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Potent Tumor-Specific Protection Ignited by Adoptively Transferred CD4+ T Cells[1]

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Abstract

Administration of α-CD25 mAb prior to an aggressive murine breast tumor inoculation provoked effective antitumor immunity. Compared to $CD4^+$ T cells purified from α-CD25 mAb-pretreated mice that did not reject tumor, $CD4^+$ T cells purified from α-CD25 mAb-pretreated mice that rejected tumor stimulated by DCs produced more IFN-γ, IL-2 and less IL-17 *in vitro*, and ignited protective antitumor immunity *in vivo* in an adoptive transfer model. Tumor Ag-loaded DCs activated naïve $CD8^+$ T cells in the presence of these $CD4^+$ T cells *in vitro*. Tumor Ag and adoptively transferred $CD4^+$ T cells were both required for inducing a long-term tumor-specific IFN-γ-producing cellular response and potent protective antitumor activity. While adoptively transferred $CD4^+$ T cells ignited effective tumor-specific antitumor immunity in wild type mice, they failed to do so in endogenous NK cell-depleted, $Gr\text{-}1^+$ cell-depleted, $CD40^{-/-}$, $CD11c^+$ DC-depleted, B $cell^{-/-}$, $CD8^+$ T cell-depleted or IFN-$\gamma^{-/-}$ mice. Collectively, the data suggests that adoptively transferred $CD4^+$ T cells orchestrate both endogenous innate and adaptive immunity in order to generate effective tumor-specific long-term protective antitumor immunity. The data also demonstrates the pivotal role of endogenous DCs in the tumor-specific protection ignited by adoptively transferred $CD4^+$ T cells. Thus, these findings highlight the importance of adoptively transferred $CD4^+$ T cells, as well as host immune components, in generating effective tumor-specific long-term antitumor activity.

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[3]Abbreviation used in this paper:

DCs dendritic cells

Treg $CD4^+CD25^+$ regulatory T cells

WT wild type

DT diphtheria toxin

APC antigen-presenting cells.

Disclosures
The authors have no financial conflict of interest.

Keywords

Adoptively transferred $CD4^+$ T cells; Antitumor immunity; Breast tumor

Introduction

$CD4^+$ T cells have emerged as an important arm of immune system in combating tumors (1-3). $CD4^+$ T cells could mediate tumor rejection by killing tumor cells *via* TRAIL, FasL or granzyme-perforin dependent pathways, by up-regulating MHC class I and II expressions on tumor cells, inhibiting tumor cell proliferation, promoting NK cell function, rescuing CTL from activation-induced cell death, inducing angiogenesis inhibitors and recruiting macrophages/eosinophils *via* IFN-γ-mediated mechanisms (2-4). A recent study further showed that $CD4^+$ T cells were powerful in tumor rejection in comparison to $CD8^+$ T cells, especially in collaboration with NK cells (5).

In most cases, $CD4^+$ T cells may orchestrate broad immune components of hosts to elicit a tumor-specific immune attack when considering the following: first, most tumor cells are MHC class $II^{-/-}$, which cannot be recognized by $CD4^+$ T cells (6); second, the unique ability of $CD4^+$ T cells to regulate other immune cells *in vivo* (7); lastly, $CD4^+$ T cell help is especially important in the situation of tumor-bearing host lacking intrinsic “danger” signals (8). $CD4^+$ T cells freshly-purified, restimulated and/or activated *in vitro* or genetically-modified *in vitro* have been used to investigate their role in antitumor immunity in multiple animal models (9-14). Accumulated data suggests that adoptively transferred $CD4^+$ T cells may enhance antitumor activity mediated by adoptively transferred $CD8^+$ T cells and/or initiate endogenous $CD8^+$ T cell-dependent or -independent antitumor immunity (15-21).

In vivo depletion of $CD4^+CD25^+$ regulatory T cells (Treg) *via* α-CD25 mAb enhanced tumor/Ag-specific T cell responses, provoked potent antitumor activity and augmented tumor vaccine-induced antitumor immunity in various murine tumor models (22). Tumor rejection was reported in naïve mice adoptively transferred by $CD4^+$ T cells purified from mice pretreated with α-CD25 mAb (23). However, the underlying mechanism(s) is unknown.

Her2/Neu is associated with many of advanced human breast tumors and a well-documented tumor-associated “oncoantigen” as a target of both cellular- and humoral-mediated antitumor immunity (24). The importance of fresh $CD4^+$ T cells was documented in BALB/c mice, showing that a foreign Ag Her2-specific $CD4^+$ T cells are essential to reject a tumor expressing Her2 in BALB/c mice (25). Although the foreign Ag (rat Her2/Neu) is not tolerated in mice (26), murine breast tumor 4T1.2-Neu shares many characteristics with many human advanced breast cancers. This includes aggressive spontaneous metastasis, inherent resistance to chemotherapy, poorly immunogenic with MHC class $II^{-/-}$, profoundly producing various immune suppression factors and cells, and stably expressing “oncoantigen” Her2/Neu (27-28). Thus, 4T1.2-Neu is an aggressive murine breast tumor.

In this study, we tested whether mice with Treg depletion could provoke antitumor immunity against 4T1.2-Neu and examined *in vivo* antitumor function of $CD4^+$ T cells freshly-purified from those mice. We undertook a series of *in vitro* and *in vivo* experiments with various endogenous immune cell-depleted and gene knockout mice to dissect the mechanism(s) underlying the effective tumor-specific protection ignited by adoptively transferred $CD4^+$ T cells in this aggressive breast tumor model.

Materials and Methods

Mice

BALB/c and BALB/c-B cell$^{-/-}$ (C.129 (B6)-*IgH-J*^{tm1Dhu}) mice were purchased from Taconic. BALB/c-CD40$^{-/-}$ (CNCr.129P2-*Cd40*^{tm1Kik}/J), BALB/c-IFN-γ$^{-/-}$ (C.129S7 (B6)-*Ifng*^{tm1Ts}/J) and BALB/c-CD11c-DTR transgenic (C.FVB-Tg(Itgax-DTR/EGFP)57Lan/J) mice were purchased from The Jackson Laboratory. All mice were housed in specific pathogen-free conditions in the University of Pittsburgh animal facility. Mice used in experiments were female, age 6-8 weeks. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

Tumor cells

Murine breast tumor cell 4T1.2-Neu (28) was maintained in DMEM (IRVINE Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone), 2mM glutamine (Life Technologies), antibiotic antimycotic solution (Sigma) and G-418 (500 μg/ml) (Invitrogen). Murine breast tumor cell 4T1.2 (27) was maintained in culture medium of 4T1.2-Neu without G-418. Murine colon carcinoma cell CT26 (ATCC) was cultured in RPMI 1640 (IRVINE Scientific) supplemented with 10% FBS, 2mM glutamine and antibiotic antimycotic solution.

Antibodies

Hybridoma cell PC61 (ATCC) was cultured in DMEM supplemented with 10% FBS, 2mM glutamine, and antibiotic antimycotic solution. The culture supernatants were harvested by centrifuging at 4°C, 2,000 rpm for 10 min and stored at -20°C. α-CD25 mAb was purified from culture supernatants using MAb purification kit following vendor's protocol (Amersham). The protein concentration of α-CD25 mAb was measured using BCA™ protein assay kit (Pierce). Purified α-CD25 mAb was stored at -20°C for further use. α-CD8 antibody (YTS 169.4.2.1 and YTS 156.7.7) was kindly provided by Dr. H. Waldmann. α-asialoGM1 antibody used to deplete NK cells was purchased from Wako Pharmaceuticals. α-Gr-1 antibody (RB6-8C5) used to deplete Gr-1^{+} cells was purchased from BD Bioscience. α-CD40 antibody (FGK45) was kindly provided by Dr. S. Schoenberger.

Generation and characterization of CD4^{+} T cells

BABL/c mice were injected i.p. with 600μg of purified α-CD25 mAb. 3 d later, these mice were inoculated s.c with 4T1.2-Neu (1×10^5) or CT26 ($5\text{-}13\times10^5$) in 20μl endotoxin-free PBS (Sigma) at the 4th mammary fat pad or at the left flank. Primary tumor (3-mm mean diameter) was observed in all mice at d 3 after tumor inoculation, and measured by electric caliper in two perpendicular diameters every other day. Mice were sacrificed for humane reasons when primary tumor reached 10mm in mean diameter, when ulceration, bleeding or both developed, or when mice became ill due to metastatic diseases. Primary tumor was rejected around d 21 after tumor inoculation in about 70% of mice that were pretreated by α-CD25 mAb. 4-8 d after tumor rejection, CD4^{+} or CD8^{+} cells were purified from splenocytes of α-CD25 mAb-pretreated mice that rejected tumor using α-mouse CD4 or CD8 micro-beads following vendor's protocols (Miltenyi Biotec). At the same time, CD4^{+} T cells were purified from splenocytes of α-CD25 mAb-pretreated mice that did not reject tumor using α-mouse CD4 micro-beads. To characterize CD4^{+} T cells, purified CD4^{+} T cells were stained by FITC-α-CD4 (GK1.5) and PE-α-CD25 (7D4) or -CD40L (MR1) and related isotype control antibodies (eBioscience and BD Bioscience) following standard protocol and analyzed by flow cytometry. To determine cytokines produced by CD4^{+} T cells, purified CD4^{+} T cells (4×10^5) were co-cultured with purified naïve BALB/c splenic CD11c^{+}DCs loaded with 4T1.2-Neu or CT26 tumor Ag (4×10^5) (29) in 200μl RPMI 1640 10%FBS at 37°C, 5% CO_2 for 2 d. The

concentration of IFN-γ, IL-2, IL-4, IL-5, IL-10 or IL-17 in the culture supernatants was determined by ELISA following vendors' protocols (eBioscience, BD Bioscience and Biolengend, Inc.).

Determination of antitumor immunity ignited by adoptively transferred T cells

Naïve BALB/c mice (3-5 mice/group) were adoptively transferred i.v with freshly-purified splenic $CD4^+$ T cells (1×10^7) from α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu (or CT26) or that did not reject 4T1.2-Neu, or freshly-purified splenic $CD8^+$ T cells (1×10^7) from α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu. 1 d later, those mice were inoculated s.c. with 4T1.2-Neu (1×10^5) or CT26 (5×10^5) as described above. Mice without $CD4^+$ T cell adoptive transfer served as non-treatment control. Mice were monitored as described above.

Activation of naïve $CD8^+$ T cells by DCs in the presence of $CD4^+$ T cells in vitro

$CD4^+$ T cells from splenocytes of α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu were obtained as described above. $CD11c^+$DCs or naïve $CD8^+$ T cells were purified from splenocytes of naïve BALB/c mice using α-mouse CD11c or CD8 micro-beads following vendors' protocols (Miltenyi Biotec). Purified $CD11c^+$ DCs were loaded with 4T1.2-Neu tumor Ag (29). $CD4^+$ T cells (4×10^5), DCs (4×10^5) and naïve $CD8^+$ T cells (4×10^5) were co-cultured in 200μl RPMI 1640 10%FBS at 37°C, 5% CO_2 for 2 d. The concentration of IFN-γ in the culture supernatants was measured by ELISA.

Detection of IFN-γ production from lymphocyte effectors and antitumor activity ignited by adoptively transferred $CD4^+$ T cells in combination with mitomycin C-treated tumor cell immunization

$CD4^+$ T cells (1×10^7) purified from splenocytes of α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu in 200 μl endotoxin-free PBS were adoptively transferred i.v. into naïve BABL/c mice (3-4 mice/group) at d -1. Mice were immunized s.c. by mitomycin C-treated 4T1.2-Neu (1×10^6) (tumor cells were pretreated with 200 μg/ml of mitomycin C (Sigma) for 2 h and washed extensively before use) at d 0. Mice with adoptive $CD4^+$ T cell transfer or mitomycin C-treated 4T1.2-Neu immunization alone served as controls. To measure IFN-γ production from lymphocyte effectors, at d 21 or 60 after immunization, mice were sacrificed and splenocytes (1×10^6) were restimulated *in vitro* with mitomycin C-treated 4T1.2-Neu, 4T1.2 or CT26 (1×10^5) in 1ml RPMI 1640 10%FBS at 37°C, 5% CO_2 for 3 d. The concentration of IFN-γ in the culture supernatants was determined by ELISA. To evaluate antitumor activity, at d 21 after tumor cell immunization, adoptive $CD4^+$ T cell transfer or both, mice were inoculated s.c. with 4T1.2-Neu (1×10^5) at the 4^{th} mammary fat pad. Mice were monitored as described above.

Depletion of endogenous $CD8^+$ T cells, NK cells, Gr-1^+ cells or $CD11c^+$ DCs in vivo

D -1 and 0 were defined as the day of adoptive $CD4^+$ T cell transfer and tumor inoculation, respectively. To deplete endogenous $CD8^+$ T cells, NK cells or Gr-1^+ cells, α-CD8 (200 μg/injection), α-NK1.1 (40 μl/injection), or α-Gr-1 (200 μg/injection) antibody was injected i.p. into naïve BALB/c mice at d -1, +1, +6 and +9. Normal BALB/c mice without injection of antibody served as control. To transiently deplete endogenous $CD11c^+$ DCs *in vivo*, BALB/c-CD11c-DTR transgenic mice were injected i.p. once with diphtheria toxin (DT) (Sigma-Aldrich) at 5 ng/g mouse body weight (30). BALB/c-CD11c-DTR transgenic mice without administration of DT served as control. Depletion was confirmed by flow cytometry and resulted in greater than 95% reduction of relevant cell types. Detailed adoptive $CD4^+$ T cell transfer and 4T1.2-Neu challenge experiments in the mice, described above, are shown in related figure legends and tables.

Statistics

Statistical analyses were performed using Student's *t* test (GraphPad Prism *version 5*). Animal survival is presented using Kaplan-Meier Survival Curves and was statistically analyzed using Log Rank Test (GraphPad Prism *version 5*). $P < 0.05$ is considered to be statistically significant.

Results

Injection of α-CD25 mAb provokes effective antitumor immunity against an aggressive spontaneous metastatic breast tumor 4T1.2-Neu

After 4T1.2-Neu inoculation at 4th mammary fat pad, primary tumor grew in all mice and was rejected in about 70% of mice that received *in vivo* administration of α-CD25 mAb prior to tumor inoculation (Table I). Injection of α-CD25 mAb was ineffective in tumor-bearing mice (Table I). Without injection of α-CD25 mAb, no mice rejected 4T1.2-Neu (Table I). The data suggests that effective antitumor immunity against 4T1.2-Neu can be provoked by *in vivo* administration of α-CD25 mAb prior to tumor inoculation.

Cytokine profiles of CD4$^+$ T cells isolated from α-CD25 mAb-pretreated mice that rejected tumor and that did not reject tumor

Splenic CD4$^+$ T cells were purified from α-CD25 mAb-pretreated mice that rejected tumor and that did not reject tumor. Both CD4$^+$ T cells stimulated by DCs produced Th1 cytokines (IFN-γ and IL-2) (Fig. 1A-B) but not Th2 cytokines (IL-4, IL-5 and IL-10) (data not shown). Compared to CD4$^+$ T cells purified from α-CD25 mAb-pretreated mice that did not reject tumor, CD4$^+$ T cells purified from α-CD25 mAb-pretreated mice that rejected tumor stimulated by DCs produced more IFN-γ and IL-2 *in vitro*. However, only IFN-γ produced by CD4$^+$ T cells from α-CD25 mAb-pretreated mice that rejected tumor was tumor-specific (Fig. 1A). Compared to CD4$^+$ T cells from α-CD25 mAb-pretreated mice that did not reject tumor, CD4$^+$ T cells from α-CD25 mAb-pretreated mice that rejected tumor produced less amount of IL-17 in the absence or presence of DC stimulation in a non-specific manner (Fig. 1C).

Adoptively transferred CD4$^+$ T cells ignite potent protective antitumor activity

In 4T1.2-Neu model, CD4$^+$ T cells from α-CD25 mAb-pretreated mice that did not rejected tumor failed to generate protective antitumor activity (Fig.1D). Although CD4$^+$ or CD8$^+$ T cells from α-CD25 mAb-pretreated mice that rejected tumor generated protective antitumor activity in an adoptive transfer model (Fig.1D), the efficacy of antitumor immunity ignited by adoptively transferred CD4$^+$ T cells was much more potent than adoptively transferred CD8$^+$ T cells (Fig.1D) (p=0.0006). As reported by others (31-32), effective antitumor immunity against CT26 can be provoked by *in vivo* administration of α-CD25 mAb prior to tumor inoculation (data not shown). Furthermore, splenic CD4$^+$ T cells purified from α-CD25 mAb-pretreated mice that rejected CT26 also generated a significant antitumor activity in this adoptive transfer model (Fig. 1E) (p=0.0025). Taken together, the data suggests that adoptively transferred CD4$^+$ T cells, which were purified from α-CD25 mAb-pretreated mice that rejected tumors, are powerful to ignite effective protective antitumor immunity.

Tumor Ag and adoptively transferred CD4$^+$ T cells are both required for a tumor-specific IFN-γ-producing cellular response and potent protective antitumor immunity

To test whether CD4$^+$ T cells could promote a tumor-specific IFN-γ-producing cellular response, BALB/c mice were adoptively transferred by CD4$^+$ T cells and subsequently immunized by mitomycin C-treated 4T1.2-Neu. 4T1.2-Neu immunization in the presence of adoptively transferred CD4$^+$ T cells induced and sustained a long-term IFN-γ-producing cellular response compared to 4T1.2-Neu immunization or adoptive CD4$^+$ T cell transfer alone (Fig.2A). Restimulation of the splenocytes with an irrelevant CT26 demonstrated that the

cellular response was tumor-specific (Fig.2A). *In vivo* tumor challenge experiments consistently demonstrated that tumor immunization in the presence of adoptive $CD4^+$ T cell transfer induced potent protective antitumor immunity compared to adoptive $CD4^+$ T cell transfer or tumor cell immunization alone (Fig.2B). The data suggests that tumor Ag and adoptively transferred $CD4^+$ T cells are both required in generating potent protective antitumor immunity.

Adoptively transferred $CD4^+$ T cell-ignited antitumor immunity is tumor-specific

To confirm the specificity of potent antitumor immunity ignited by adoptively transferred $CD4^+$ T cells, mice were adoptively transferred by $CD4^+$ T cells and challenged by 4T1.2-Neu, a relevant 4T1.2 or an irrelevant CT26. Mice adoptively transferred by $CD4^+$ T cells effectively rejected 4T1.2-Neu but failed to reject 4T1.2 and CT26 (Table II-a). The data suggests that adoptively transferred $CD4^+$ T cell-ignited antitumor immunity is tumor-specific.

Both endogenous innate and adaptive immunity are involved in potent antitumor immunity ignited by adoptively transferred $CD4^+$ T cells

We next examined which immune components play a role in generating antitumor immunity ignited by adoptively transferred $CD4^+$ T cells against this aggressive solid tumor. First, we examined the role of innate immune cells such as NK cells and Gr-1^+ cells. In this experimental model, Gr-1^+ or NK cell-depleted mice could not effectively reject tumor (Table II-b). Next, the role of adaptive immune lymphocyte subsets was determined. B $cell^{-/-}$ mice could not effectively generate antitumor activity in mice adoptively transferred by $CD4^+$ T cells (Table II-c). Depletion of endogenous $CD8^+$ T cells abrogated effective antitumor immunity ignited by adoptively transferred $CD4^+$ T cells (Table II-c). Finally, we tested the role of effector mechanisms such as IFN-γ. IFN-$\gamma^{-/-}$ mice adoptively transferred by $CD4^+$ T cells could not effectively reject tumor (Table II-d). Taken together, the data suggests that adoptively transferred $CD4^+$ T cells orchestrate both innate and adaptive immunity in order to generate potent tumor-specific immune responses against this aggressive breast tumor.

DCs play a pivotal role in effective antitumor immunity ignited by adoptively transferred $CD4^+$ T cells

We further determined the role of DCs in adoptively transferred $CD4^+$ T cell-ignited antitumor immunity *in vitro* and *in vivo*. Although purified splenic $CD4^+$ T cells from α-CD25 mAb-pretreated mice that rejected tumor or tumor Ag-loaded DCs alone could not activate naïve $CD8^+$ T cells, naïve $CD8^+$ T cells were activated in the presence of both $CD4^+$ T cells and DCs *in vitro* (Fig.3A), indicating that DCs are required in adoptively transferred $CD4^+$ T cell-ignited $CD8^+$ T cell-dependent antitumor immunity. Consistently, $CD40^{-/-}$ mice lost the ability to reject tumor in this experimental model (Fig.3B), further suggesting that adoptively transferred $CD4^+$ T cells may act on antigen-presenting cells (APC) such as $CD11c^+$ DCs *via* CD40 signaling. To confirm their importance, we showed that $CD11c^+$ DCs are required for successful antitumor activity ignited by adoptively transferred $CD4^+$ T cells by using mice carrying a transgene encoding a diphtheria toxin receptor (DTR) under the control of the murine CD11c promoter, which allows inducible ablation of $CD11c^+$ DCs *in vivo* (30). In $CD4^+$ T cell adoptive transfer and tumor challenge experiment, survival was significantly increased in mice without transient depletion of $CD11c^+$ DCs compared to mice with transient depletion of $CD11c^+$ DCs (Fig.3C). It is worth mentioning that the efficacy of tumor rejection in BALB/c-CD11c-DTR transgenic mice is lower than in BALB/c WT mice (Figs. 1D, 2B, 3B-C and Table II). It is possible that BALB/c-CD11c-DTR mice somehow resist adoptive $CD4^+$ T cell transfer, to a certain degree, due to the transgenic manipulation. Taken together, the data suggests that DCs are critical in adoptively transferred $CD4^+$ T cell-ignited antitumor activity.

Adoptively transferred CD4$^+$ T cells ignite tumor-specific memory antitumor immunity

To test whether adoptively transferred CD4$^+$ T cells generate memory antitumor activity, d 40 after initial 4T1.2-Neu rejection, mice were challenged by 4T1.2-Neu, 4T1.2 or CT26. As shown in Table III, mice that rejected initial 4T1.2-Neu also successfully rejected secondary 4T1.2-Neu but failed to reject 4T1.2 or CT26. This data showed that adoptively transferred CD4$^+$ T cells generated tumor-specific memory immune response against this tumor.

Discussion

4T1.2-Neu is MHC class II$^{-/-}$ (28), therefore, adoptively transferred CD4$^+$ T cells are unlikely to attack this tumor on their own. From this, it was hypothesized that adoptively transferred CD4$^+$ T cells regulate endogenous immune components to generate antitumor immune responses against this tumor.

Tumor cell immunization (providing Ag) alone did not generate a tumor-specific IFN-γ-producing cellular response and failed to trigger antitumor immunity (Fig. 2A-B). However, tumor cell immunization induced a tumor-specific IFN-γ-producing T cell response and potent antitumor activity in the presence of adoptive CD4$^+$ T cell transfer (providing signal(s)) (Fig. 2A-B). Indeed, tumor inoculation (providing Ag) 1 d later after adoptive CD4$^+$ T cell transfer resulted in potent antitumor immunity (Fig. 1D), whereas tumor inoculation 21 d later after adoptive CD4$^+$ T cell transfer (may be unable to provide strong signal(s) due to insufficient adoptively transferred CD4$^+$ T cells at this time point) was dependent on tumor cell immunization to generate potent antitumor activity (Fig. 2B).

It is likely that both signal(s) provided by adoptively transferred CD4$^+$ T cells and Ag provided by tumor cells are required to generate tumor-specific immunity against this tumor. The question becomes which signal(s) are provided by adoptively transferred CD4$^+$ T cells. First, although CD40-independent antitumor responses have been reported (33), CD40 ligation (mimicking activated CD4$^+$ T cells by using functional α-CD40 mAb) has been shown to generate, enhance and restore antitumor CTL function in some tumor models (34-38). In 4T1.2-Neu (data not shown) and relevant 4T1 models (39), injection of functional α-CD40 mAb did not induce tumor rejection. This observation indicates that adoptively transferred CD4$^+$ T cells not only provide CD40L signaling, but also other undefined signals/factors, to generate antitumor immunity. Second, multiple experiments suggested that IL-2 and IFN-γ benefited antitumor immune responses. Therefore, there is the possibility that *in vivo* IL-2 and IFN-γ produced by adoptively transferred CD4$^+$ T cells act on CD8$^+$ T cells or APC to initiate help of CD8$^+$ T cells in this model (2-3). However, recent data showed that IL-2 and IFN-γ also had a role in immune suppression. In this study, CD4$^+$ T cells, which were purified from α-CD25 mAb-pretreated mice that rejected tumor and that did not reject tumor, produced IL-2 and IFN-γ stimulated by DCs *in vitro* (Fig. 1A-B). Although DCs significantly promoted IL-2 production by both CD4$^+$ T cells in a non-specific manner (Fig. 1B), IL-2 production did not correlate tumor-specific antitumor activity *in vivo* in the adoptive transfer model (Fig. 1D,Table IIa). The observation suggests that IL-2 produced by adoptively transferred CD4$^+$ T cells may not be responsible for effective tumor-specific antitumor immunity. CD4$^+$ T cells from α-CD25 mAb-pretreated mice that rejected tumor produced a high amount of tumor-specific IFN-γ *in vitro* (Fig. 1A) and generated effective tumor-specific antitumor activity *in vivo* (Fig. 1D,Table IIa), indicating that tumor-specific IFN-γ produced by adoptively transferred CD4$^+$ T cells may have a positive impact on effective antitumor immunity. Third, CD4$^+$ T cells purified from α-CD25 mAb-pretreated mice that rejected tumor were CD4$^+$CD25$^-$ (data not shown). It was hypothesized that the lack of Treg results in antitumor activity of adoptively transferred CD4$^+$ T cells. However, CD4$^+$ T cells purified from α-CD25 mAb-pretreated mice that did not reject tumor were also CD4$^+$CD25$^-$ (data not shown), which did not generate antitumor activity in the adoptive transfer model. Furthermore, adoptively transferred CD4$^+$CD25$^-$ T cells

purified from naïve BALB/c or tumor-bearing mice could not activate antitumor immunity (data not shown). The data indicates that the lack of Treg in adoptively transferred $CD4^+$ T cells may not be responsible for effective antitumor immunity. Fourth, $CD4^+$ T cells from α-CD25 mAb-pretreated mice that did not reject tumor enhanced the capacity to produce IL-17 *in vitro* (Fig. 1C) but failed to generate antitumor activity *in vivo* (Fig. 1D). In contrast, $CD4^+$ T cells from α-CD25 mAb-pretreated mice that rejected tumor reduced the capacity to produce IL-17 *in vitro* (Fig. 1C) but generated effective tumor-specific antitumor activity *in vivo* (Fig. 1D,Table IIa). This observation indicates that IL-17 produced by adoptively transferred $CD4^+$ T cells may be involved in the failure of antitumor immunity. This finding is consistent with a recent report suggested that IL-17 produced by $CD8^+$T cells directly accounted for promoting 4T1 (original parental cell line of 4T1.2-Neu) tumor growth (40) even though another recent report found that Th17-polarized cells were superior in mediating destruction of advanced B16 melanoma (41). Taken together, adoptively transferred $CD4^+$ T cells serve as the source of signal(s) to induce antitumor immunity in the presence of tumor Ag. However, the theory that signal(s) provided by adoptively transferred $CD4^+$ T cells accounts for the potent antitumor activity needs further investigation even though IFN-γ (positive) and IL-17 (negative) may be involved in $CD4^+$ T cell-activated antitumor immunity.

Our *in vivo* data confirmed that adoptively transferred $CD4^+$ T cells induced $CD8^+$ T cell-dependent antitumor response *via* acting on DCs (Table IIc, Fig.3), but their role in igniting antitumor immunity is complicated. First, NK cells and $Gr\text{-}1^+$ cells were required for tumor rejection in this model (Table II-b). Adoptively transferred $CD4^+$ T cells may directly force NK cells to acquire the capacities of antitumor cells or may supply IFN-γ to maintain an endogenous Th1 response (42). NK cells may also directly kill tumor cells resulting in enhanced tumor Ag capture by resting DCs and subsequently promoting tumor-specific T cell priming. Gr-1 is widely expressed on myeloid populations which can serve as APC. Thus, *in vivo* administration of α-Gr-1 antibody may wipe out APC populations that are critical in adoptively transferred $CD4^+$ T cell-activated immunity, resulting in a dampened tumor-specific immune response (Table II-b). Second, B cells were required in this model (Table II-c). It was showed that vaccination with activated rat Her2/Neu induces a $CD4^+$ T cell-dependent protective antibody response which may be unique to BALB/c mice (43). In addition to adoptively transferred $CD4^+$ T cells may induce class switching of B cells for production of antitumor antibodies (44), they may also activate B cells to generate cellular-mediated antitumor immunity against this tumor because 'activated' B cells can prime both $CD4^+$ and $CD8^+$ T cells (45-47). Third, endogenous IFN-γ was necessary in adoptively transferred $CD4^+$ T cell-ignited antitumor activity (Table II-d). IFN-γ not only contributes to antitumor activity but also reduces antitumor responses through various mechanisms, under different experimental models and/or conditions (48-51). Previous studies have indicated that IFN-γ was critical in fighting off this tumor and mediated immunoglobulin class switch for producing anti-Her2/Neu antibodies of the IgG2a and IgG2b subclasses (44, 52), which were reported to play a pivotal role in cancer prevention. Thus, IFN-γ produced by endogenous immune cells is required in tumor rejection in this model. Taken together, it is likely that adoptively transferred $CD4^+$ T cells interact with many different types of endogenous immune components to reject this aggressive breast tumor.

Recent studies showed that $CD4^+$ T cell help was nonspecific in certain cases (53). Alternatively, in this model, the help provided by adoptively transferred $CD4^+$ T cells is tumor-specific. Mice adoptively transferred by 4T1.2-Neu-$CD4^+$ T cells rejected 4T1.2-Neu but not 4T1.2 or CT26 (Table II-a). Notably, an IFN-γ-producing T cell response enhanced by adoptive 4T1.2-Neu-$CD4^+$ T cell transfer was not specific to CT26 (Fig. 2A,39), despite the sharing of a murine virus Ag gp70 (54). Interestingly, splenocytes from mice that were immunized by 4T1.2-Neu in the presence of adoptively transferred 4T1.2-Neu-$CD4^+$ T cells responded to 4T1.2 restimulation (Fig.2A). However, mice adoptively transferred by 4T1.2-Neu-$CD4^+$ T

cells failed to reject 4T1.2 (Table II and III). The data suggests that cellular-mediated immunity alone might be insufficient to reject this tumor. B cells needed in the potent antitumor immunity ignited by adoptively transferred $CD4^+$ T cells suggests that antitumor antibody response might be required in tumor rejection. Either 4T1.2 or 4T1.2-Neu can be the target of cellular-mediated immune responses. However, 4T1.2-Neu provides a well-demonstrated Neu target of antibody-mediated immune responses, which might explain the demonstrated specificity of adoptively transferred $CD4^+$ T cell-ignited antitumor activity.

In summary, our data show that adoptively transferred $CD4^+$ T cells orchestrate broad immune components of hosts in order to ignite tumor-specific long-term antitumor immunity. This is endogenous $CD8^+$ T cell-dependent tumor rejection. These findings highlight the importance of adoptively transferred $CD4^+$ T cells, as well as host immune components, in generating effective tumor-specific long-term antitumor activity. Although adoptively transferred $CD4^+$ T cells successfully ignited potent tumor-specific protective antitumor immunity, they failed to eradicate an established 4T1.2-Neu (data not shown). Considerable Treg elevation induced by this tumor may attenuate antitumor immunity ignited by adoptively transferred $CD4^+$ T cells (55, data not shown). Further investigation of whether combination of adoptive $CD4^+$ T cell transfer and Treg manipulation could eradicate an established 4T1.2-Neu is being actively pursued.

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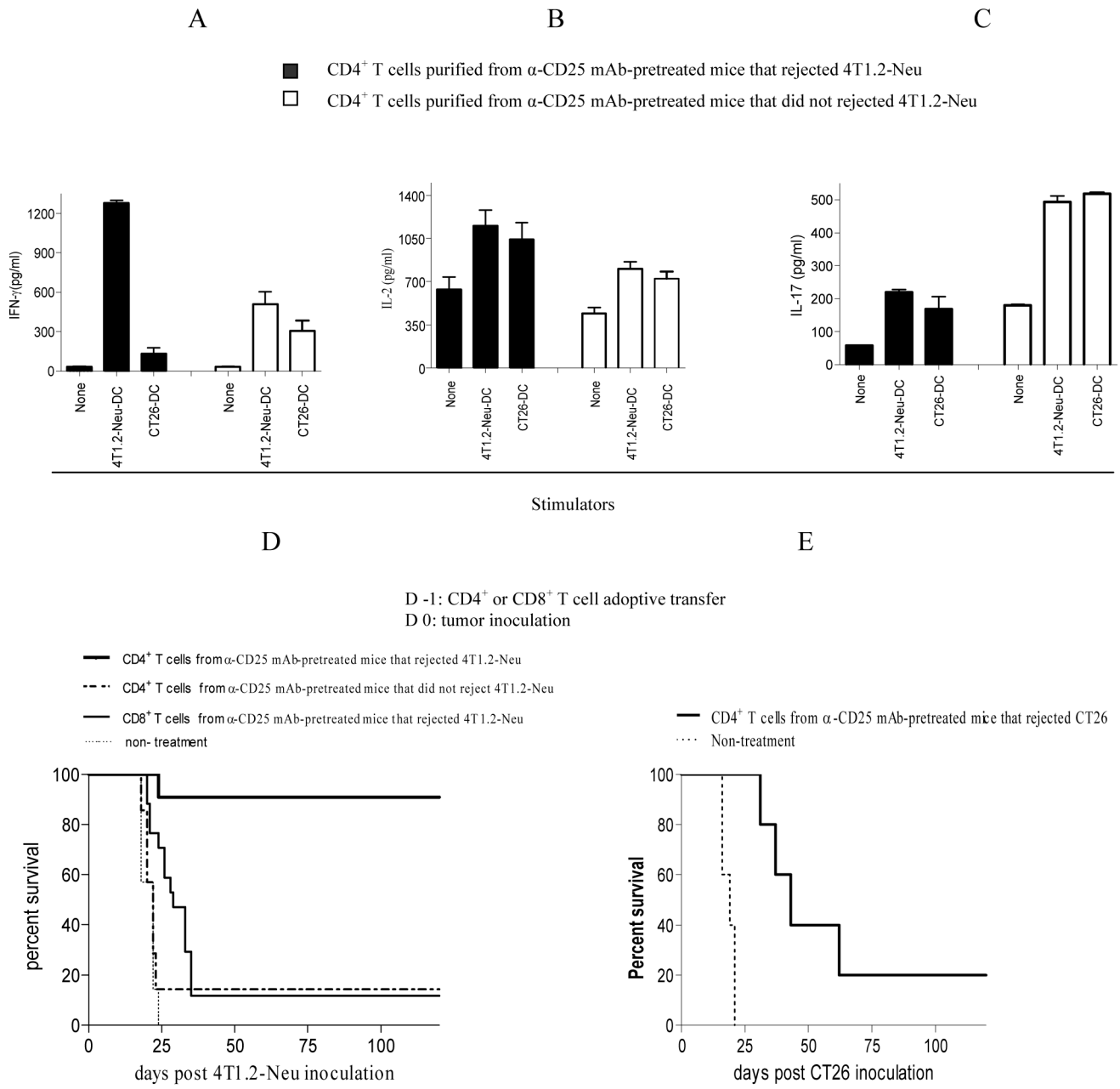

FIGURE 1.

Protective antitumor immunity is ignited by adoptively transferred $CD4^+$ T cells freshly-purified from α-CD25 mAb-pretreated mice that rejected tumors. $CD4^+$ T cells isolated from α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu and that did not reject 4T1.2-Neu were cultured *in vitro* in the absence or presence of stimulators (4T1.2-Neu or CT26-loaded DCs) for 2 d. The concentration of IFN-γ (A), IL-2 (B) or IL-17 (C) in culture supernatant was determined by ELISA. Data represents three independent experiments. A. $CD4^+$ T cells from α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu: 4T1.2-Neu-DCs *vs.* none or CT26-DCs: $p<0.0001$; $CD4^+$ T cells from α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu *vs.* $CD4^+$ T cells from α-CD25 mAb-pretreated mice that did not reject 4T1.2-Neu: $p=0.0002$ (4T1.2-Neu-DC stimulation). B. $CD4^+$ T cells from α-CD25 mAb-pretreated mice that rejected

4T1.2-Neu: 4T1.2-Neu-DCs *vs.* none (p=0.0027); $CD4^+$ T cells from α-CD25 mAb-pretreated mice that did not reject 4T1.2-Neu: 4T1.2-Neu-DCs *vs.* none (p<0.0001); $CD4^+$ T cells from α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu *vs.* $CD4^+$ T cells from α-CD25 mAb-pretreated mice that did not reject 4T1.2-Neu: p=0.0171 (4T1.2-Neu-DC stimulation). C. $CD4^+$ T cells from α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu *vs.* $CD4^+$ T cells from α-CD25 mAb-pretreated mice that did not reject 4T1.2-Neu: p<0.0001 (both non-stimulation and DC-stimulation). $CD4^+$ or $CD8^+$ T cells purified from α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu or CT26 tumor were adoptively transferred i.v. into naïve BALB/c mice on d -1. Mice were inoculated s.c. with 4T1.2-Neu (D) at the 4^{th} mammary fat pad or CT26 (E) at the left flank at d 0. Non-treatment mice and mice that were adoptively transferred with $CD4^+$ T cells purified from α-CD25 mAb-pretreated mice that did not reject 4T1.2-Neu tumor served as controls. In all experiments, primary tumor was observed at d 3 after tumor inoculation and assessed by palpation every other day. Mice that rejected primary tumor demonstrated long-term survival without metastatic tumors. Data represents two to three independent experiments. $CD4^+$ T cells from α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu (n=11) *vs.* non-treatment (n=7): p<0.0001; $CD8^+$ T cells from α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu (n=17) *vs.* non-treatment (n=7): p=0.0008; $CD4^+$ T cells from α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu (n=11) *vs.* $CD4^+$ T cells from α-CD25 mAb-pretreated mice that did not reject 4T1.2-Neu (n=7): p=0.0002; $CD4^+$ T cells from α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu (n=11) *vs.* $CD8^+$ T cells from α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu (n=17): p=0.0006; $CD4^+$ T cells from α-CD25 mAb-pretreated mice that rejected CT26 (n=5) *vs.* non-treatment (n=5): p=0.0025.

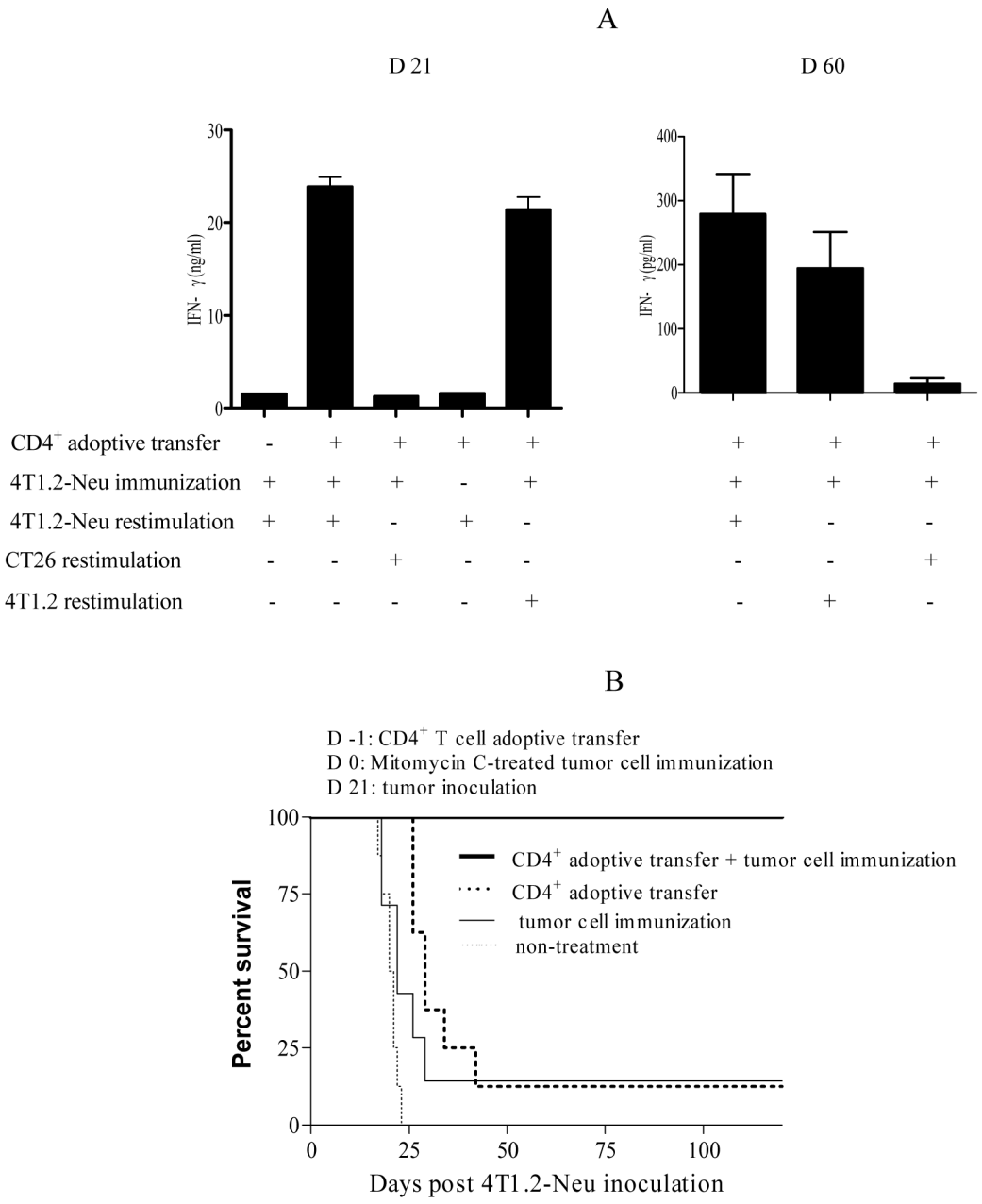

FIGURE 2.

Tumor Ag and adoptively transferred CD4+ T cells are both required to ignite a long-term tumor-specific IFN-γ-producing cellular response and potent protective antitumor immunity. A. CD4+ T cells purified from α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu were adoptively transferred i.v. into naïve BALB/c mice. 1 d later, mice were immunized s.c. with mitomycin C-treated 4T1.2-Neu. Mice with injection of mitomycin C-treated 4T1.2-Neu or CD4+ T cells alone served as controls. 21 or 60 d later, mice were sacrificed and splenocytes were restimulated *in vitro* with mitomycin C-treated 4T1.2-Neu, 4T1.2 or CT26 for 3 d. The concentration of IFN-γ in the culture supernatants was determined by ELISA. One representative of three independent experiments with similar results is presented. B. CD4+ T cells purified from α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu were adoptively

transferred i.v. into naïve BALB/c mice at d -1. Mitomycin C-treated 4T1.2-Neu were injected s.c. at d 0. 21 d later, mice were inoculated s.c. with 4T1.2-Neu at the 4^{th} mammary fat pad. Primary tumor was observed at d 3 after tumor inoculation. Mice that rejected primary tumor demonstrated long-term survival without metastatic tumors. Data represents two independent experiments. $CD4^+$ plus immunization *vs.* $CD4^+$ or immunization alone: $p<0.005$.

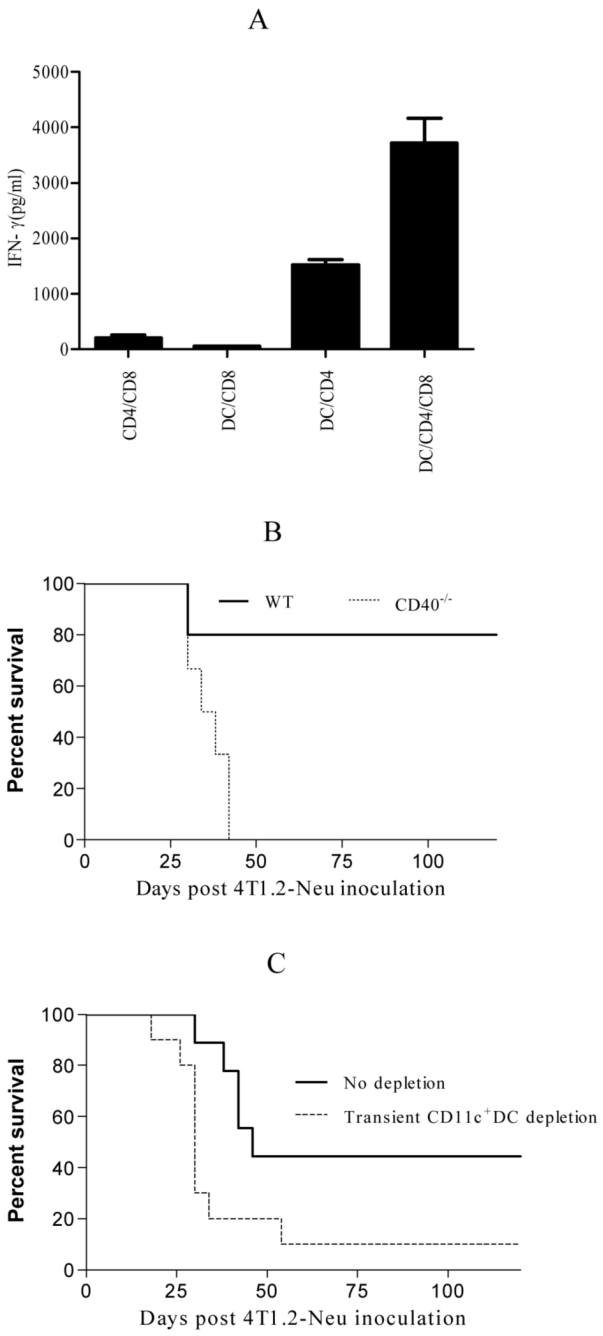

FIGURE 3.

DCs play a pivotal role in effective antitumor immunity ignited by adoptively transferred $CD4^+$ T cells. A. $CD4^+$ T cells were obtained from α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu. DCs or $CD8^+$ T cells were purified from splenocytes of naïve BALB/c mice. Purified $CD11c^+$ DCs were loaded with 4T1.2-Neu. $CD4^+$ T cells, 4T1.2-Neu-loaded DCs and naïve $CD8^+$ T cells were co-cultured for 2 d. The concentration of IFN-γ in the culture supernatants was measured by ELISA**.** One representative of four independent experiments is shown. CD4/DC *vs.* CD4/DC/CD8: p=0.0170. B. $CD4^+$ T cells (1×10^7) purified from α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu were adoptively transferred i.v. into WT or $CD40^{-/-}$ BALB/c mice at d -1. Mice were inoculated s.c. by 4T1.2-Neu (1×10^5) at the 4^{th}

mammary fat pad at d 0. Data represents two independent experiments. WT (n=6) *vs.* $CD40^{-/-}$ (n=5): p=0.0213. C. BALB/c CD11c-DTR transgenic mice were injected i.p. with DT. After 24 h, $CD4^+$ T cells (1×10^7) purified from α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu were adoptively transferred i.v. into transient $CD11c^+$ DC-depleted or non-depleted (control) mice. 1 d later, mice were inoculated s.c. by 4T1.2-Neu (1×10^5) at the 4^{th} mammary fat pad. Data represents two independent experiments. No depletion (n=10) *vs.* transient $CD11c^+$ depletion (n=9): p=0.0254. In all experiments, primary tumor was observed at d 3 after tumor inoculation. Mice that rejected primary tumor demonstrated long-term survival without metastatic tumors.

Table I

In vivo administration of α-CD25 mAb prior to tumor inoculation provokes potent antitumor immunity against an aggressive spontaneous metastatic murine breast tumor 4T1.2-Neu

α-CD25 mAb treatment	Tumor rejection (%)
d 3 prior to tumor inoculation	7/10 (70%)
d 3 after tumor inoculation	0/10 (0%)
none	0/10 (0%)

Mice were injected i.p. with purified α-CD25 mAb at 3 d, either prior to or after, 4T1.2-Neu s.c. inoculation at the 4^{th} mammary fat pad. In all experiments, primary tumor was observed at d 3 after tumor inoculation and assessed by palpation every other day. Mice pretreated with α-CD25 mAb rejected primary tumor around 3 week after tumor inoculation. Data represents two independent experiments.

Table II

Adoptively transferred $CD4^+$ T cells orchestrate endogenous immune components to ignite potent tumor-specific protective antitumor immunity

Mice	$CD4^+$ adoptive transfer	Tumor inoculation	Tumor rejection (%)
[a]WT	Yes	4T1.2-Neu	7/9 (78%)
WT	No	4T1.2-Neu	0/9 (0%)
WT	Yes	CT26	0/6 (0%)
WT	No	CT26	0/5 (0%)
WT	Yes	4T1.2	0/11 (0%)
WT	No	4T1.2	0/5 (0%)
[b]WT	Yes	4T1.2-Neu	6/8 (75%)
NK cell-depleted	Yes	4T1.2-Neu	1/5 (20%)
Gr-1^+cell-depleted	Yes	4T1.2-Neu	1/6 (17%)
[c]WT	Yes	4T1.2-Neu	6/8 (75%)
B $cell^{-/-}$	Yes	4T1.2-Neu	1/10 (10%)
$CD8^+$ cell-depleted	Yes	4T1.2-Neu	0/12 (0%)
[d]WT	Yes	4T1.2-Neu	5/6 (83%)
IFN-$\gamma^{-/-}$	Yes	4T1.2-Neu	2/10 (20%)

$CD4^+$ T cells (1×10^7) purified from α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu were adoptively transferred i.v. into wild type (WT), endogenous $CD8^+$-depleted, NK-depleted, Gr-1^+-depleted, B $cell^{-/-}$ or IFN-$\gamma^{-/-}$ BALB/c mice at d -1. Mice were inoculated s.c. with 4T1.2-Neu (1×10^5) at the 4^{th} mammary fat pad at d 0. Mice that were not adoptively transferred with $CD4^+$ T cells served as control. CT26 (1.3×10^6, inoculation s.c. at left flank) and 4T1.2 (1×10^5, inoculation s.c. at 4^{th} mammary fat pad) served as tumor-specific controls. In all experiments, primary tumor was observed at d 3 after tumor inoculation and assessed by palpation every other day. Mice that rejected primary tumor demonstrated long-term survival without metastatic tumors. Data represents two to three independent experiments. WT *vs*. NK-depleted, Gr-1^+-depleted, B $cell^{-/-}$, $CD8^+$-depleted or IFN-$\gamma^{-/-}$: $p<0.05$.

Table III

Adoptively transferred $CD4^+$ T cells generate tumor-specific memory antitumor immunity

Mice	Secondary tumor inoculation	Tumor rejection (%)
Rejected initial 4T1.2-Neu	4T1.2-Neu	8/8 (100%)
Naïve	4T1.2-Neu	0/10 (0%)
Rejected initial 4T1.2-Neu	4T1.2	0/8 (0%)
Naïve	4T1.2	0/5 (0%)
Rejected initial 4T1.2-Neu	CT26	0/6 (0%)
Naïve	CT26	0/6 (0%)

$CD4^+$ T cells (1×10^7) purified from α-CD25 mAb-pretreated mice that rejected 4T1.2-Neu were adoptively transferred i.v. into BALB/c mice at d -1. Mice were inoculated s.c. with 4T1.2-Neu (1×10^5) at the 4^{th} mammary fat pad at d 0. 40 d after initial tumor rejection, mice that rejected initial 4T1.2-Neu were inoculated by 4T1.2-Neu. Naïve BALB/c mice served as control. CT26 and 4T1.2 described in Table II served as tumor-specific controls. In all experiments, primary tumor was observed at d 3 after tumor inoculation. Mice that rejected primary tumor demonstrated long-term survival without metastatic tumors. Data represents two to three independent experiments.