Adoptive transfer of HER2/neu-specific T cells expanded with alternating gamma chain cytokines mediate tumor regression when combined with the depletion of myeloid-derived suppressor cells. *Cancer Immunol. Immunother. 58: 941-953.*

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Appendix 1

Cancer Immunol Immunother (2009) 58:941–953
DOI 10.1007/s00262-008-0609-z

ORIGINAL ARTICLE

Adoptive transfer of HER2/neu-specific T cells expanded with alternating gamma chain cytokines mediate tumor regression when combined with the depletion of myeloid-derived suppressor cells

Johanna K. Morales · Maciej Kmieciak · Laura Graham · Marta Feldmesser · Harry D. Bear · Masoud H. Manjili

Received: 3 September 2008 / Revised: 1 October 2008 / Accepted: 3 October 2008 / Published online: 1 November 2008
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Abstract Adoptive immunotherapy (AIT) using ex vivo-expanded HER-2/neu-specific T cells has shown initial promising results against disseminated tumor cells in the bone marrow. However, it has failed to promote objective responses against primary tumors. We report for the first time that alternating gamma chain cytokines (IL-2, IL-7 and IL-15) ex vivo can expand the neu-specific lymphocytes that can kill breast tumors in vitro. However, the anti-tumor efficacy of these neu-specific T cells was compromised by the increased levels of myeloid-derived suppressor cells (MDSC) during the premalignant stage in FVBN202 transgenic mouse model of breast carcinoma. Combination of AIT with the depletion of MDSC, in vivo, resulted in the regression of neu positive primary tumors. Importantly, neu-specific antibody responses were restored only when AIT was combined with the depletion of MDSC. In vitro studies determined that MDSC caused inhibition of T cell proliferation in a contact-dependent manner. Together, these results suggest that combination of AIT with depletion or inhibition of MDSC could lead to the regression of mammary tumors.

J. K. Morales and M. Kmieciak have equally contributed to this work.

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Keywords Adoptive immunotherapy · Breast cancer · Myeloid-derived suppressor cells · HER-2/neu · Gamma chain cytokines

Introduction

To date, several vaccination strategies have been successfully employed to induce tumor-specific CD8+ and CD4+ T cell responses. However, such immunological responses have rarely been robust enough to achieve tumor regression [7, 48]. To overcome this obstacle by increasing the frequency of tumor-specific T cells, adoptive immunotherapy (AIT) treatments with effector cells activated and expanded ex vivo have been introduced and evaluated against a variety of cancers [8, 36]. Although AIT trials have exhibited partial effects in the case of melanoma and metastatic breast carcinoma, these therapies failed to exhibit objective responses against HER-2/neu positive breast tumors [2, 29]. Among a variety of approaches for the expansion of anti-tumor T cells, ex vivo, stimulation of T cells with bryostatin-1 (B) and ionomycin (I) has been shown to be promising in specifically activating anti-tumor effector T cells by mimicking signals through the T cell receptor, thus causing the expansion of tumor-specific effector T cells in the absence of the nominal antigen, ex vivo [6]. T cells activated in this manner and expanded in the presence of IL-2 were able to cause regression of MCA-105 pulmonary metastasis in vivo, as well as of established 4T07 mammary tumors transfected with IL-2 [5, 41]. However, IL-2 expansion may lead to the induction of regulatory T cells (T regs) and cause activation-induced cell death in T cells [20, 33, 47]. Alternatively, the gamma chain cytokines IL-7 and IL-15 are attractive candidates for AIT because of their role in the maintenance and proliferation of effector and memory T

cells. IL-7 has been implicated as a pro-survival cytokine for early memory CD8+ T cell selection and maintenance, while IL-15 can protect effector CD8 + T cells from apoptosis, as well as augment their cytotoxic effects [21, 22, 30, 38, 40, 43]. Additionally, unlike IL-2, IL-7 and IL-15 do not cause activation-induced cell death, nor are they needed for the maintenance of T regs [42].

One major obstacle hindering the clinical response of cancer immunotherapy is the presence of suppressor cells. In tumor-bearing hosts, myeloid-derived suppressor cells (MDSC) have emerged as a significant mediator of immune suppression in various types of cancer. For example, MDSC accumulate in several mouse models of cancer, including the MCA-26 colon carcinoma, the 4T1 mammary carcinoma, and the neu-overexpressing mouse mammary carcinoma (MMC) [4, 19, 27]. In humans, the presence of MDSC has been associated with head and neck cancer, renal cell carcinoma, and breast cancer [10, 32, 45]. Importantly, increased levels of circulating MDSC have recently been correlated with disease stage and extensive metastatic tumor burden in patients with breast cancer [10]. It was reported that MDSC accumulate in response to tumor-derived soluble factors VEGF, GM-CSF, and M-CSF [14, 16] and inhibit anti-tumor T cell responses, possibly through the production of soluble factors nitric oxide, arginase-1, reactive oxygen species, and inhibitory cytokines such as IL-10 [3, 28, 32, 35, 46]. In particular, arginine depletion and the production of reactive oxygen species can result in downregulation of the TCR zeta chain as well as T cell arrest in the G_0 phase of the cell cycle [13, 14] and impairment of IL-2 signaling [28], respectively. However, the role of MDSC in inhibiting adoptively transferred tumor-specific effector T cells has not yet been clearly defined.

The FVBN202 mouse model of spontaneously arising mammary carcinomas provides a clinically relevant model for investigating the immunotherapy of HER2/neu positive mammary tumors. These mice develop mammary carcinomas within 4-12 months of age due to the overexpression of the rat neu oncogene in their mammary glands under the regulation of the mouse mammary tumor virus (MMTV) promoter [18]. In these mice, atypical mammary hyperplasia develops prior to the occurrence of spontaneous mammary tumors and is accompanied by an increase in MDSC in the peripheral blood and spleen of FVBN202 mice during the premalignant stage, rendering animals resistant to neu-targeted immunotherapy [19, 26]. Furthermore, inoculation of MMC into FVBN202 mice causes a rapid and pronounced increase in MDSC [19]. In contrast, parental FVB mice do not express the rat neu oncogene and therefore are able to generate a robust immune response against challenge with neu-expressing MMC. These mice subsequently reject MMC, thus generating a pool of T cells with proven effectiveness against these tumors. These T cells derived from parental FVB mice are therefore ideal candidates for evaluating the efficacy of protocols for expanding neu-specific T cells in the absence of the nominal antigen, ex vivo. In addition, their FVBN202 transgenic counterpart provides an ideal model in which the effects of increased endogenous MDSC on adoptively transferred effector T cells can be evaluated. This study addresses, for the first time, the consequences of increased MDSC on the efficacy of AIT in the FVBN202 mouse model of breast carcinoma, as well as the novel finding of a restored neu-specific antibody response following the depletion of MDSC, in vivo. These findings suggest that AIT in breast cancer patients may be improved if it is combined with the inhibition or depletion of MDSC and that MDSC may also play a role in the suppression of B cell responses.

Materials and methods

Mouse model

Parental FVB (Jackson Laboratories) and FVBN202-transgenic female mice (Charles Riveer Laboratories) were used between 6 and 10 weeks of age throughout these studies. FVBN202 mice overexpress an unactivated rat *neu* transgene under the regulation of the MMTV promoter [18]. These mice develop premalignant mammary hyperplasia similar to ductal carcinoma in situ (DCIS) prior to the development of spontaneous carcinoma [24]. These studies have been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University.

Tumor cell lines

The MMC cell line was established from a spontaneous tumor harvested from an FVBN202 transgenic mouse as previously described [23]. The antigen negative variant (ANV) cell line was derived from a relapsed MMC tumor in the FVB strain as previously described and is characterized by a loss of neu expression [23]. Both cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Mice were challenged with 3×10^6 MMC cells i.d. where indicated.

Recombinant neu protein

The cDNAs coding for sub-domain II of the extracellular domain of rat neu (ECDII:187-332 aa) were amplified by PCR using the following primers: 5′-GTGGAATTCACCA

ATCGTTCCCGGGCC-3′(sense) and 5′-TCAAGCTTCA CAACGCTGTGTTCC-3′ (antisense). Restriction sites are underlined. The resulting fragments were cleaved with EcoR I and Hind III and ligated into the *Eco*RI–*Hin*d III fragment of pRSET A to generate the constructs. The recombinant proteins were expressed in *E. coli* using IPTG as an inducer of expression. Purification of His-tagged protein was performed under denaturing conditions using the Guanidinium Lysis Buffer. After elution, proteins were dialyzed in 20 mM Tris, pH 9.0 overnight (4°C). Dialyzed proteins were concentrated using 10,000 MW cut-off columns (Viva Spin), filter-sterilized and protein concentration was determined using Bradford assay.

Flow cytometry

Flow cytometry analysis was performed as previously described by our group [23]. Briefly, splenocytes were homogenized into a single cell suspension and 10^6 cells were aliquoted into each sample tube. Non-specific binding to Fc receptors was blocked with anti-CD16/CD32 antibody (Biolegend) for 20 min on ice. Cells were stained with surface antibodies towards various markers and incubated on ice in the dark for 20 min. Cells were washed twice and fixed with 1% paraformaldehyde or were washed again in 1X Annexin V buffer (BD Pharmingen) and the Annexin V staining protocol was followed. Samples were run on a Beckman Coulter FC 500 and analyzed using Summit version 4.3 software.

Cytotoxicity assay

Neu-specific effector lymphocytes were cultured with MMC at 10:1 and 20:1 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 (Peprotech) in six well culture dishes. After 24 h, 3 mL fresh media was added to the existing 3 mL of media. After 48 h, cells were harvested and stained for neu (anti-c-ErbB2/c-Neu, Calbiochem), Annexin V and PI according to the manufacturer's protocol (BD Pharmingen). Flow cytometry was used to analyze the viability of neu positive cells.

IFN-γ ELISA

Neu-specific effector lymphocytes were cultured in complete medium at a 10:1 ratio with irradiated MMC cells or ANV cells (15,000 rad) for 24 h. Supernatants were collected and stored at −80°C until used. IFN-γ was detected using a Mouse IFN-γ ELISA Set (BD Pharmingen) according to the manufacturer's protocol. Results are reported as the mean values of duplicate wells.

Expansion of effector T cells from FVB mice

FVB parental mice were inoculated with 5×10^6 MMC cells and splenocytes harvested after 20–25 days. Splenocytes (10^6 cells/mL) were stimulated in complete medium containing 15% FBS with bryostatin-1 (5 nM) and ionomycin (1 μM) along with 80 U/mL of IL-2 (Peprotech) for 16 h. Cells were washed three times and cultured at 10^6 cells/mL in complete medium with 40 U/mL IL-2 and media was changed every other day for a total of 7 days. Cells expanded with alternating gamma chain cytokines were cultured on day 1 with 10 ng/mL IL-7 and 10 ng/mL IL-15 (Peprotech). On day 2, 40 U/mL of IL-2 was added. Medium was changed on days 3 and 5, each time culturing with 10 ng/mL each of IL-7 and IL-15 and injections were done on day 7. Aliquots of cells were taken at indicated time points and samples were stained with combinations of the following antibodies and assessed by flow cytometry: FITC-CD25, FITC-CD62L, FITC-CD8, PE-CD44, PE-CD69, PE-CD8, PE/Cy5-CD4, PE/Cy5-CD8, PE/Cy5-CD69 from Biolegend and PE-CD25, PE-CD4 from BD Pharmingen. All antibodies were used at the manufacturer's recommended concentration.

Adoptive immunotherapy

Twenty-four hours prior to AIT, FVBN202 mice were treated with Cyclophosphamide (CYP, 100 mg/kg) by i.p. injection in order to induce lymphopenia. Mice were challenged i.d. with 3×10^6 MMC cells and then received 70×10^6 T cells by tail vein injection later the same day. Tumor growth was monitored by digital caliper and tumor volumes were calculated by: V(volume) = [L(length) × W(width)^2]/2. Blood was collected from the orbital sinus periodically to determine antibody responses and levels of CD11b + Gr1+ cells by flow cytometry. At the termination of the experiment, splenocytes were harvested and stained using the same antibodies as indicated above.

Depletion of MDSC in vivo

Monoclonal antibody against the surface antigen Gr1 was purified from the RB6-8C5 hybridoma by collecting supernatant from the CELLine CL 1,000 flask (IBS Integra Biosciences) according to the user manual. Supernatant was stored at −80°C until IgG purification using a MEP-Hypercell column. Where indicated, mice were injected i.p. with 250 μg of anti-Gr1 antibody for a total of 6 times at 3-day intervals, followed by a final injection of 200 μg on day 25. Depletion was verified by flow cytometry of the peripheral blood for the CD11b and Gr1 surface markers. The dose of anti-Gr1 antibody was proven completely effective in mice bearing MMC tumors that were 25 mm^3 or smaller.

Isolation of MDSC in vitro

Gr1+ cells were isolated using an EasySep PE Selection kit from StemCell Technologies. The protocol from the manufacturer was followed using splenocytes homogenized from MMC tumor-bearing FVBN202 mice labeled with 2 μg/mL of PE-Gr1 or PE-CD11b antibody from Biolegend. Purity of Gr1 + cells was confirmed by flow cytometry and was >90%.

In vitro T cell proliferation and BrdU labeling

T cell stimulations were done in 96 well plates. Plates were coated with 10 μg/mL of anti-CD3 (BD Pharmingen) and were washed three times with PBS after 24 h to remove any unbound antibody. Splenocytes (10^6 cells/mL in complete media), were labeled by adding 10 μM BrdU (BD Pharmingen) directly to the culture medium. Soluble anti-CD28 antibody (BD Pharmingen) was also added to the culture medium at 1 μg/mL. Cells were plated at 2×10^5 cells/well and were allowed to proliferate for 72 h at 37°C, 5% CO_2. Staining for BrdU was done following the protocol from the manufacturer (BD Pharmingen) using the FITC-conjugated anti-BrdU flow kit. Where indicated, MDSC were depleted from the splenocyte populations using the PE Selection protocol above with either PE-Gr1 or PE-CD11b antibodies (Biolegend). Isolated MDSC were added to wells where indicated at a 1:2 MDSC to splenocyte ratio either in the absence of a transwell insert, or in the top chamber of a transwell insert with 8.0 μm pore (Corning Life Sciences).

ELISA

Blood was collected from mice via the retro-orbital sinus, allowed to sit at room temperature for 10 min, and then spun for 10 min at 10,000 rpm. Serum was harvested and stored at −80°C until used. For measuring the antibody response against neu, 96 well plates were coated with 10 μg/mL of the ECDII and incubated overnight at 4°C. Plates were washed with PBS + 0.05% Tween-20 and blocked with 2% skim milk for 1 h. After washing, five-fold serial dilutions of the sera were added (100 μL/well) and incubated for 2 h at room temperature. Horse-radish-peroxidase (HRP)-conjugated anti-mouse IgG1 from Caltag was added at a 1:2,000 dilution for 1 h. Plates were washed and reactions developed by adding 100 μL/well of the TMB Microwell peroxidase substrate (Kierkegaard and Perry). The reaction was stopped with 2 M H_2SO_4, and the OD read at 450 nm. Mean antibody titers were then calculated.

Results

Bryostatin-1/Ionomycin (B/I) stimulation followed by IL-2 expansion generates highly activated neu-specific effector T cells

Since parental FVB mice recognize the rat neu protein as a foreign antigen and are subsequently able to reject MMC, whereas FVBN202 mice often tolerate neu protein and are unable to reject MMC, FVB mice were used as donors for AIT transfers into FVBN202 recipients in these studies. Since B/I selectively activates effector/memory T cells regardless of their antigen specificity [41], we sensitized FVB mice with MMC cells in order to increase the pool of neu-specific effector/memory T cells prior to T cell harvest for B/I activation ex vivo. We first compared the populations of CD4+ and CD8+ T cells from these donors immediately after harvest, after activation with B/I, and after a 7-day expansion with IL-2. Representative data from duplicate experiments are presented in Fig. 1a. FVB donor splenocytes contained 35 and 9% CD4+ and CD8+ T cells, respectively. These populations were similar immediately after B/I activation (27 and 12% CD4+ and CD8+, respectively) but were greatly increased after 7 days of culture with IL-2 (55 and 32% CD4+ and CD8+, respectively). Furthermore, IL-2 treatment increased the absolute number of viable T cells by 9.5-fold over the cell number that was cultured after B/I expansion (Fig. 1b). Annexin V+ CD4+ populations remained nearly constant during ex vivo expansion, starting at 20% on day 0 compared with 24% after B/I activation and cytokine treatment (Fig. 1c). The CD8+ T cells, however, showed a marked increase in Annexin V+ staining from day 0 to 7, with the fresh splenocytes being only 13% Annexin V+, while the post-B/I and post-cytokine values were 23 and 54%, respectively (Fig. 1c).

In order to determine T cell phenotypes, flow cytometry was performed for memory T cells (CD44+ CD62L+) and effector T cells (CD44+ CD62L−), as well as for the activation marker CD25, and the very early activation marker CD69, in both the CD4+ and CD8+ populations (Fig. 1d). As expected, B/I activation greatly increased the effector phenotype in the CD4+ population from 16 to 79% and in the CD8+ population from 5 to 66% while naïve (CD44− CD62L+) and memory (CD44+ CD62L+) CD4+ and CD8+ T cells were greatly decreased (Fig. 1d). After IL-2-induced expansion, both CD4+ and CD8+ T cells showed a marked increase in CD44+ CD62L+ memory T cells (74 and 58% in the CD4+ and CD8+ compartments, respectively), while maintaining increased levels of CD44+ CD62L− effector T cells compared to pre-B/I treatment (16 vs. 22% and 5 vs. 38%, respectively). Additionally, the expression of CD25 on CD4+ and CD8+ T cells was markedly increased to over

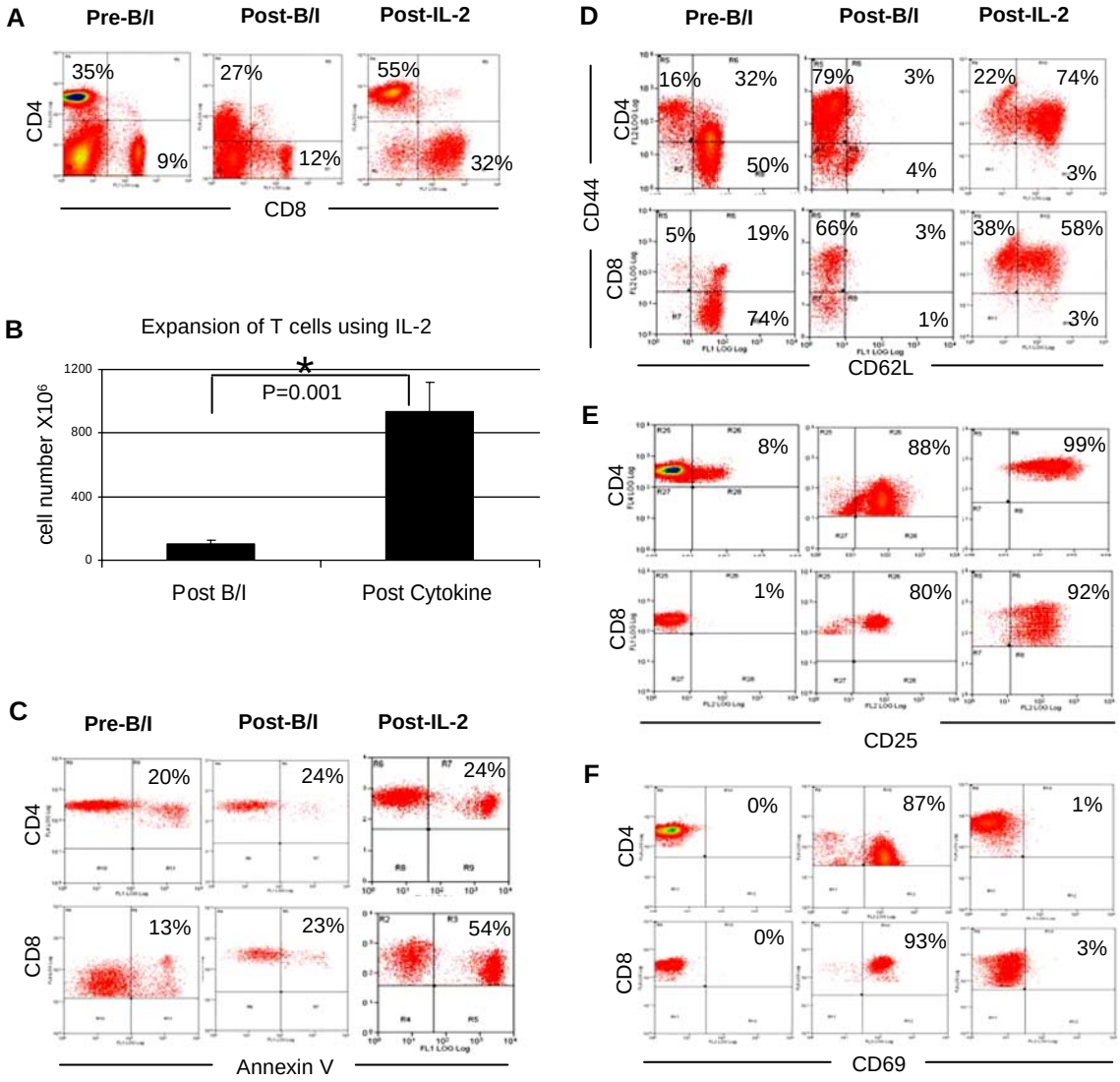


Fig. 1 Phenotype analysis of neu-specific anti-tumor T cells before and after activation with B/I and expansion with IL-2. **a** Flow cytometry analysis of total CD4+ and CD8+ populations in the lymphocyte region prior to B/I activation (pre-B/I), 16 h after B/I activation (post-B/I), and 7 days after expansion in the presence of IL-2 (post-IL-2). Representative data from two independent experiments are shown. **b** Absolute numbers of viable cells were determined from three independent experiments before and after expansion with IL-2 as determined by trypan blue exclusion using a hemocytometer. **c** Viability of T cell subsets as determined by Annexin V negative population within gated CD4+ or CD8+ lymphocyte regions. **d–f** Flow cytometry analysis performed on gated CD4+ or CD8+ T cells to determine percentage of memory T cells (CD44+ CD62L+), effector T cells (CD44+ CD62L−), early effector T cells (CD25+) and very early effector T cells (CD69+)

80% in both cases after B/I activation, and increased to over 90% in both populations after IL-2 expansion (Fig. 1e). The very early activation marker, CD69, was greatly increased from about 0.1–0.2% in fresh splenocytes to 87% in CD4+ T cells and 93% in CD8+ T cells after B/I activation. This value, however, dropped again after a 7-day culture with IL-2, with 0.8% CD69 expression in CD4+ T cells, and, notably, 3% remaining in the CD8+ T cells. However, most cells retained a late effector phenotype (CD44+ CD69−) on day 7 (Fig. 1f).

To confirm anti-tumor efficacy in vitro, T cells derived from MMC sensitized FVB mice prior to (Pre-B/I) or after a 7-day ex vivo expansion (post-cytokine) were co-cultured with MMC target cells (E:T ratio of 10:1) for 48 h followed by staining with antibodies directed towards neu, Annexin V, and PI. Control wells were seeded with MMC in the absence of T cells (Medium) (Fig. 2a). Gating on neu positive cells and analyzing the percentages of Annexin V and PI positive cells allowed for the determination of specific killing of the neu positive MMC cells by T cells. Viability of MMC in the absence of T cells was 86% (Annexin V and PI negative) while it dropped to 44% in the presence of the freshly isolated T cells (Pre-B/I). Viability of MMC was further decreased to 27% when cultured with B/I-activated,

IL-2-expanded T cells (post-cytokine) (Fig. 2a) Absolute numbers of viable MMC also reflect increased anti-tumor efficacy of B/I-activated, IL-2-expanded T cells compared to freshly isolated T cells ($P = 0.026$) (Fig. 2b). No killing was detected against the neu negative tumor variant, as determined by trypan blue exclusion (data not shown). Increased anti-tumor efficacy of the B/I-activated, IL-2-expanded T cells was due to an increased frequency of the neu-specific T cells compared to the freshly isolated T cells, as determined by IFN-γ ELISA (data not shown).

T cell expansion using an alternating gamma chain cytokine regimen increases T cell expansion and viability as well as anti-tumor efficacy in vitro

In order to select the best method of T cell expansion for AIT we sought to determine if expansion of T cells in the presence of alternating gamma chain cytokines may have advantages over expansion in the presence of IL-2 alone in terms of the expansion rates, viability, phenotype, and anti-tumor efficacy in vitro. Therefore, we expanded T cells using alternating gamma chain cytokines, i.e., adding a combination of IL-7 and IL-15 (10 ng/mL) on days 1, 3, and 5 with a one-time "pulse" of IL-2 (40 U/mL) on the second day. This type of expansion increased the percentage of CD8+ T cells (32% in Fig. 1a vs. 47% in Fig. 3a) and showed an 11-fold expansion in overall viable T cell number as compared to a 9.5-fold expansion with IL-2 treatment (Fig. 1b vs. 3b). Alternating gamma chain cytokines also greatly enhanced the viability of T cells on day 7 of culture when compared to expansion with IL-2 alone (Fig. 3c shows 13% Annexin V+ CD4+ T cells and 11% Annexin V+ CD8+ T cells compared with 24% Annexin V+ CD4+ T cells and 54% Annexin V+ CD8+ T cells in Fig. 1c). Of note, there were more CD44+ CD62L− effector T cells in both the CD4+ and CD8+ compartments after expansion using alternating gamma chain cytokines as compared to expansion with IL-2 alone (33 vs. 22% CD4+ T cells and 48 vs. 38% CD8+ T cells, Figs. 1d, 3d). While the percentage of CD4+ CD25+ T cells and CD4+ CD69+ T cells remained unchanged, CD8+ CD25+ T cells decreased from 92 to 69% and CD8+ CD69+ T cells decreased from 3 to 0.3% in comparing the IL-2-expanded T cells with alternating cytokine-expanded T cells, respectively (Figs. 1d, 3d). The cytotoxic effect of T cells against MMC, in vitro, was also greater using alternating gamma chain cytokines compared to that using IL-2 (27% viable MMC in Fig. 2a vs. 14% viable MMC in Fig. 3e). Absolute numbers of viable MMC also reflects a slight increase in anti-tumor efficacy of B/I-activated, alternating cytokine-expanded T cells compared to IL-2-expanded T cells (0.5×10^6 in Fig. 2b vs. 0.4×10^6 in Fig. 3e). Cells expanded in alternating cytokines also exhibited a strong IFN-γ response when stimulated with irradiated neu positive MMC (15,000 rad), but not with neu-negative ANV cells ($P = 0.006$), thus confirming the neu specificity of these cells (Fig. 3f).

Adoptive transfer of T cell subsets expanded ex vivo with alternating gamma chain cytokines inhibits tumor growth when combined with the depletion of MDSC in vivo

We hypothesized that an immunosuppressive environment characterized by a drastic increase in CD11b+ Gr1+ MDSC in the peripheral blood and spleens of FVBN202 mice

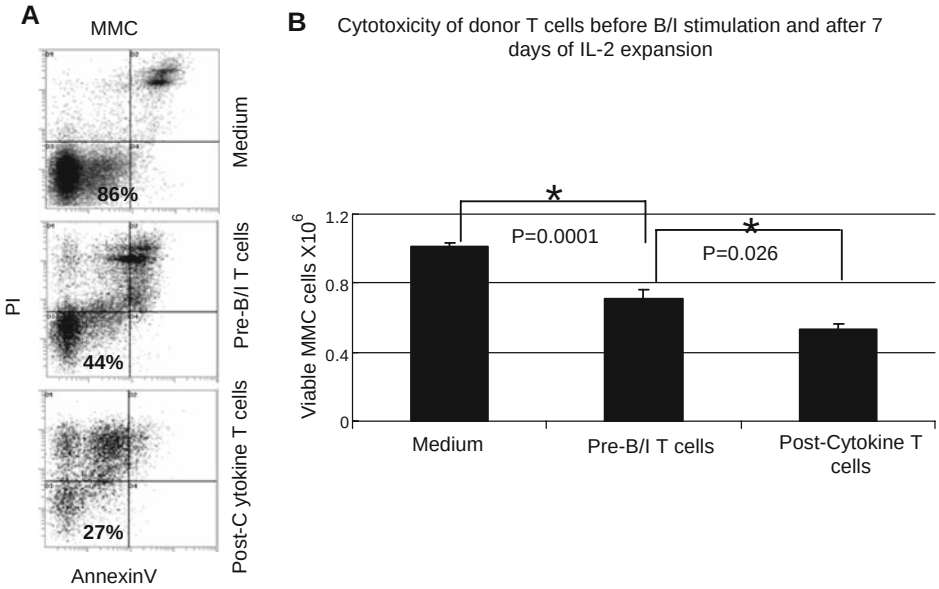


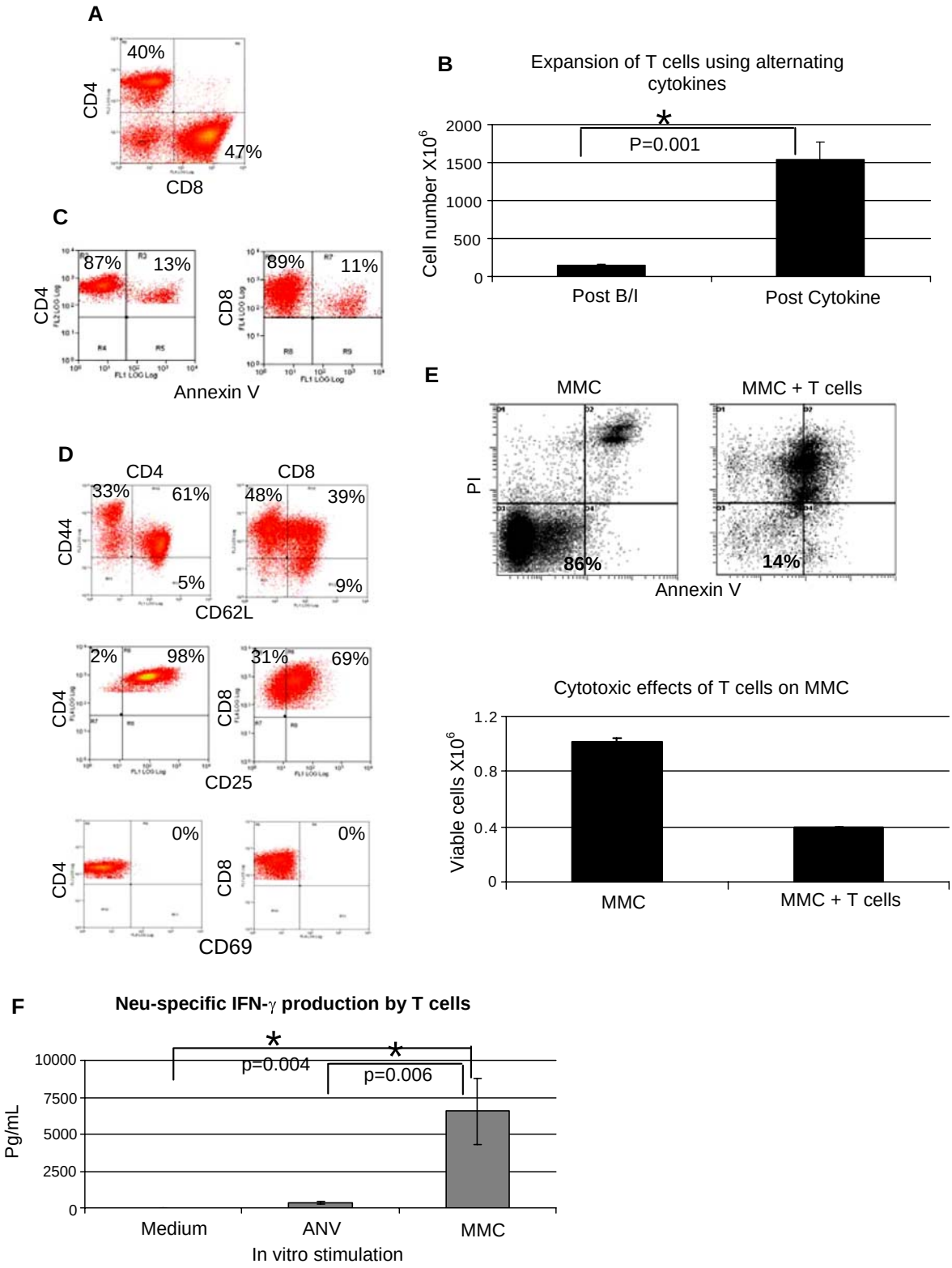
Fig. 2 Anti-tumor efficacy of neu-specific effector T cells before and after activation with B/I and expansion with IL-2. **a** Annexin V and PI analyses of gated neu positive MMC after 48 h of culture alone (*top*), or with neu-specific lymphocytes before (*middle*) or after (*bottom*) B/I activation and 7 days expansion in the presence of IL-2. Representative data of gated neu positive cells are shown from two independent experiments. **b** Cell counts using trypan blue exclusion for quantification of the total number of viable MMC. Data are averages of 2–4 experiments ± SEM

would inhibit the anti-tumor efficacy of neu-specific T cells prepared from MMC sensitized FVB donor mice. To test this hypothesis, FVB mice were inoculated with MMC (5×10^6 cells/mouse) and donor splenocytes were prepared 21 days after tumor challenge, when animals had completely rejected MMC. T cells were activated with B/I and were then expanded in the presence of alternating gamma chain cytokines as described above (Fig. 3). All FVBN202 mice were treated with i.p. injection of CYP (100 mg/kg) in order to create lymphopenia. Flow cytometry of the peripheral blood before, and 24 h after, CYP injection, showed no effect of this drug on the CD11b+ Gr1+ population. FVBN202 mice were challenged with MMC (3×10^6) 24 h after CYP treatment. MMC-challenged mice then received no treatment, the alternating gamma chain cytokine-expanded T cells alone (i.v. injection of 70×10^6 lymphocytes/mouse), or AIT combined with the depletion of MDSC by i.p. injection of an anti-Gr1 antibody starting 6 days after tumor inoculation and continuing every 3 days for a total of six injections, followed by a final injection of 200 μg on day 25 (Fig. 4a). AIT alone offered no protection against MMC (Fig. 4a). However, the in vivo depletion of MDSC improved the efficacy of AIT and caused significant tumor inhibition ($P = 0.001$ for week 4 and $P = 0.0003$ for week 5). The efficacy of MDSC depletion was above 98% 9 days after tumor challenge (data not shown). Flow cytometry analysis of blood collected from each group 24 days after the tumor challenge showed that the group receiving Gr1 depletions had a significantly reduced percentage of MDSC (Fig. 4b). However, it is noteworthy that 38% of the granulocytes were still CD11b + Gr1 + at this time, a problem that we believe to be caused by slightly increased tumor burden in these mice leading to increased recruitment of MDSC (Fig. 4b). Our preliminary studies showed that the optimal dose of anti-Gr1 antibody was effective in depleting MDSC, however, we were not able to increase the frequency of antibody injections because of toxicity of the antibody (data not shown). Flow cytometry analysis of MDSC levels in the blood on day 35 (10 days after the last injection of anti-Gr1 antibody) showed that levels of MDSC were fully replenished in the Gr1 depletion group (data not shown). AIT using either CD4 + or CD8+ T cells alone, as well as the administration of anti-Gr1 antibody alone, did not cause tumor inhibition (data not shown).

Since the ECD-specific antibody response is also involved in the protection against neu positive mammary tumors [11, 12, 25], we sought to determine whether FVBN202 mice mounted antibody responses against the ECD following AIT. Serum taken from mice receiving adoptive transfer of T cells expanded with alternating gamma chain cytokines with and without the in vivo depletion of MDSC, along with control mice, indicated that only mice that were depleted of Gr1+ cells were able to mount an antibody response against the ECD ($P = 0.006$) (Fig. 4c).

The presence of MDSC inhibits CD3/CD28-induced proliferation of T cells in vitro in a contact-dependent manner

To determine the mechanisms by which MDSC suppress anti-tumor immune responses, splenocytes were isolated from MMC tumor-bearing and tumor-free FVBN202 mice. The tumor-bearing mice had a large influx in MDSC in their spleens as compared to tumor-free mice [19]. Splenocytes were labeled with the thymidine analog BrdU and stimulated with antibodies against CD3 (10 μg/mL, plate-bound) and CD28 (1 μg/mL, soluble), or were left unstimulated to serve as a control. After 3 days, cells were stained using anti-CD4 and anti-CD8 antibodies and analyzed for BrdU uptake by flow cytometry. We found higher proliferation of CD4+ T cells from tumor-free mice compared to those from tumor-bearing animals (Fig. 5a, 91 vs. 59%, $P = 0.002$). The same trend was seen for CD8+ T cells (Fig. 5a, 93 vs. 70%). Similar trends were detected while analyzing absolute numbers of CD4+ and CD8+ T cells (Fig. 5b). Fig. 5b shows that the average number of BrdU+ CD4+ and BrdU+ CD8+ T cells from tumor-free mice is 3.3-fold higher than that of BrdU+ CD4+ and BrdU+ CD8+ T cells from tumor-bearing mice ($P = 0.003$ for CD4+ and $P = 0.0004$ for CD8+ T cells). Using lymphocytes derived from FVB donors we also found similar patterns of the MDSC-mediated suppression of T cell proliferation in vitro (data not shown). To confirm that the suppression of T cell proliferation in FVBN202 splenocytes was caused by the presence of elevated MDSC, we depleted MDSC in vitro, from the splenocytes of the tumor-bearing animals. As seen in Fig. 5a, the depletion of MDSC (tumor-bearing-MDSC) significantly restored the proliferative responses of both CD4+ (87% BrdU+) and CD8+ (92% BrdU+) T cells over those seen in total splenocytes ($P = 0.028$ for CD4+ and $P = 0.009$ for CD8+ T cells). Similar trends were found while comparing the absolute numbers of CD4+ and CD8+ T cells, showing a 3.1-fold increase in proliferation in the CD4+ population ($P = 0.027$) and 2.5-fold increase in the CD8+ population ($P = 0.012$) over total splenocytes from tumor-bearing mice (Fig. 5b). To determine if the anti-proliferative effects of MDSC on T cells were contact-dependent, CD11b+ cells were depleted from the splenocytes of tumor-bearing and tumor-free mice and equal numbers of lymphocytes (5×10^5) were stimulated in the lower chamber of a plate containing a Transwell insert. CD11b+ cells depleted from tumor-bearing mice were then added to the lower chamber of the plate, or were added to the top chamber of the Transwell insert, where they were separated from the lymphocytes by an 8.0 υm pore. Staining for BrdU incorporation on day 3 showed potent inhibition when



CD11b+ cells were added back to the cultures, but only in the absence of a Transwell insert (Fig. 5c). There was a total lack of inhibition observed when CD11b+ cells were separated from the T cells by a transwell insert (Fig. 5c). Similar trends were detected while analyzing the absolute numbers of CD4+ and CD8+ T cells (data not shown).

◀ **Fig. 3** Characterization of neu-specific anti-tumor effector T cells expanded ex vivo using "alternating" gamma chain cytokines. **a** Flow cytometry analysis was performed to determine the percentage of CD4+ and CD8+ T cells after B/I activation and 7 days expansion with alternating gamma chain cytokines. Representative data from three experiments are shown. **b** Absolute numbers of lymphocytes were determined by trypan blue exclusion cell counts using a hemocytometer. Data are mean of three experiments ± SD. **c** Viability of T cell subsets as determined by Annexin V negative population within gated CD4+ or CD8+ lymphocyte regions. **d** Flow cytometry analysis was performed on gated CD4+ or CD8+ T cells to determine the percentage of memory T cells (CD44+ CD62L+), effector T cells (CD44+ CD62L−), early effector T cells (CD25+) and very early effector T cells (CD69+) after B/I activation and 7 days expansion with alternating cytokines. **e** MMC cells were cultured for 48 h with medium (MMC) or in the presence of the neu-specific lymphocytes activated with B/I and expanded with alternating gamma chain cytokines (MMC+ T cells). Annexin V and PI analyses were performed on gated neu positive MMC. Cell counts using trypan blue exclusion were done for quantification of the total number of viable MMC. **f** IFN-γ secretion by T cells in the presence or absence of irradiated neu + MMC or neu-ANV. Results are the average of duplicates ± SD

Discussion

The adoptive transfer of tumor-specific T cells that have been activated and expanded in vitro is a promising means of systemically treating residual cancers after resection of the primary tumor. The presence of immune suppressor cells has, however, become a substantial obstacle to the success of these treatments. In particular, MDSC represent a potent population of suppressor cells that are elevated in many different types of cancer, including neu positive mammary tumors in FVBN202 mice, and have been associated with suppression of T cell responses by multiple mechanisms. T regs were also reported to suppress anti-tumor immune responses. We looked at T regs in the blood, spleen, lymph nodes and the tumor site of FVBN202 mice and found no changes in the number of CD4+ CD25+ Foxp3+ T regs (data not shown). Since we wanted to determine whether MDSC could suppress robust T cell responses even against allogeneic antigen, we used FVB mice as donors of T cells. Although using such an allogeneic system will reduce clinical application of the proposed AIT regimen, failure of such effector T cells in the rejection of MMC tumor cells attests to the strength of in vivo suppression by MDSC, and upon proving that MDSC depletion does indeed facilitate an otherwise ineffective AIT, we would next hope to use these procedures with the expansion of splenocytes from tumor sensitized FVBN202 mice. We also showed for the first time that alternating gamma chain cytokine conditions are more effective than IL-2 alone for the expansion of neu-specific anti-tumor effector T cells. Having compared IL-2 with alternating gamma chain cytokines (IL-7 and IL-15 with a one-time dose of IL-2), we found that the latter was superior to the former in expanding

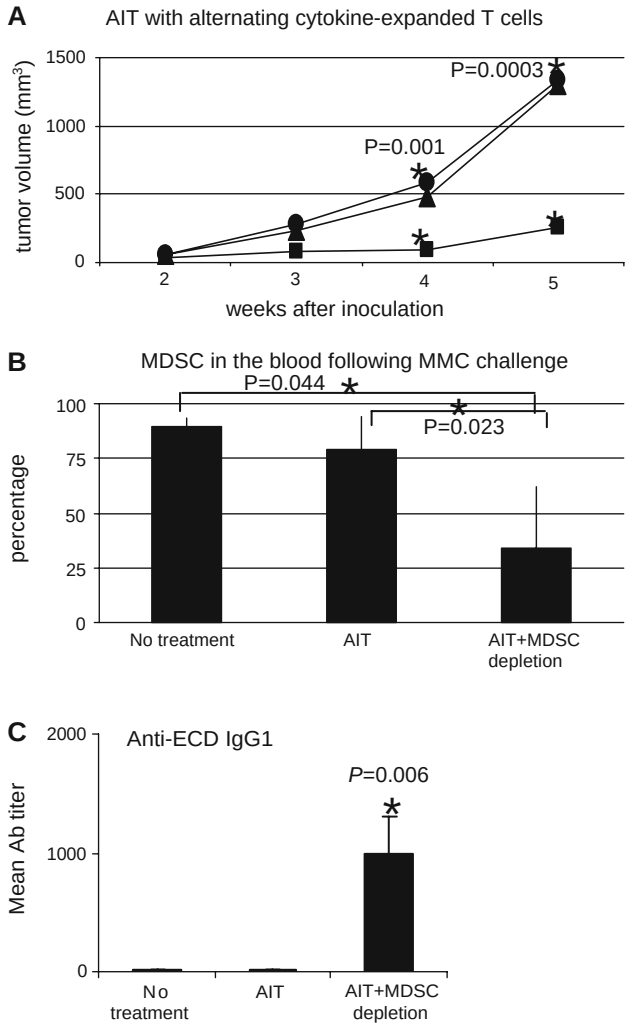


Fig. 4 AIT after expansion with "alternating" gamma chain cytokines combined with the depletion of MDSC in vivo can result in tumor inhibition and restoration of the neu-specific antibody response. **a** Tumor growth measurements of mice that received CYP followed by MMC challenge (3×10^5) were given no treatment (*circles*), 70×10^6 adoptively transferred T cells (*triangles*), or 70×10^6 adoptively transferred T cells followed by administration of 250 μg of anti-Gr1 antibody on days 6, 9, 12, 15, 18, and 21 and 200 μg on day 25 after tumor innoculation for depletion of MDSC (*squares*). Data points represent the averaged tumor volumes of 4–6 mice per group. **b** Efficacy of MDSC depletions as measured by flow cytometry analysis of blood using antibodies against CD11b and Gr1. *Values* are the average percentage of CD11b+ Gr1+ cells from the granulocyte regions of 4–6 mice per group 24–30 days after tumor inoculation ± SD. **c** Mean IgG1 antibody titers against ECDII in serum collected from animals ($n = 4$) 21 days after MMC inoculation

T cells with a higher overall viability, as well as a higher proportion of effector T cell phenotypes, which could readily exhibit anti-tumor activity. In fact, alternating gamma chain cytokine-expanded T cells showed a higher anti-tumor efficacy than IL-2-expanded T cells, as evaluated by cytotoxicity assay in vitro. However, adoptive transfer of such effector T cells did not overcome the pre-existing

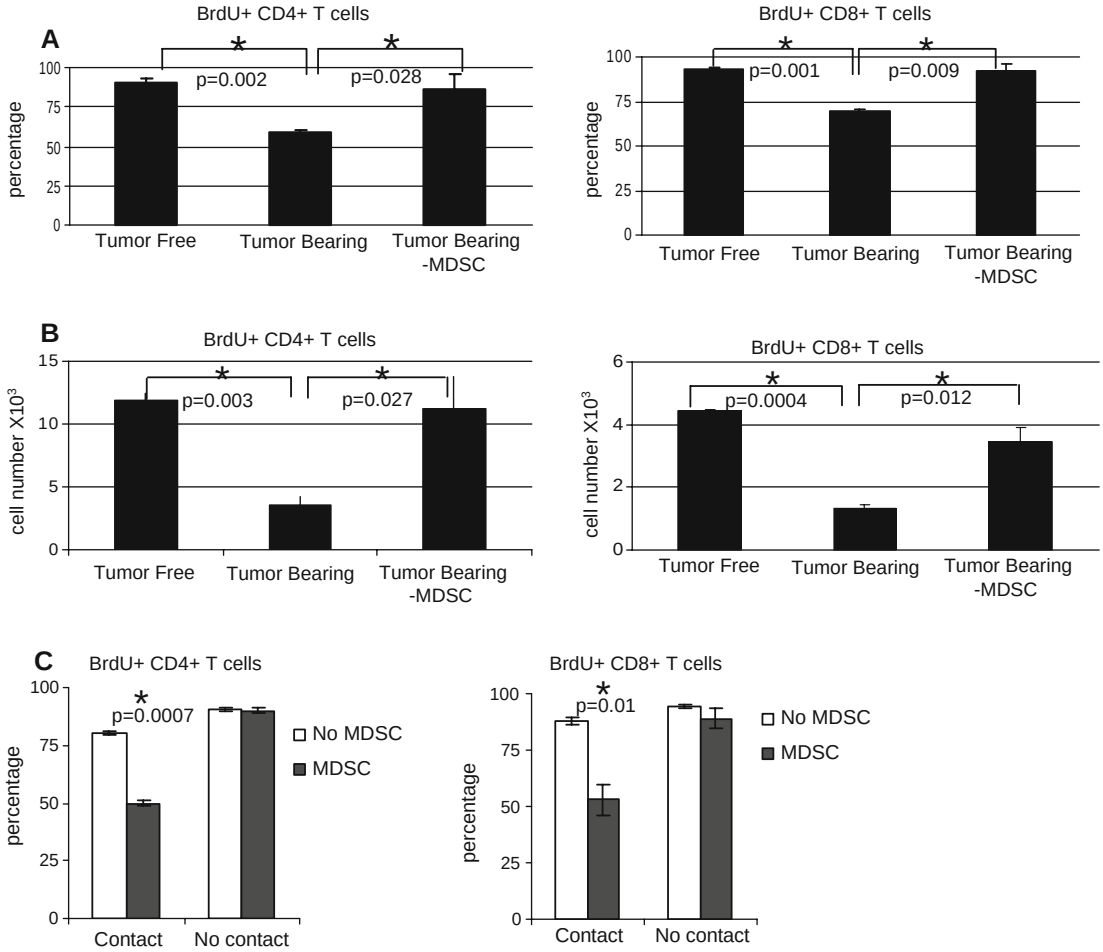


Fig. 5 MDSC-mediated inhibition of T cell proliferation in a contact-dependent manner. **a**–**b** Percentage and absolute numbers of CD4+ and CD8+ cells that were positive for BrdU incorporation after a 3-day culture with anti-CD3 and anti-CD28 antibodies using total splenocytes from tumor-free or tumor-bearing FVBN202 mice with and without MDSC depletion in vitro. Data are averages of two separate experiments ± SD. **c** Splenocytes from FVBN202 mice were depleted of CD11b+ cells, and added to the bottom of a transwell culture dish and pulsed with BrdU. Where indicated, MDSC from the tumor-bearing FVBN202 mice were added, either directly to the splenocytes (Contact) or in the top chamber of a transwell insert (No contact). Cells were stimulated with anti-CD3 and anti-CD28 antibodies for 3 days and analyzed for BrdU incorporation in the CD4+ and CD8+ populations by flow cytometry as before. Data are presented from duplicate experiments

immune suppressive microenvironment in FVBN202 animals. We have previously shown that these mice have increased levels of MDSC, even at the premalignant stage due to mammary hyperplasia [19, 24]. Interestingly, the combination of depleting MDSC in vivo with AIT using neu-specific T cells resulted in a robust tumor regression following AIT. Complete rejection of MMC tumors by the effector T cells, however, was hindered by the fact that antibody-mediated MDSC depletion in FVBN202 mice was incomplete. This was due to increasing numbers of MDSC in tumor-bearing mice over time following the cessation of injection of anti-Gr1 antibody, as preliminary depletions (9 days after tumor challenge) were nearly 100% effective (data not shown). To achieve MDSC depletion, we were not able to inject the rat anti-mouse Gr1 antibody more than six times because of its toxicity. Because of such limitations in the control of MDSC in vivo, we were able to perform prophylactic studies only. Therapeutic efficacy of this strategy on established tumors remains to be determined using alternative drugs such as gemcitabine for selective elimination of MDSC [26]. Sporadic spontaneous tumor development after a long latency in FVBN202 mice makes it difficult to test whether AIT combined with the inhibition of MDSC could also protect animals against spontaneous tumor development.

Although other groups have reported the role of MDSC in suppression of anti-tumor T cell responses [3, 15, 16], we report for the first time that MDSC also suppress humoral immune responses following AIT so that depletion of MDSC in vivo restored anti-neu antibody responses in FVBN202 mice. This is very important because it has been reported that collaboration of humoral and cellular immune

responses is required for optimal elimination of HER-2/neu positive tumors [2]. In addition, a novel HER-2/neu-specific antibody, Pertuzumab, is currently in phase III trial and has been shown to have anti-tumor function through recognizing and blocking the dimerization domain of HER-2/neu, ECDII [1, 37]. Curiously, there was no antibody response in the groups that received AIT alone, indicating that the presence of adoptively transferred CD4+ T cells alone is not sufficient to facilitate IgG1 isotype switching by the recipients' B cells because of the presence of MDSC. It is unclear at this point whether the restoration of the antibody response results from lifting MDSC suppression of adoptively transferred helper T cells, or whether MDSC directly suppress B cell responses. We performed multiplex cytokine array analysis of the sera collected from the experimental groups above following AIT but did not detect any changes in the level of IL-4 (unpublished observation). The role of MDSC in the suppression of the humoral response should be further investigated and could have many applications beyond cancer immunotherapy, since increased MDSC have also been seen in some parasitic infections such as Trypanosoma cruzi [17] and in cases of polymicrobial sepsis [9].

In order to further confirm that failure of AIT to induce regression of MMC in FVBN202 mice was indeed due to inhibition of T cell function by MDSC, we performed in vitro assays to assess MDSC-mediated inhibition of T cell proliferation. Consistent with the fact that tumor-bearing animals exhibit about a fourfold increase of MDSC in the granulocyte region of their splenocytes [19], total splenocytes from tumor-bearing mice showed a marked reduction in the number and percentage of proliferating T cells. Significantly, depleting MDSC from the culture using either an anti-CD11b or an anti-Gr1 antibody caused the restoration of TCR-mediated T cell proliferation. These observations suggest that MDSC suppress TCR-induced proliferation of T cells.

The possible contact-dependent mechanism of T cell suppression by MDSC has been an area of some debate. Using different tumor models, most groups have found suppression of T cells by MDSC to be mediated by soluble factors such as arginase-1, nitric oxide, reactive oxygen species and peroxynitrites [34, 39]. In particular, arginase-1 has been implicated in downregulation of the TCR zeta chain and the induction of this enzyme has been linked to tumor-derived soluble factors such as prostaglandin E2 as well as IL-4 and IL-13 [13, 34, 35]. Furthermore, arginine-depleted conditions can cause TCR zeta downregulation in the absence of cell-to-cell contact [35]. We were therefore surprised to see that in our model, cell-to-cell contact was required for the suppression of T cell proliferation. Although other mechanisms may be involved in suppression of cytotoxic responses by T cells, we report here that no suppression of T cell proliferation was seen when MDSC were added to the top chamber of a transwell insert. Nagaraj et al. [31] have recently shown that direct cell-to-cell contact between CD8+ T cells and MDSC causes nitration of tyrosines in the TCR–CD8 complex, therefore disrupting binding of specific antigen-MHC class I complexes to the TCR's of OT-1 transgenic T cells. It is unclear, however, if the effects of this nitration may be exaggerated due to transgenic expression of the TCR and what role this mechanism may play in a TCR non-transgenic model. Furthermore, Gabrilovich et al. [15] have reported that blocking the MHC class I molecules expressed on the surface of MDSC can reverse suppression of CD8+ T cells, which involved MDSC production of nitric oxide, but reported that these cells did not suppress CD4+ T cells responses towards MHC class II presented peptides. In 2000, Kusmartsev et al. showed inhibition of CD3/CD28 T cell activation by MDSC isolated from mice bearing MCA-26 colon carcinomas. However, this suppression was reversed by the addition of a superoxide dismutase mimetic and a nitric oxide synthase inhibitor and a possible role of contact was not investigated, and proliferation was not determined in separate populations of CD4+ and CD8+ T cells [27]. In contrast, we show here that MDSC from mice bearing HER2/neu + mammary carcinomas inhibit the proliferation of both CD4+ and CD8+ T cells in contact-dependent manner. Therefore, although contact between MDSC and CD8+ T cells has been speculated to be important in inhibiting the IFN-γ response of CD8+ T cells towards specific peptide, we show here that contact is also necessary to inhibit CD3/CD28 T cell stimulation and can affect the proliferation of both the CD4+ and CD8+ T cells populations.

Furthermore, we have shown that endogenous MDSC inhibit T cell proliferation in the splenocytes of tumor-bearing FVBVN202 mice. Therefore, it is likely that elevated levels of MDSC during the premalignant stage may generate an immunosuppressive microenvironment in these animals that could inhibit anti-tumor efficacy of AIT. We have previously shown that increased MDSC in FVBN202 mice during the premalignant stage was associated with mammary gland hyperplasia, and this was correlated with the failure of pre-existing neu-specific immune responses to prevent spontaneous mammary carcinomas in these mice [19, 24]. We have also reported the existence of an immune suppressive microenvironment at the tumor lesions of FVBN202 mice, as evidenced by increased levels of IL-10, IL-10 receptor, SOCS-1, and SOCS-3 [44]. Such a microenvironment could then inhibit AIT unless it is combined with the depletion of MDSC, suggesting that MDSC may be the key cells initiating the cascade of events leading to tumor-specific immune suppression.

Acknowledgments This work was supported by NIH R01 CA104757 grant (M. H. Manjili) and flow cytometry shared resources facility supported in part by the NIH grant P30CA16059. We thank Daniel Conrad and Jamie Sturgill for their assistance with purification of the anti-Gr1 antibody. We gratefully acknowledge the support of VCU Massey Cancer Centre and the Commonwealth Foundation for Cancer Research, Department of Defense Grant BC083048.

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