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ORIGINAL ARTICLE

Lung cancer patients' CD4$^+$ T cells are activated in vitro by MHC II cell-based vaccines despite the presence of myeloid-derived suppressor cells

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Abstract
Background Advanced non-small cell lung cancer (NSCLC) remains an incurable disease. Immunotherapies that activate patients' T cells against resident tumor cells are being developed; however, these approaches may not be effective in NSCLC patients due to tumor-induced immune suppression. A major cause of immune suppression is myeloid-derived suppressor cells (MDSC). Because of the strategic role of CD4$^+$ T lymphocytes in the activation of cytotoxic CD8$^+$ T cells and immune memory, we are developing cell-based vaccines that activate tumor-specific CD4$^+$ T cells in the presence of MDSC. The vaccines are NSCLC cell lines transfected with costimulatory (CD80) plus major histocompatibility complex class II (MHC II) genes that are syngeneic to the recipient. The absence of invariant chain promotes the presentation of endogenously synthesized tumor antigens, and the activation of MHC II-restricted, tumor-antigen-specific CD4$^+$ T cells.
Methods Potential vaccine efficacy was tested in vitro by priming and boosting peripheral blood mononuclear cells from ten NSCLC patients who had varying levels of MDSC. CD4$^+$ T cell activation was quantified by measuring Type 1 and Type 2 cytokine release.
Results The vaccines activated CD4$^+$ T cells from all ten patients, despite the presence of CD33$^+$CD11b$^+$ MDSC. Activated CD4$^+$ T cells were specific for NSCLC and did not cross-react with tumor cells derived from non-lung tissue or normal lung fibroblasts.
Conclusions The NSCLC vaccines activate tumor-specific CD4$^+$ T cells in the presence of potent immune suppression, and may be useful for the treatment of patients with NSCLC.

Keywords Lung cancer · Major histocompatibility complex class II · CD4$^+$ T lymphocytes · Cell-based cancer vaccine · Myeloid-derived suppressor cells

Abbreviations

Ii	Invariant chain
MDSC	Myeloid-derived suppressor cells
NSCLC	Non-small cell lung cancer
XRT	Radiotherapy

Introduction

Lung cancer is a major cause of morbidity throughout the world [16], with more than 1 million new cases diagnosed

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annually [28]. Despite the existing treatments of surgery, radiotherapy, chemotherapy, and multimodal therapies, the long term survival of patients remains low [24, 28, 32]. Because of the high mortality rate, alternative strategies for the treatment of lung cancer are being developed, including novel agents aimed at activating a patient's immune response to lung cancer cells. Some of these immune-enhancing approaches have shown preclinical efficacy, and have modest effects in a small subset of patients in clinical trials; however, none have been curative and the majority of patients do not respond [24, 28, 32]. Many of these treatments are "active" immunotherapies that require patients to be immunocompetent. However, individuals with cancer are frequently immunosuppressed, and this immune suppression may block the ability to respond to the therapy [8, 11, 19].

Various populations of cells and soluble factors produced by cells have been identified in cancer patients and shown to mediate immune suppression. These include, myeloid-derived suppressor cells (MDSC) [34, 46], T regulatory cells (T regs) [37], and soluble factors such as TGFβ [22]. MDSC are a heterogeneous population of cells of myeloid origin that are present in many cancer patients, including patients with non-small cell lung cancer (NSCLC) [1]. They suppress immunity through multiple pathways, including the inhibition of T cell activation [1, 6, 21, 39], the blocking of NK cell cytotoxicity [42], the induction of T regulatory cells [14], the production of IL-10 [38], and by decreasing macrophage production of IL-12 [38].

We are focusing on developing immunotherapies for the treatment of advanced stages of lung cancer. Because $CD4^+$ T cells are critical for activating $CD8^+$ T cells and for the induction of immunological memory [15, 35], we have designed cell-based vaccines that activate tumor-specific $CD4^+$ T cells. Since >70% of lung cancer patients have NSCLC [24], we are focusing our efforts on this group of patients. To activate $CD4^+$ T cells, we have genetically engineered lung cancer cell lines to express major histocompatibility complex class II (MHC II) molecules and a co- stimulatory molecule (CD80) in the absence of invariant chain (Ii), an MHC II chaperone protein that is present in professional antigen presenting cells (APC), such as dendritic cells (DC). Ii promotes the presentation of exogenous antigen, and its absence facilitates the presentation of endogenously synthesized antigen [23]. Therefore, tumor cells that express syngeneic MHC II and costimulatory molecules and do not express Ii, should activate MHC II syngeneic $CD4^+$ T cells to endogenously synthesized tumor antigens. We have called these genetically modified tumor cells "MHC II vaccines," and previous studies using uveal melanoma and breast cancer cells demonstrate that they activate a novel repertoire of tumor-specific $CD4^+$ T cells [45] from healthy donors [9] and from uveal melanoma patients [5]. Since the role of the activated $CD4^+$ T cells is to provide help to $CD8^+$ T cells and to facilitate the development of immune memory, the activated $CD4^+$ T cells do not need to directly react with patients' tumor cells. Therefore, MHC II and/or CD80 on the patients' tumor cells is not required.

Since MDSC have been found in patients with NSCLC [1], we are determining their effects on NSCLC patients' responsiveness to MHC II lung cancer vaccines. We show that despite varying levels of MDSC in NSCLC patients, $CD4^+$ T cells from these individuals are activated in vitro by the MHC II lung cancer vaccines, suggesting that NSCLC patients may be candidates for active immunotherapy with the MHC II vaccines.

Materials and methods

Cells

SUM159PT and MCF10CA1 (hereafter called MCF10) mammary carcinomas and OMM2.3 uveal melanoma cells were maintained as described [5, 9, 44]. The human NSCLC cell lines H358 (bronchioloalveolar adenocarcinoma) and HTB-177 (large cell carcinoma; hereafter called H177), and normal lung fibroblasts were obtained from the American type culture collection. Tumor lines were cultured in tumor medium (RPMI supplemented with 10% heat inactivated fetal calf serum, 1.5 g/l sodium bicarbonate, 10 mM HEPES, 1.0 mM sodium pyruvate, 1% penicillin/streptomycin (Biosource, Rockville, MD, USA), 2 mM glutamax (BRL/Life Sciences, Grand Island, NY, USA) and 1% gentamycin). Fibroblasts were cultured in fibroblast medium (DMEM supplemented with 10% heat inactivated fetal calf serum, 1.5 g/l sodium bicarbonate, 10 mM HEPES, 1.0 mM sodium pyruvate, 1% penicillin/streptomycin, 2 mM glutamax, and 0.1 mM non-essential amino acids (BRL/Life Sciences). MCF10 cells transduced with DR7, CD80, or Ii (MCF10/DR7/CD80, MCF10/DR7/CD80/Ii), Jurkat, and Sweig cells were maintained as previously described [45]. HLA genotypes of the NSCLC cell lines are shown in Table 1. Procedures with human materials were approved by the Institutional Review Boards of the participating institutions.

IFNγ treatment

A total of 2×10^5 cells were incubated at 37°C, 5% CO_2 for 48 h in six well plates in 3 ml of their culture medium containing 200 U/ml recombinant human IFNγ (Pierce Biotechnology, Rockford, IL, USA).

Antibodies and immunofluorescence

Monoclonal antibodies W6/32 (pan HLA-A, B, C), L243 (pan HLA-DR), and PIN1.1 (Ii) were prepared as described

Table 1 HLA haplotypes of the cell lines and patient and healthy donors used in these studies

Patient/healthy donor/cell line	HLA-A[a]	HLA-B[a]	HLA-DR[b]	Lung cancer stage[c]	Treatment
Patient 1	A29, A32	B44, B60	DR4,DR7	IV	Cisplatin, TLK-286, alimta, erbitux
Patient 3	A1	B8, B57	DR7, DR15	IIIB	
Patient 4	A1, A2	B15, B60	DR1, DR8	IV	Carboplatin, gemcitabine
Patient 5	A3	B7, B−	DR15	IB	Carboplatin, Taxol
Patient 6	A2, A24	B13, B51	DR4, DR7	IV	Carboplatin, Taxol, XRT[d]
Patient 7	A2, A3	B7, B62	DR4	IV	None
Patient 8	A3, A11	B−, B35	DR1	IIIB	Carboplatin, Taxol, XRT
Patient 9	A2	B44, B62	DR4, DR16	IV	Cisplatin, TLK-286, alimta
Patient 10	A-, A11	B7, B44	DR7, DR15	IV	Cisplatin, TLK-286, alimta, taxotere, tarceva
Patient 11	A1, A3	B35, B57	DR1, DR7	IV	None
Donor BC123104	A33, A36	B−, B44	DR1, DR7		
Donor BC011405	A23, A68	B44, B*	DR7, DR15		
Cell line H358	A*03	B35	DR1, DR−		
Cell line H177	A24, A68	B35, B51	DR1, DR4		

[a] Patients' and healthy donors' PBMC and the cell lines constitutively express MHC I

[b] Patients' and healthy donors' PBMC, but not the cell lines, constitutively express MHC II

[c] TNM stage

[d] Radiotherapy

[9]. CD80-PE, CD19-FITC, mouse FITC (IgG2a) and PE isotype (IgG2a) controls, and streptavidin-PerCP were from BD PharMingen (San Jose, CA, USA). CD4-FITC, CD8-FITC, and goat-anti-mouse magnetic beads were from Miltenyi Biotech (Auburn, CA, USA). CD33-FITC and CD11b-PE were from SeroTec (Raleigh, NC, USA). CD3-PE, CD56-FITC, CD68-PE, CD11c-PE, DEC205-FITC, and CD15-biotin were from eBioscience (San Diego, CA, USA). Goat-anti-mouse IgG-FITC was from ICN (Costa Mesa, CA, USA). Live tumor cells or peripheral blood mononuclear cells (PBMC) were stained for cell surface markers (CD4, CD8, CD56, CD3, CD11b, and CD80), and fixed and permeabilized cells were stained for internal markers (FoxP3 or Ii). Immunofluorescence and flow cytometry were performed as described [9].

Western blots

Western blots for Ii were performed as described [5, 44] using PIN1.1 mAb followed by sheep-anti-mouse-HRP (Amersham) at a 1:5,000 dilution.

Patient and healthy donor PBMC, HLA typing, and HLA nomenclature

Blood samples from healthy donors BC123104, BC011405, BC120204, BC100504, and BC100706 and lung cancer patients 1, 3, 4, 5, 6, 7, 8, 9, 10, and 11, were obtained by venipunture and the resulting cells purified by Ficoll gradient and stored in liquid nitrogen as described [9]. PBMC that were >80% viable after thawing were used. PBMC and lung cancer cell lines were HLA-typed and analyzed using MicroSSP™ HLA Class I and II ABDR DNA typing trays and analysis software (One Lambda, Inc., Canoga Park, CA, USA) according to the manufacturer's instructions. Stage of disease, treatments of NSCLC patients, and HLA genotypes of patients and healthy donors used in the T cell activation studies are shown in Table 1. HLA genotypes are referred to by their short hand form (e.g. HLA-DRB1*0701 is HLA-DR7).

CD4, CD8 and CD33 cell depletions

Peripheral blood mononuclear cells were depleted for $CD4^+$, $CD8^+$, or $CD33^+$ cells using magnetic beads, LD or LS columns, and QuadroMACS separation system according to the manufacturer's instructions (Miltenyi Biotech) as described [9]. Depleted populations were confirmed by flow cytometry and <5% of depleted populations were present in the final populations.

Purification of peripheral blood $CD11b^+CD33^+$ MDSC

$CD11b^+CD33^+$ cells were positively purified from patients' PMBC using CD33-magnetic beads according to the manufacturer's instructions (Miltenyi Biotech). Briefly, PBMC were washed twice with degassed cold MACS buffer (0.5% BSA in PBS with 2 mM EDTA) and $1-2 \times 10^7$ washed cells were incubated with anti-human CD33 microbeads (1:5 dilution) for 15 min at 4°C. The resulting cells were

resuspended in MACS buffer, centrifuged at 300*g* for 10 min, and the pelleted cells resuspended in 1 ml and passed through an LS column for positive selection. Cells released from the column were 87–90% $CD11b^+CD33^+$ and contained less than 1–2% $CD3^+$, $CD11c^+$, $Dec205^+$, $CD68^+$ or $CD56^+$ cells as assayed by flow cytometry. $CD33^+$ $CD11b^+$ cells were stained with H&E using a Diff-Quick kit (Dade Behring Inc., Newark, DE, USA) and observed under 100× oil immersion magnification using a Leica DM IRB microscope.

DNA constructs and transfection

Human lung cancer lines were stably transfected with pLHCX/CD80, pLNCX2/DR7, pLNCX2/DR4, and/or pLNXC2/DR1 [9] constructs by Nucleofector™ technology according to the manufacturer's instructions (Amaxa Biosystems). Briefly, $1–2 \times 10^6$ tumor cells were resuspended in 100 µl of Nucleofector solution V with 8–10 µl of DNA (1.5–2 µg), transferred to a cuvette, and immediately subjected to nucleofection using program T-20. Nucleofected cells were then removed from the cuvette by addition of 500 µl RPMI medium and cultured in 3 ml of complete tumor cell culture medium in a six well plate. After 48 h of culture, transfected cells were grown for 3–4 days in complete culture medium supplemented with hygromycin (CD80 transfectants; Calbiochem, San Diego, CA, USA) or G418 (MHC II transfectants; Sigma, St. Louis, MO, USA). Stable transfectants were obtained by multiple rounds of drug treatment followed by magnetic bead sorting using L243 and CD80 primary mAbs and goat-anti-mouse beads as described [9]. Stable transfectants were grown in the same culture medium as their parental cells. CD80 and MHC II levels were assessed by flow cytometry periodically over the course of a year and remained constant.

T cell activation by anti-CD3 and anti-CD28

Round bottom 96-well plates (BD Falcon™, Franklin Lakes, NJ, USA) were coated overnight at 4°C with 100 µl of 1 µg/ml anti-CD3 antibody (BD Pharmingen) diluted 1:1 with PBS. Unbound anti-CD3 antibody was removed by washing with excess PBS and 1×10^5 PBMC in 200 µl of T cell medium (Iscove's modified Dulbecco's medium, 5% human AB serum (Gemini Bio-Products, Woodland, CA, USA), 1% penicillin, 1% streptomycin, 2 mM glutamax, 1.0 mM sodium pyruvate, 5×10^{-5} M β-mercaptoethanol, 10 mM HEPES, 1% gentamycin) containing soluble anti-CD28 antibody at 2 µg/ml were added. Cells were cultured at 37°C for 72 h, and each well then pulsed with 1 µCi ^{3}H-thymidine in 50 µl of T cell medium. Sixteen to eighteen hours later the cells were harvested onto glass fiber filter mats using a Packard Micromate 196 cell harvester. Filter mats were sealed in plastic bags with 5 ml of betaplate scintillation fluid (PerkinElmer, Shelton, CT, USA) and counted using a Wallac 1,450 Microbeta liquid scintillation counter (PerkinElmer). Data are expressed as cpm (mean ± SD) of triplicate wells.

T cell activation by MHC II lung vaccine cells

PBMC were in vitro activated with MHC II vaccines as previously described [5]. Briefly, for priming, 2.5×10^6 PBMC were cultured for three days with 2.5×10^5 irradiated (50 Gys) vaccine cells in 2 ml T cell medium/well in 24-well plates. Non-adherent cells were then harvested, washed and replated with IL-15 (20 ng/ml) in a 24-well plate at 1×10^6 cells/2 ml T cell medium. Five days later, non-adherent cells were harvested, washed, replated in 24-well plates at 1×10^6 cells/2 ml of T cell medium, and rested for 24 h. The primed cells were then boosted with live stimulator cells at a ratio of 1:2 (2.5×10^4 vaccine cells: 5×10^4 primed PBMC/200 µl/well) in flat bottom 96 well plates. For T cell proliferation assays after priming, PBMC and irradiated (50 Gys) H358/DR7/CD80 cells were cultured for 72 h and then pulsed with 1 µCi ^{3}H-thymidine/well. Sixteen to eighteen hours later the cells were harvested and counted. For some experiments Miltenyi-purified $CD33^+CD11b^+$ cells were added at the start of the boosting phase at a ratio of 1:1 primed PBMC:$CD11b^+CD33^+$ cells. Percent $CD4^+$ T cell yield = 100% × [(number of $CD4^+$ T cells on day 0 before priming/number of $CD4^+$ T cells on day 9 at boosting)].

Cytokine measurements

IL-2, IL-5, IL-10, IL-12, and some IL-4 levels were measured at the University of Maryland, Baltimore cytokine analysis facility by multiplex analysis, using a Luminex 100 system and fluorescent microbeads [http://www.cytokines.com]. IFN-γ and some IL-4 levels were measured by ELISA as previously described [44].

Statistical analysis

Mean, SD, and statistical significance as measured by Student's *t* test were calculated using Excel (Windows XP professional).

Results

Lung cancer patients have high levels of immunosuppressive $CD11b^+CD33^+$ cells

Studies by others with patients having a variety of different tumors have demonstrated that $CD33^+CD11b^+CD14^-$,

$CD11b^+CD14^-CD15^+$, $Lin^-CD33^+HLA\text{-}DR^-$, $CD14^+HLA^{low/-}$cells of the blood are immune suppressive [1, 10, 47]. These cells have been called MDSC [12]. To test if NSCLC patients have MDSC, PBMC from ten NSCLC patients and from five healthy individuals were stained with antibodies to CD33, CD11b, and CD15, as well as antibodies identifying $CD4^+$ T cells, $CD8^+$ T cells, B cells, NK cells, and NKT cells. Figure 1a shows representative flow cytometry profiles of cells from three patients (patients 3, 6, and 11) and healthy donor BC123104. These patients have varying levels of $CD11b^+CD33^+$ cells, most of which also express CD15, consistent with the interpretation that the $CD11b^+CD33^+$ population in the peripheral blood of NSCLC patients is predominantly MDSC. These cells, as visualized by H&E staining are a heterogenous mixture of cells with myeloid characteristics (Fig. 1b). Table 2 summarizes the percent of each cell type from ten patients and five healthy donors. $CD11b^+CD33^+$ cells in the healthy donors are 12–16% of PBMC, while NSCLC patients have between 12–53% $CD11b^+CD33^+$ cells. Percentages of B cells ($CD19^+$), NK cells ($CD56^+$), and NKT cells ($CD56^+CD3^+$) did not significantly differ between patients and healthy donors. In contrast, T cells ($CD3^+$), $CD4^+$ T cells ($CD4^+CD3^+$), and $CD8^+$ T cells ($CD8^+CD3^+$) decline as percentages of $CD11b^+CD33^+$ cells increase. The level of T regulatory cells as measured by $CD4^+CD25^+Foxp3^+$ cells for both NSCLC and healthy donors ranged from 1.7–3.2% (data not shown).

To determine if the $CD11b^+CD33^+$ population is suppressive, PBMC from >>NSCLC patients 6, 7, and 11 were activated in vitro with anti-CD3 and anti-CD28 antibodies in the presence or absence of the $CD11b^+CD33^+$ cells (Fig. 1c). Depletion of $CD11b^+CD33^+$ cells significantly increased T cell proliferation, consistent with $CD11b^+CD33^+$ cells being suppressive.

Bronchioloalveolar and large cell carcinoma cells transfected with HLA-DR7 and CD80 stably express these molecules and do not co-express Ii

MHC II and Ii are transcriptionally coordinately regulated by the MHC class II transactivator, CIITA [40]. Since previous studies have established that MHC II vaccines are most efficacious if they do not contain Ii [2, 45], NSCLC cell lines were tested for their constitutive expression of MHC II and Ii, and screened for induction of MHC II and Ii following incubation with IFNγ. H358, a bronchioloalveolar carcinoma, and H177, a large cell carcinoma, did not constitutively express MHC II or Ii, whereas control breast cancer cells transfected with the Ii gene (MCF10/DR7/Ii) express high levels of p33 Ii (Fig. 2a, b). Since IFNγ induces Ii and MHC II [18], the NSCLC cells were incubated in vitro with IFNγ and their expression of MHC II and Ii assessed by flow cytometry. Neither H358 nor H177 cells were induced to express MHC II or Ii by IFNγ and were therefore chosen as base lines for NSCLC vaccines. Following transfection with plasmids encoding HLA-DR7, HLA-DR1, or HLA-DR4, and the costimulatory molecule CD80, the H358 and H177 cell lines stably expressed HLA-DR and CD80 as assayed by flow cytometry (Fig. 2c).

MHC II lung cancer vaccine cells prime and boost tumor-specific type 1 $CD4^+$ T cells from healthy donors

To test if the MHC II lung cancer vaccine cells prime and boost HLA-DR syngeneic $CD4^+$ T cells, PBMC from HLA-DR7 healthy donor BC123104 were primed in vitro with irradiated MHC II vaccine cells (H358/DR7/CD80 vaccine) and boosted with H358, H358/DR7, or H358/DR7/CD80 cells (Fig. 3a). Vaccine cells and responding PBMC were syngeneic for MHC II alleles to optimize an MHC class II-restricted response. Previous studies established that allogeneic MHC class I differences between vaccine cells and responding T cells do not activate IFNγ-producing $CD4^+$ T cells [5], so the vaccine cells and PBMC were not matched for MHC I alleles. T cell activation was assessed by quantifying IFNγ in the culture supernatants (Fig. 3b). Maximal PBMC activation was achieved by priming and boosting with H358/DR7/CD80 vaccine cells, whereas, priming with H358/DR7/CD80 and boosting with H358 cells that lack either DR7 or CD80 is significantly less effective. Priming with unmodified parental cells (H358) followed by boosting with H358, H358/DR7, H358/CD80, or H358/DR7/CD80 cells did not activate T cells. Similar results were obtained using PBMC from another HLA-DR7 healthy donor (BC011405), as well as from an HLA-DR4 donor primed and boosted with H358/DR4/CD80 cells (data not shown). Therefore, genetically modified bronchioloalveolar NSCLC cells efficiently activate PBMC from healthy donors, provided they co-express HLA-DR and CD80 molecules.

To identify the population of T cells that is activated by the MHC II vaccine cells, PBMC from healthy donor BC123104 were depleted for $CD8^+$ or $CD4^+$ T cells prior to priming and boosting with H358/DR7/CD80 vaccine cells (Fig. 3c). Depletion of $CD4^+$ T cells abolished IFNγ release, while depletion of $CD8^+$ T cells had no effect. Therefore, the vaccine specifically activates MHC II-restricted, $CD4^+$ T cells.

T cells have been termed “type 1” or “type 2” depending on the spectrum of cytokines they produce, and type 1 responses are considered beneficial for anti-tumor immunity [17]. To determine which type of response is induced by the vaccines, PBMC from healthy donor BC123104 were primed with irradiated H358/DR7/CD80 cells and

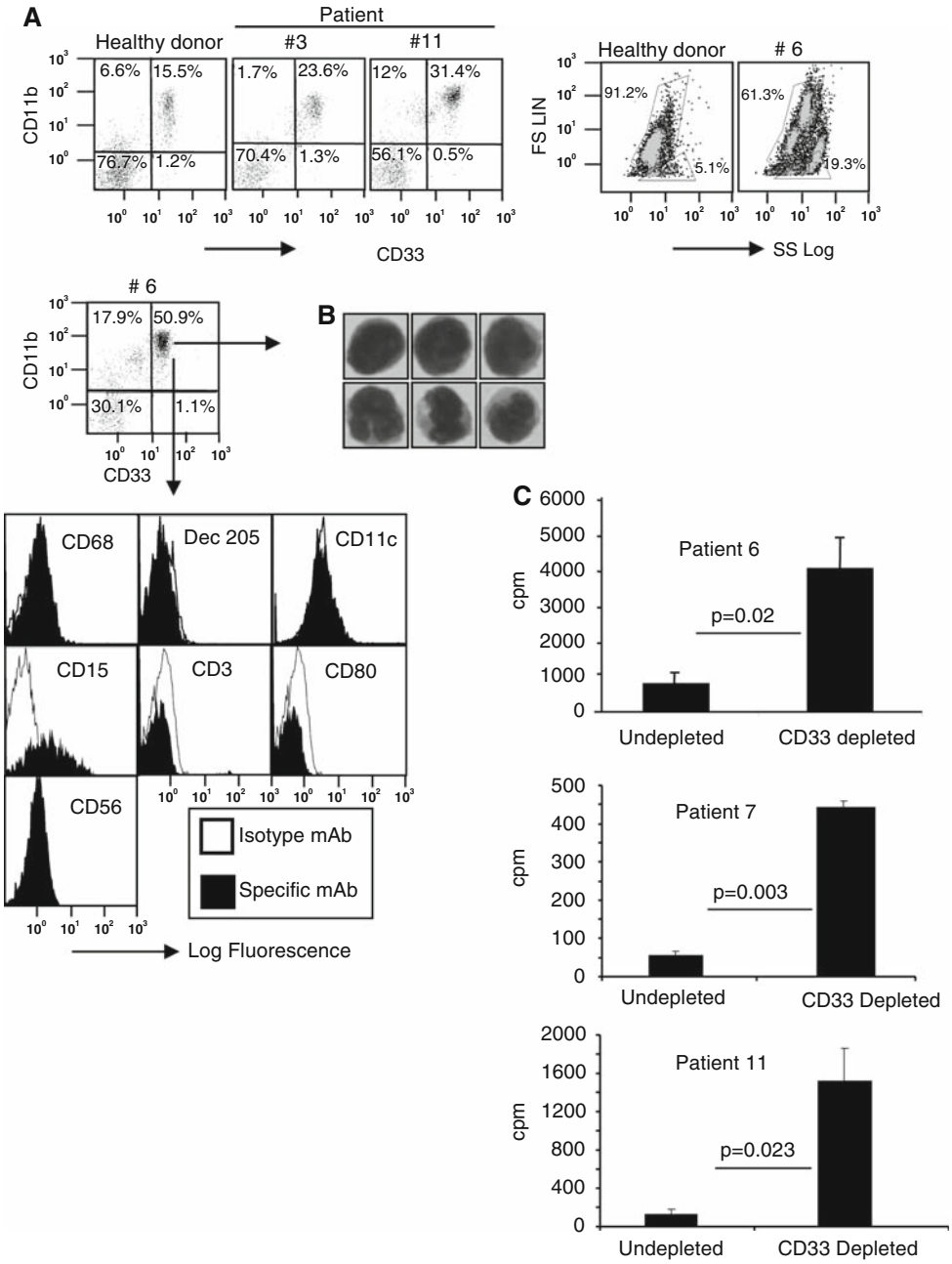

Fig. 1 Lung cancer patients have immunosuppressive $CD11b^+CD33^+$ cells in their peripheral blood. **a** PBMC from healthy donor BC123104 and from NSCLC patients 3, 11 and 6 were stained with mAbs for CD11b, CD33, CD15, CD3, CD68, CD11c, DEC205, CD56, and CD80, or isotype control mAbs. Viable cells were gated (largest gate in the *right-hand panels*) and analyzed by flow cytometry. **b** H&E stained, MACS purified $CD11b^+CD33^+$ cells. **c** PBMC from lung cancer patients 6, 7, and 11 were either depleted or not depleted for $CD11b^+CD33^+$ MDSC cells and activated with anti-CD3 and anti-CD28 mAbs. T cell proliferation was assessed by ^{3}H-thymidine uptake. The ratio of T cells ($CD3^+CD4^+$ plus $CD3^+CD8^+$) to $CD11b^+CD33^+$ MDSC was 1:5, 1:0.5, and 1:0.8 for patients 6, 7, and 11, respectively. The data of **a** and **b** are one of two independent experiments using PBMC from two healthy donors and ten patients. The data of **c** are one of two independent experiments using PBMC from three patients

boosted with parental H358, H358/DR7, or H358/DR7/CD80 cells, and the resulting supernatants analyzed for cytokines by multiplex luminex assay (Fig. 3d). The high level of IFNγ production is indicative of a type 1 response, with minimal type 2 cytokine (IL-4, IL-5, and IL-10) production. Therefore, the vaccine activates a type 1 $CD4^+$ T cell response.

The MHC II vaccines are designed to activate $CD4^+$ T cells that are specific for NSCLC so they can be used as therapeutic agents in NSCLC patients. Since the vaccination strategy uses established cell lines such as H358, it is necessary for these cell lines to activate $CD4^+$ T cells that cross-react with the patient's tumor cells. Cross-reactivity could occur if established cell lines such as H358 share antigens with other NSCLC cells. To test this hypothesis PBMC from HLA-DR7 healthy donor BC123104 were primed with H358/DR7/CD80 vaccine cells and boosted with the priming vaccine, or with $DR7^+CD80^+$ vaccine cells prepared from a large cell carcinoma of the lung (H177/DR7/CD80), a breast adenocarcinoma (MCF10/DR7/CD80), or a uveal melanoma (OMM2.3/DR7/CD80) (Fig. 3e, f). PBMC primed with the H358/DR7/CD80 vaccine were efficiently boosted by the priming vaccine cells as well as by vaccine cells prepared from the large cell carcinoma of the lung (H177/DR7/CD80). In contrast, boosting with either the breast carcinoma or uveal melanoma

Table 2 Percent MDSC, T cells, B cells, NK, and NKT cells in the blood of NSCLC patients and healthy donors

Cells	Healthy donor[a]	Percent cells ± SD for patient									
		1[b]	3[b]	4	5	6	7	8	9	10	11[b]
$CD3^+$	67 ± 11	35 ± 8.2	59 ± 2	43.5	68.5	19.6	50	29.2	33.6	39.7	35 ± 10
$CD3^+CD4^+$	37 ± 13	12 ± 0.8	38 ± 6.2	14.4	51.9	10.4	18.2	16.8	21.3	23.6	14 ± 4
$CD3^+CD8^+$	20 ± 4	18 ± 3.5	17 ± 2	25.8	18.1	7.9	20.2	7.7	16.2	25.7	16 ± 1
$CD56^+$	3 ± 2	4 ± 1	6.3 ± 4	7.4	11.9	0.8	0.2	11.3	10	2.9	1.5 ± 0.1
$CD3^+CD56^+$	0.7 ± 0.5	1.2 ± 0.2	0.4 ± 0.1	1.0	3.6	0.1	0.1	0.6	5.9	0.3	0.35 ± 0.2
$CD19^+$	7 ± 3.5	3.8 ± 1.6	3.2 ± 1	2.4	10.6	6.0	4.7	1.4		6.1	2.1 ± 1
$CD11b^+CD33^+$	11 ± 6	14.0	25 ± 2	14	12	53 ± 2	18	32	32.4	28	37 ± 8

[a] Average ± SD of the two healthy donors listed in Table 1 plus three additional healthy donors

[b] Average of 2–3 independent experiments

vaccines gave minimal responses. Similar results were obtained using PBMC from healthy donor BC011405 (data not shown). Therefore, MHC II vaccines prepared from a bronchioloalveolar carcinoma cell line activate $CD4^+$ T cells that are specific for lung cancer cells and cross-react with two other subtypes of lung cancer cells, and do not cross-react with HLA-DR-matched tumor cells derived from non-lung tumors (data not shown).

MHC II lung cancer vaccines activate PBMC from lung cancer patients despite the presence of MDSC

Although the MHC II NSCLC vaccines activate tumor-specific $CD4^+$ T cells from healthy donors, the presence of $CD11b^+CD33^+$ in NSCLC patients may block T cell activation. To assess this possibility, $HLA\text{-}DR7^+$ PBMC from NSCLC patient 6 (52% $CD11b^+CD33^+$ cells), patient 3 (25% $CD11b^+CD33^+$ cells), patient 1 (14% $CD11b^+CD33^+$cells), and healthy donor BC123104 (15% $CD11b^+CD33^+$ cells) were primed with H358/DR7/CD80 or H177/DR7/CD80 vaccine cells and boosted with the priming vaccine cells, with parental tumor cells, or with breast cancer cells (Fig. 4a–c). Although there were quantitative differences in the responses of the patients, overall the MHC II vaccines induced robust $CD4^+$ T cell IFNγ responses from both the NSCLC patients and healthy donor. Similar responses were seen for PBMC from the other six NSCLC patients (data not shown) which contained $CD11b^+CD33^+$ cells ranging from 14–37%. Therefore, $CD11b^+CD33^+$ suppressor cells in lung cancer patients do not prevent the vaccines from activating tumor-specific, IFNγ-secreting $CD4^+$ T cells.

$CD11b^+CD33^+$ cells are maintained through the priming phase of the cultures; however, they decrease in number with time (Srivastava and Ostrand-Rosenberg, unpublished results). Therefore, the lack of suppression in Fig. 4a–d may be due to an absence of functional $CD11b^+CD33^+$ cells during the boosting phase. To test this possibility, PBMC from patient 9 (32.4% $CD11b^+CD33^+$) were primed and boosted with the H358/DR4/CD80 vaccine, and autologous purified $CD11b^+CD33^+$ cells were added at the beginning of the boosting phase at a ratio of 1:1 activated T cells to $CD11b^+CD33^+$ cells (Fig. 4e). The 1:1 ratio was used because the experiments of Fig. 1c indicated that this ratio gave complete suppression. The additional $CD11b^+CD33^+$ cells did not impair T cell activation as measured by IFNγ release, and the production of type 2 cytokines was not different in the presence or absence of the additional $CD11b^+CD33^+$ cells (IL-4: <44.4 pg/ml ± $CD11b^+CD33^+$ cells; IL-10: 10.2 ± 0.77 vs. 11.2 ± 1.77 pg/ml for without or with additional $CD11b^+CD33^+$ cells, respectively), demonstrating that the vaccines activate type 1 $CD4^+$ T cells despite the presence of suppressive $CD11b^+CD33^+$ cells.

Since the results of Fig. 1c indicated that $CD11b^+CD33^+$ cells inhibited T cell proliferation, we tested if $CD11b^+CD33^+$ cells affected the proliferation and yield of activated $CD4^+$ T cells by testing PBMC from NSCLC before and after removal of $CD11b^+CD33^+$ cells. PBMC from healthy donor BC123104 (15% $CD11b^+CD33^+$ cells), undepleted PBMC from patient 11 (31% $CD11b^+CD33^+$ cells), and $CD11b^+CD33^+$-depleted PBMC from patient 11 (<2% $CD11b^+CD33^+$ cells), were co-cultured with H358/DR7/CD80 vaccine cells and proliferation of the primed T cells was measured (Fig. 4d). PBMC from the healthy donor and from the $CD11b^+CD33^+$-depleted patient's PBMC proliferated well in response to priming with the vaccine cells, whereas, there was minimal proliferation by the non-depleted patient's PBMC. Therefore, $CD11b^+CD33^+$ cells inhibit the vaccine-induced proliferation of T cells during priming, and removal of the $CD11b^+CD33^+$ cells restores T cell proliferation.

To determine if the deficit in proliferation during priming reduces the final yield of T cells, the number of $CD4^+$ T cells in the PBMC of healthy donor BC123104 and patient 6 was quantified on day nine after expansion with IL-15

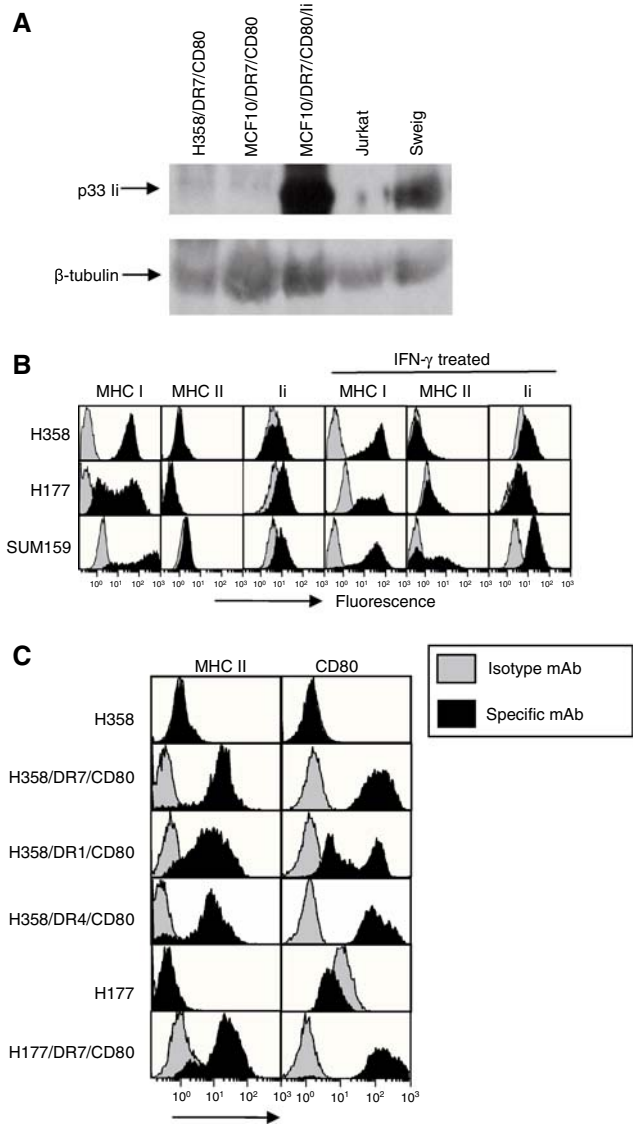

Fig. 2 Bronchioloalveolar adenocarcinoma (H358) and large cell carcinoma (H177) cells transfected with costimulatory molecule CD80 and HLA-DR7 or HLA-DR1 genes express cell surface HLA-DR and CD80 and do not express Ii and are not inducible by IFNγ for Ii or HLA-DR. **a** Western blots of NSCLC and control cells stained for Ii. **b** IFNγ- treated or untreated tumor cells were stained for cell surface MHC I (mAb W6/32), MHC II (mAb L243), or CD80 (anti-human CD80 mAb), and analyzed by flow cytometry. **c** H358 and H177 NSCLC cells were transfected with pLNCX2/DR7, pLNCX2/DR1, pLNCX2/DR4, and/or pLHCX2/CD80 plasmids using nucleofection technology (Amaxa), stained for HLA-DR and CD80, and analyzed by flow cytometry. Profiles are for transfectants that have been in cultures for >9–12 months

and immediately before boosting. Although patient 6 initially had fewer $CD4^+$ T cells than the healthy donor, the yield of activated $CD4^+$ T cells from patient 6 was similar to the yield from the healthy donor (55% vs. 44% for patient 6 and the healthy donor, respectively). Therefore, although $CD11b^+CD33^+$ MDSC inhibit the initial proliferation of $CD4^+$ T cells, the MHC II lung cancer vaccines prime and boost IFNγ-secreting, tumor-specific $CD4^+$ T cells and overcome the initial proliferation deficit.

MHC II NSCLC vaccines activate T cells that react with malignant lung cancer cells, and not with normal lung fibroblasts

Since the MHC II NSCLC vaccines are made from lung cancer cells, they contain antigens that are potentially expressed by non-malignant cells. To determine if the vaccines activate $CD4^+$ T cells against non-malignant cells, HLA-$DR7^+$ PBMC from patient 10 (28% $CD11b^+CD33^+$ cells) and healthy donor BC123104 were primed with H358/DR7/CD80 vaccine cells and boosted with the priming vaccine cells, parental H358 cells, or non-malignant lung fibroblasts (Fig. 4e, f). Strong responses were produced against the vaccine; however, virtually no response was elicited by the fibroblasts. Similar results have been observed with PBMC from patient 5 and healthy donor BC011405 (data not shown). Therefore, the MHC II NSCLC vaccines activate T cells that are reactive with malignant NSCLC cells, and do not activate T cells reactive with non-malignant cells from the lung.

Discussion

Despite advances in chemotherapy and radiation therapy, advanced lung cancer remains an incurable disease. The specificity and potential potency of the immune system for recognizing and destroying malignant cells has prompted investigators to explore novel strategies for activating patients' T lymphocytes against cancer cells. Although several of these strategies have shown promise in experimental animals with small tumors, they have been disappointing in clinical trials (reviewed in [24, 28, 32]). One reason for these results may be tumor-induced immune suppression, a condition that accompanies tumor progression and exists in most patients with advanced cancer [11]. A major cause of immune suppression in cancer patients is MDSC, a cell population that blocks adaptive and innate immunity [1, 34]. Here, we report that despite the presence of MDSC in the peripheral blood of NSCLC patients, our novel MHC II lung cancer vaccines activate tumor-specific $CD4^+$ T cells from NSCLC patients.

Although MDSC were first observed over 23 years ago when they were called "natural suppressor cells" [41], they have only recently been acknowledged as a complicating factor for immunotherapy [1, 34]. MDSC interfere with adaptive immunity by inhibiting the activation of $CD4^+$ and $CD8^+$ T cells [6, 20, 39]. In addition, they block innate immunity by inactivating natural killer cells [42] and they skew immunity towards a tumor-promoting type 2 pheno-

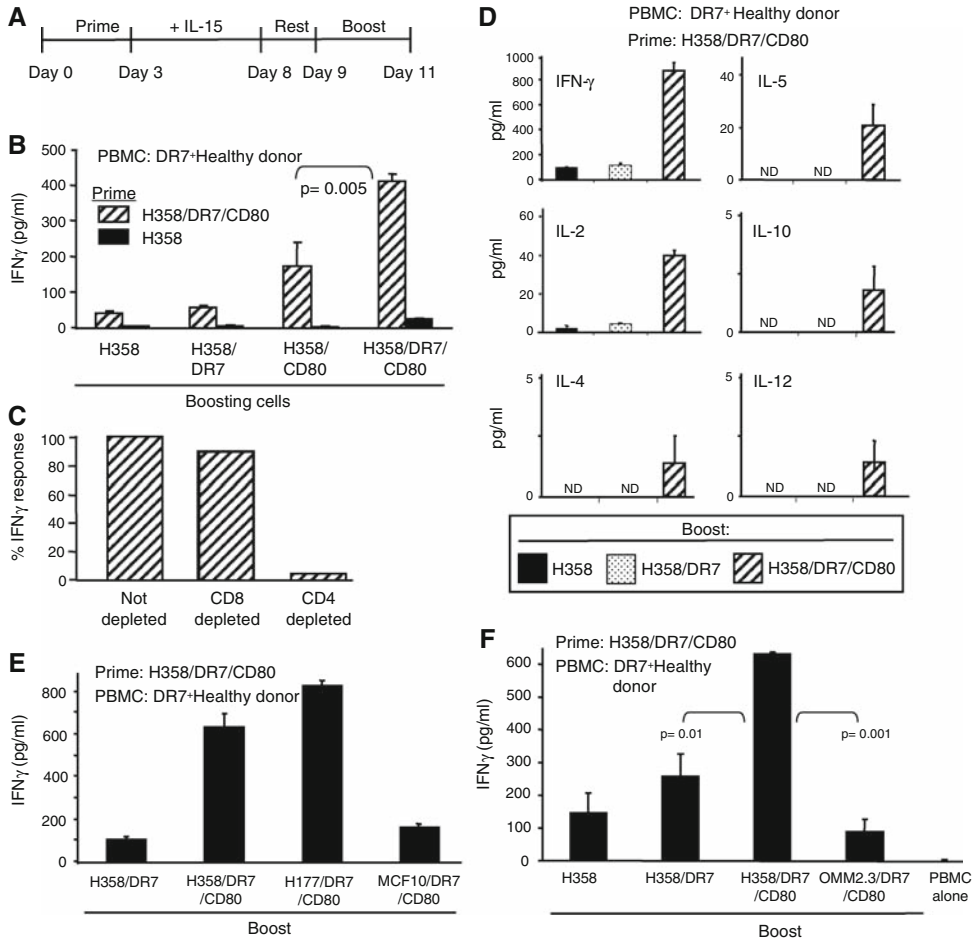

Fig. 3 Major histocompatibility complex class II (*MHC II*) lung cancer vaccines prime and boost type 1 $CD4^+$ T cells from healthy donors that react with lung cancer cells and do not react with non-lung tumor cells. **a** Time line for in vitro priming and boosting of PBMC. **b** HLA-$DR7^+$ healthy donor PBMC BC123104 were primed with irradiated H358/DR7/CD80 (MHC II vaccine) or H358 cells and boosted with the indicated cells. IFNγ production was measured by ELISA. **c** HLA-$DR7^+$ PBMC from healthy donor BC123104 were either not depleted or depleted for $CD4^+$ or $CD8^+$ T cells before priming. IFNγ release by non-depleted PBMC was 450–500 pg/ml. Non-depleted PBMC contained 16–20% $CD8^+$ T cells and 40–50% $CD4^+$ T cells. PBMC depleted for $CD4^+$ and $CD8^+$ T cells contained <1 and <2% $CD4^+$ and $CD8^+$ T cells, respectively. **d** Supernatants from primed and boosted PBMC from panel B were assayed for cytokines by multiplex luminex analysis. **e**, **f** PBMC from healthy donor BC123104 were primed with the H358/DR7/CD80 MHC II lung cancer vaccine and boosted with the indicated cells. IFNγ production by activated $CD4^+$ T cells was measured by ELISA. Data for each panel are representative of two to three independent experiments with PBMC from healthy donors BC123104 and BC011405

type through their production of IL-10 and their down-regulation of IL-12 production by macrophages [38]. Since the accumulation and retention of MDSC is regulated by tumor-secreted factors as well as by factors produced by the tumor micro-environment [20], patients with advanced cancer can have high levels of MDSC. As a result, immunotherapies which require an active immune response in the recipient, must overcome significant immune suppression.

A variety of experimental immunotherapies have been tested in advanced stage (IIIB and IV) NSCLC patients. These include peptide vaccines prepared from single [3] or multiple [24] peptides derived from NSCLC cells, as well as DC pulsed with peptides derived from NSCLC cells [13]. The glycoprotein MUC-1 [7] and epidermal growth factor [33], both of which are found on many tumor cells including NSCLC cells, have also been tested as therapeutic vaccines. These vaccines did not cause significant side-effects; however, their therapeutic efficacy remains to be determined.

Two cell-based vaccines have also been tested. GM-CSF-transduced autologous NSCLC cells induced a 10% response rate in advanced stage patients [27]. However, this treatment may not be practical for large numbers of patients because it requires the transduction of individual patients' tumor cells [26]. More promising is a vaccine generated by transfecting allogeneic NSCLC cells with the costimulatory molecule CD80 [30, 31]. Nineteen patients with advanced disease were treated with this vaccine. One patient had a

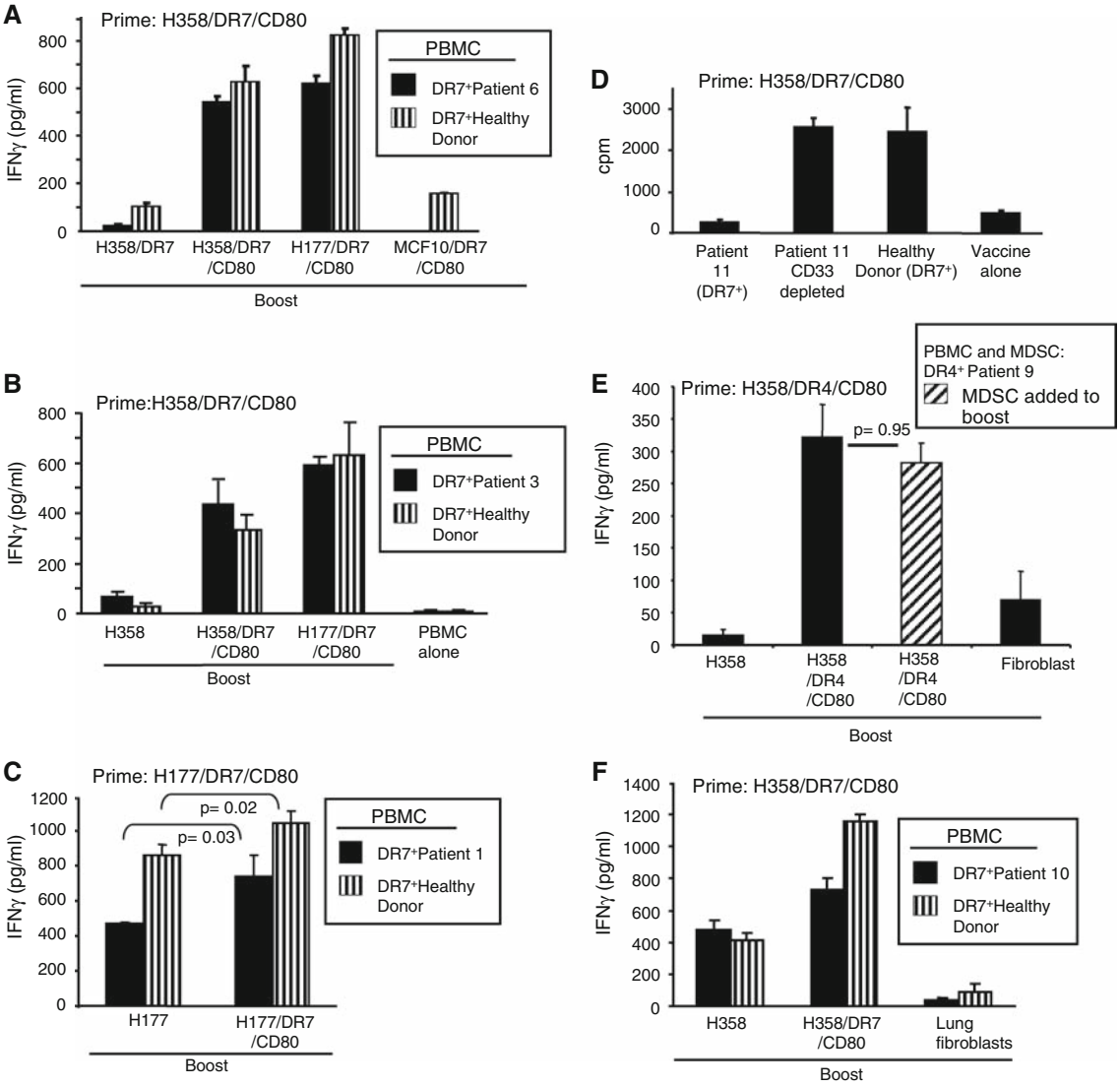

Fig. 4 Major histocompatibility complex class II lung cancer vaccines prime and boost tumor-specific T cells from lung cancer patients' PBMC despite the presence of $CD11b^+CD33^+$ MDSC. HLA-$DR7^+$ PBMC from healthy donor BC123104 or BC011405 and from patient 6 (**a**), patient 3 (**b**), or patient 1 (**c**) were primed with the H358/DR7/CD80 or H177/DR7/CD80 MHC II lung cancer vaccine and boosted with the indicated cells. IFNγ production by activated $CD4^+$ T cells was measured by ELISA. **d** HLA-$DR7^+$ PBMC from healthy donor BC123104 or from patient 11 (depleted or not depleted for $CD11b^+CD33^+$ cells) were primed with H358/DR7/CD80 vaccine cells and T cell proliferation following priming was measured by ^{3}H-thymidine uptake. **e** HLA-$DR4^+$ PBMC from patient 9 were primed with H358/DR4/CD80 vaccine cells and boosted with the priming vaccine, parental H358 cells, or lung fibroblasts in the absence or presence of purified autologous $CD11b^+CD33^+$ cells. **f** HLA-$DR7^+$ PBMC from healthy donor BC123104 and from patient 10 were primed with H358/DR7/CD80 vaccine cells and boosted with the priming vaccine cells, parental H358 cells, or non-malignant lung fibroblasts. IFNγ production by activated $CD4^+$ T cells was measured by ELISA. Data for panels **d**, and **e** are from one of two independent experiments. Data for panels **a**, **b**, **c**, and **f** are representative of one to two independent experiments with PBMC from ten NSCLC patients

partial response and five had stable disease at the end of the initial 18 month observation period. A recent review reports that four of these patients remain alive with an overall survival time of 36–63 months [32], compared with the 8–12 month survival time for late stage NSCLC patients who fail initial chemotherapy [36]. The MHC II vaccines described in the current report are similar to this CD80 vaccine in that both are MHC I allogeneic NSCLC cells and are transfected with CD80. The MHC II vaccines have the additional benefit of activating tumor-specific $CD4^+$ T cells because they express syngeneic HLA-DR alleles. This added activity may not only improve the immediate therapeutic effect of the vaccine, but may also provide long-term protection against tumor progression and/or recurrence.

Our finding that the MHC II vaccines activate T cells in the presence of MDSC suggests that this cell-based vaccine may be efficacious in late stage NSCLC patients who are immune suppressed. Further support is provided by studies

with a vaccine consisting of NSCLC cells transduced with an anti-sense construct for TGFβ [25]. TGFβ is an immune suppressive cytokine that is present in many cancer patients, and the prognosis for NSCLC patients with high levels of plasma TGFβ is poor [19]. Treatment of advanced stage NSCLC patients with an anti-sense TGFβ vaccine produced a 15% partial response rate, suggesting that inactivation of TGFβ may facilitate the development of therapeutic tumor immunity. Since the MHC II vaccines activate T cells despite the presence of MDSC and some MDSC suppress via the production of TGFβ [43], the MHC II vaccines may by-pass TGFβ-induced suppression and therefore induce immunity in late stage NSCLC patients.

To maximize potential recipients and maintain feasibility, we are preparing MHC II vaccines from established, allogeneic tumor cells [5]. This approach will only be effective if different lung tumor cells share common antigens. Our findings that T cells primed with an MHC II bronchioloalveolar adenocarcinoma vaccine cross-react with a large cell lung carcinoma, combined with the findings of Raez et al. using allogeneic CD80 vaccines [31, 32] support this assumption.

A concern with any cell-based vaccine, including the MHC II vaccines described here, is the potential for autoimmunity due to tumor cell-expressed antigens that are shared with non-malignant cells. Autoimmunity has not been observed in the mouse models used to test the MHC II vaccine concept [4, 29], and there was no response to non-malignant breast cells in a MHC II vaccine mammary carcinoma system [44] or against normal lung fibroblast cells as reported here. Collectively, these observations suggest that autoimmunity against normal lung tissue is unlikely to occur. Therefore, the MHC II lung cancer vaccines are active despite the presence of tumor-induced immune suppression, and may be useful immunotherapeutic reagents for NSCLC patients.

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