This information is current as of April 14, 2017.

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*J Immunol* 2011; 186:3327-3335; Prepublished online 11 February 2011; doi: 10.4049/jimmunol.1000652
http://www.jimmunol.org/content/186/6/3327

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2011/02/11/jimmunol.1000652.DC1

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NK Cell Adoptive Transfer Combined with Ontak-Mediated Regulatory T Cell Elimination Induces Effective Adaptive Antitumor Immune Responses

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Previous work from our laboratory showed that hydrocortisone (HC) combined with IL-15 induces expansion of activated human NK cells. We set up an experimental tumor model to evaluate the use of adoptively transferred, HC plus IL-15 (HC/IL-15)–activated and –expanded murine NK cells in the treatment of syngeneic mice carrying established lung metastases of the CT26 transplantable tumor. We also examined the effect of denileukin diftitox (Ontak) on the depletion of regulatory T cells to enhance the in vivo antitumor immunity induced by the adoptively transferred NK cells. Our results clearly demonstrate that murine DX5+ NK cells are largely expanded in the presence of IL-15 plus HC while retaining intact their functional status. Moreover, when intravenously infused, they mediated significant antitumor responses against CT26 lung tumors in syngeneic BALB/c animals that were further enhanced upon pretreatment of the tumor-bearing animals with Ontak. Total splenocytes and isolated splenic T cells from NK-treated mice responded in vitro against CT26 tumor cells as evidenced by IFN-γ–based ELISPOT, proliferation, and cytotoxicity assays. Importantly, animals treated with Ontak plus adoptive transfer of HC/IL-15–expanded NK cells significantly retarded CT26 tumor growth after a rechallenge with the same tumor s.c. in their flanks. Taken altogether, our data suggest that NK cell adoptive transfer can trigger adaptive antitumor T cell responses, and regulatory T cell depletion by Ontak is mandatory for enabling HC/IL-15–activated NK cells to promote long-lasting adaptive antitumor immunity. The Journal of Immunology, 2011, 186: 3327–3335.

Natural killer cells are key components of the innate immune system, playing a critical role in early host defense against viral, bacterial, and other infections, as well as against cancer. They exert their effector function by direct killing of virally infected cells and tumor cells and by producing immunoregulatory cytokines and chemokines, thereby effecting adaptive immune responses (1). NK cell function is regulated by activating and inhibitory receptors expressed on their surfaces, by cytokines and chemokines (2) (3), as well as by cross-talk with other populations of the immune system, such as dendritic cells (4), effecter CD4+ T cells (5), and regulatory T cells (Tregs) (6).

The ability of NK cells to kill tumor cells has made them an attractive tool for cancer immunotherapy. Initial studies, ~25 y ago, were modestly successful in using NK cells for the treatment of several types of malignancies and were mainly restricted to the use of ex vivo-generated lymphokine-activated killer cells (7–10) or in vivo cytokine infusions aiming at the expansion and activation of NK cells against autologous tumor cells (11–14).

Recently, NK cells re-emerged as a powerful tool in cancer immunotherapy after reports on their biology and function (15–17).

One major obstacle in NK cell-based immunotherapy protocols is the limiting number of NK cells that can be obtained for infusion (16, 18). We have reported (19) novel data on the effect of glucocorticoids (GCs), along with stimulatory signals from IL-15 or IL-2, on human NK cell expansion and function. In contrast to previous reports on the inhibitory effects of GCs on NK cell function (20), we have demonstrated that NK cells, activated with IL-15 in the presence of hydrocortisone (HC), not only retain their functional potential but are also protected from apoptosis, and their proliferative rate is significantly elevated (19), thus making feasible their large-scale production from a limited volume of peripheral blood and their potential application in clinical protocols. Furthermore, expanding NK cells in the presence of HC, thus rendering them preconditioned to the presence of GCs, might be advantageous because their i.v. injection into a recipient exposes them to a microenvironment where active cortisol is naturally present at concentrations similar to the ones used in vitro. Whether IL-15 plus HC expanded NK cells still retain their functional capacity in vivo remains to be established.

Currently, Tregs are thoroughly being investigated in the context of cancer immunotherapy. The identification of a better-characterized regulatory subpopulation among CD4+ cells, constitutively expressing high levels of CD25, CTLA-4, GITR, Foxp3, and lacking CD127 expression, displaying anergy when stimulated by TCR cross-linking in vitro, and actively inhibiting CD4+CD25+ T cells, CD8+ T cells, dendritic cells (DCs), B, NK, as well as NK cells in a cell–cell contact and dose-dependent manner (21, 22), led to a recent intensification of research on the role of these cells in tumor development, growth, and escape from immunosurveillance. Increased frequency of Tregs at the tumor site has been documented in several murine models, suggesting that the tumor itself actively promotes the increase of...
Tregs, either by activating naturally occurring Tregs or by converting non-Tregs into Tregs (23). In humans, increased frequency of Tregs either at the tumor site, the tumor-draining lymph nodes, or in the circulation have been reported in patients with several types of malignancy (21, 24). Treg depletion or functional inhibition can lead to the induction of efficient antitumor activity. This can be achieved either by pharmacological agents such as cyclophosphamide (25), fludarabine (26), cyclooxygenase-2 inhibitors (27), temozolomide (27), or by directly targeting Tregs with Abs against the IL-2 receptor or against CTLA-4 (28) or with IL-2 conjugated with toxin (denileukin diftitox; Ontak) (29, 30). Elimination of Tregs has proved promising either alone or in combination with other immunotherapeutic interventions in preclinical models or in clinical studies (31).

Single immunotherapeutic modalities for cancer treatment, such as NK cell adoptive transfer alone, have proved to date to have limited success in the clinical setting (32), and therefore combination with other immune interventions has become obvious. In the current study, we sought to establish an improved NK cell-based immunotherapy protocol in a syngeneic mouse metastatic tumor model, taking advantage of the increased ex vivo expansion rate of NK cells activated with IL-15 in the presence of HC. We initially tested the effect of HC on IL-15–activated murine NK cell survival, expansion, and functional potential. Having confirmed that mouse NK cells respond in vitro upon activation with IL-15 in the presence of HC, we used the murine CT26 metastatic lung model (33) for the evaluation of the in vivo antitumor potential of these in vitro HC plus IL-15 (HC/IL-15)–expanded NK cells either as a single therapeutic modality or combined with elimination of Tregs by Ontak treatment.

Materials and Methods

**Mice**

BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in a pathogen-free animal facility and were used at 6–8 wk of age. Animal experiments were performed under strict guidelines according to the Hellenic Animal Committee.

**Cell lines**

The murine colon carcinoma cell line CT26 and the murine lymphoma cell line YAC-1 were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, sodium pyruvate (1 mM), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.83% ammonium chloride (lysing solution), mouse DX5+ cells were purified in a two-step process as follows: Briefly, goat anti-mouse IgG microbeads were initially used to eliminate spleen leukocytes of B cells by constitutive passing through an LS and an LD column. Subsequently, the microbeads were used at 6–8 wk of age. Animal experiments were performed under strict guidelines according to the Hellenic Animal Committee.

**Isolation and culture of murine spleen-derived DX5+ cells**

Fresh DX5+ cells were obtained from untreated BALB/c mice. Mouse were euthanized by cervical dislocation, and their spleens were excised. Leukocytes were subsequently obtained by pressing tissue through 70-µm nylon filters (Becton Dickinson, Cowley, U.K.) into chilled PBS. After RBC lysis with ammonium chloride 0.83% (lysing solution), mouse DX5+ cells were purified in a two-step process as follows: Briefly, goat anti-mouse IgG microbeads were subsequently used to eliminate spleen leukocytes of B cells by constitutive passage through an LS and an LD column. Subsequently, the DX5+ cell population was obtained by labeling with anti-mouse DX5 microbeads and passing through an LS column (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. The purity of the isolated NK cell population (DX5+CD3+) was always >90%.

Isolated DX5+ cells were seeded into a 6- or 24-well plate at 1 x 10^6 cells/ml in complete medium, which consisted of α-MEM (Life Technologies) with 20% FCS, supplemented with l-glutamine (2 mM), non-essential amino acids (1%), 50 µg/ml gentamicin, sodium pyruvate (1 mM), 2-mercaptoethanol (50 µM), recombinant human IL-15 (R&D Systems, Abingdon, U.K.) at 20 ng/ml, and the indicated concentration of HC (Sigma, St. Louis, MO). Every 3–4 d, half of the medium was discarded and replenished with medium containing freshly added cytokine and HC, thereby adjusting cell density to 0.5 x 10^6 cells/ml. The effect of different HC concentrations on the proliferation rate of DX5+ cells, incubated for the indicated time period with IL-15 in the presence of graded concentrations of HC, was determined by cell counting, using a Neubauer chamber, every 5 d. Dilutions of the cell cultures were taken into consideration to calculate the final fold-increase of cells at each time point. Viability was assessed by trypan blue dye exclusion.

CYTOTOXICITY ASSAY

Cytotoxic activity of cultured cells or freshly isolated splenocytes was assessed in a standard 4-h 51Cr-release assay, as previously described (19), against the NK-sensitive cell line YAC-1 and/or the CT26 colon carcinoma cell line. In brief, 10^4 target cells were labeled with 100 µCi sodium 51Cr chromate (5 Ci/mmol; Amersham, Little Chalfont, U.K.) for 1 h at 37°C. Effector cells were incubated with target cells at the indicated ratios. Spontaneous 51Cr release was measured by incubating target cells in the absence of effector cells. Maximum 51Cr release was determined by adding 1% Triton X-100 (Sigma). Spontaneous lysis did not exceed 10% of maximum release. The amount of 51Cr released was measured in a gamma-counter (Packard, Downers Grove, IL) and percentage lysis was calculated according to the following formula: percentage specific lysis = (experimental 51Cr release − spontaneous 51Cr release)/(maximum 51Cr release − spontaneous 51Cr release) x 100.

**Quantification of IFN-γ production by DX5+ cells**

For cytokine production determinations, either in culture supernatants or by intracellular staining, cells recovered from cultures with IL-15 in the presence or absence of HC were washed twice with HBSS (Life Technologies) and incubated for an additional 48 h in fresh medium containing recombinant human IL-15 (20 ng/ml), recombinant mouse IL-12 (10 ng/ml; R&D Systems), and IL-18 (10 ng/ml; R&D Systems) and the corresponding HC concentration. Supernatants were collected by centrifugation and stored at −75°C until use. IFN-γ was quantified by ELISA (R&D Systems) according to the manufacturer’s recommendations. For intracellular staining, brefeldin A (AppliChem, Darmstadt, Germany) was added during the last 4 h of incubation at a final concentration of 10 µg/ml. Cells were then harvested, washed with FACS buffer, fixed for 10 min in 4% paraformaldehyde, permeabilized with BD Perm/Wash, stained with an anti–IFN-γ mAb conjugated with PE (BD Pharmingen), and analyzed on a FACSCalibur (Becton Dickinson, Mountain View, CA) using CellQuest software (BD Biosciences, Franklin Lakes, NJ).

**Induction and evaluation of pulmonary metastases**

On day 0, age- and sex-matched BALB/c mice were inoculated i.v. with 5 x 10^5 to 5 x 10^6 CT26 colon carcinoma cells. After 1–3 wk, depending on the number of tumor cells received, mice exhibited discomfort signs such as cachexia, loss of weight, and dyspnea and eventually died. At that time, or as otherwise stated, animals were sacrificed, and the lungs were stained with Higgins Black Magic ink (15% v/v) and bleached with Fekete’s solution (formaldehyde [10% v/v], glacial acetic acid [5% v/v] in ethanol [60% v/v]), thus enabling the enumeration of white metastatic nodules on a black lung background.

**NK cell adoptive transfer**

In the first series of experiments, BALB/c mice were simultaneously inoculated i.v. with 5 x 10^5 CT26 cells and 4 x 10^5 syngeneic DX5+ cells, which had been expanded in vitro for at least 10 d prior to in vivo administration with IL-15 in the absence or presence of 10^7 or 10^8 M HC. Animals were either euthanized on day 10 and the number of tumor metastases was scored or monitored for survival.

In the experiments assessing cellular immunotherapy with adoptively transferred NK cells, animals were first (day 0) inoculated i.v. with the minimal tumorigenic dose of CT26 (5 x 10^5 tumor cells) and randomly distributed among the various groups. On day 8 post-tumor inoculation, animals were given i.v. either 5 µg of denileukin diftitox (Ontak; Eisai, Woodcliff Lake, NJ) per mouse in 100 µl saline or saline alone (control). For the survival experiments, on day 10, half of the animals from each group (control or Ontak) received four i.v. injections of HC/IL-15-expanded DX5+ cells at weekly intervals, the first consisting of 4 x 10^5 cells, followed by three subsequent inoculations of 1 x 10^6 NK cells. Remaining were treated with saline only at the same time intervals. For the determination of NK cell engraftment and Treg enumeration within the lungs, animals received only the first injection of 4 x 10^6 cells or saline on day 10 and subsequently sacrificed 3 d later.
Flow cytometry and quantification of absolute cell numbers

The mAbs anti-CD3 and anti-CD4 conjugated with FITC, anti-CD49b (anti-DX5), anti-CD25 and anti-CD11b conjugated with PE, PerCP-conjugated anti-CD45, anti-CD62L conjugated with allophycocyanin, and fluorescein-conjugated isotype-matched Abs were purchased from BD Pharmingen. The following mAbs were purchased from eBioscience: FITC-conjugated NKP46 (CD335) and CD94, PE-conjugated CXCR4 (CD184), CXCR3 (CD183), and CD27, PerCP-Cy5.5 (PerCP combined with the cyanine dye Cy5.5)-conjugated CD3e, allophycocyanin-conjugated DX5 (CD49b), NKG2D, and CD69. Allophycocyanin-conjugated anti-FoxP3 was also purchased from eBioscience.

Surface mAb staining was done in PBS containing 0.01% sodium azide and 1% FCS (FACS buffer) for 15 min at room temperature, followed by two washes with ice-cold FACS buffer and fixation with 1% paraformaldehyde in PBS.

Absolute counts of NK cells were performed using TrueCount tubes (Becton Dickinson), according to the manufacturer’s instructions, with the appropriate modifications. In brief, lung tissues were excised, weighed out, suspended in the appropriate volume of cold FACS buffer to obtain 100 mg lung tissue/ml, and homogenized to get a single-cell suspension. One hundred microliters of this suspension was stained for 15 min at room temperature with pretitrated combinations of anti-CD45–PerCP, anti-CD49b–PE, and anti-CD3–FITC, followed by 10-min incubation with lyzing solution. For the determination of Treg frequency, lung single-cell suspensions were first stained for surface CD4, CD25, and CD45 expression, followed by FcRp intracellular staining, according to the manufacturer’s instructions (eBioscience).

Annexin V–PE was purchased from BD Biosciences; NK cell apoptosis was determined by annexin V and 7-aminoactinomycin D uptake on gated DX5+ or NKp46+/CD3+ cells according to the manufacturer’s instructions.

Cells were analyzed on a FACSCalibur using CellQuest software.

Generation of DCs from murine bone marrow

DCs were generated from murine bone marrow (BM) cells as follows: On day 0, BM cells were flushed with RPMI 1640 medium supplemented with 10% FCS, 2 mM t-glutamine, 50 µg/ml gentamicin, and 5 × 10−3 M 2-mercaptoethanol (complete RPMI) medium from the tibiae and femurs of wild-type BALB/c mice, passed through a 70-µm nylon filters (Becton Dickinson) into chilled PBS. After RBC lysis with ammonium chloride 0.83% (lysing solution), BM cells were cultured in 6-well plates at 105 cells/ml in 3 ml complete RPMI medium with the addition of 20 ng/ml murine GM-CSF and 10 ng/ml IL-4 (Imgenex, San Diego, CA) according to the manufacturer’s instructions. The purity of the isolated T cell population (CD3DX5+) was >90%.

IFN-γ ELISPOT assay

IFN-γ–producing splenocyte frequencies were determined by an ELISPOT assay. Briefly, total splenocytes from untreated and therapeutically-treated BALB/c mice were collected and used as responders 20 and 60 d after CT26 i.v. injection, respectively. Splenocytes from naive mice were also used as controls. Cells were plated in polystyrene flask ELISPOT plates (BD Biosciences) in quadruplicate, at 3 × 105 cells/well, for 24 h at 37°C and stimulated with CT26 lysate, corresponding to 3 × 105 cells, in a final volume of 200 µl/well complete RPMI 1640 medium supplemented with 10 IU/ml IL-2. When T cells were used as responders, they were pooled and plated at 3 × 105 cells/well in polystyrene flask ELISPOT plates (BD Biosciences) and stimulated with the CT26 lysate, as above, in the presence of DCs at a ratio of 5:1 (T/DC). IFN-γ spots were developed according to the manufacturer’s instructions and counted in an automated 4-plate ELISPOT reader (EliExpert) using the Eli.Analyze Software (version V5.0; A.EL.VIS, Hannover, Germany). Results were expressed as percent area occupied by spots.

Proliferation assay

Measurement of murine total or T cell-enriched splenocyte proliferation was performed using a standard [3H]thymidine incorporation assay. Briefly, on day 60 post-tumor inoculation, isolated total splenocyte or T cell-enriched splenocyte cultures were set up similarly to those described earlier in 96-well flat-bottom plates for 72 h. [3H]Thymidine (30–40 Ci/mmol; Amersham, Cardiff, U.K.) was added at 1 μCi/well for the last 16 h of incubation. Proliferative response was calculated according to the following formula: Δcpm = cpm (experimental, against CT26 lysate) – cpm (background).

CFSE proliferation assay

Splenic CD8+ and CD4+ T cell proliferation was performed by CFSE-labeling, as described (34). In brief, 20 × 106 total splenocytes were resuspended in PBS containing 5% (v/v) FCS and stained with stock CFSE solution for 5 min at room temperature to a final CFSE concentration of 5 µM. Cultures were set up in 48-well plates (Costar) containing 2.5 × 105 CFSE-labeled splenocytes/well stimulated with CT26 lysates (equivalent to 3 × 105 cells) plus 10 IU/ml IL-2 in a total volume of 250 µl/well. Seven days later, cells were harvested and stained with rat anti-mouse mAbs anti-CD49b (DX5)–PE, anti-CD3–PerCP-Cy5.5, and anti-CD8–allophycocyanin.

Establishment of subcutaneous tumors

Eight days after the completion of the therapeutic protocol (i.e., on day 39, see earlier), experimental groups of treated versus naive BALB/c mice were s.c. inoculated with 1 × 105 CT26 cells on the right flank. Ten days later, once palpable tumors were established, tumor size was measured with external calipers and was, thereafter, monitored every 4 d. Tumor area (mm2) was calculated from the greatest longitudinal (length) and the greatest transverse diameter (width) of each individual tumor. The observation was terminated by euthanasia 30 d later.

Statistical analysis

For survival curve plots and statistical analysis, GraphPad Prism version 5 (GraphPad Software, San Diego, CA) was used. Two-tailed Student’s t test for unpaired values statistical analysis was performed, and a p value < 0.05 was considered significant.

Results

Effect of HC on murine NK cells

In our previous study, we have shown that HC, at doses ranging from 10−4 M to 10−7 M, greatly enhances human NK cell expansion in cultures with IL-15 (19). Therefore, we sought to investigate whether HC could exert a similar effect on murine DX5+ cells in cultures supplemented with IL-15. We found that the number of DX5+ cells increased significantly (p < 0.001) in the presence of IL-15 and HC at concentrations ranging from 10−7 M to 10−9 M (Fig. 1A). HC at 10−6 M or more had a detrimental effect on murine spleen DX5+ cells (data not shown). Additionally, DX5+ cells grown in cultures supplemented with IL-15 and HC, at 10−7 M to 10−9 M, exhibited higher viability than that of control DX5+ cells cultured with IL-15 alone, as evidenced by trypan blue dye exclusion (Fig. 1B) and annexin V staining (Fig. 1C, 1D).

We next investigated the in vitro cytotoxic potential of these HC/IL-15–activated cells against YAC-1 and CT26 tumor targets. DX5+ cells activated and expanded for 10 d with HC and IL-15 exhibited similar cytolytic activity as that of NK cells cultured in the presence of IL-15 alone (Fig. 1E, 1F).) Furthermore, NK cells grown with HC/IL-15 retained their ability to secrete IFN-γ upon additional stimulation for 48 h with IL-12 and IL-18 (Fig. 1G, 1H).

Phenotypic analyses (Supplemental Fig. 1) revealed that culture of DX5+ cells with IL-15, both with or without HC, favored the expansion of the CD11b−low/CD27+ immature NK population (35) with similar NKG2D, NKP46, CD94, and CD69 expression. However, NK cells expanded with HC/IL-15 expressed higher
CXCR4, CXCR3, and CD62L, suggesting an increased migratory capacity of these cells as opposed to NK cells expanded with IL-15 alone.

**HC/IL-15–expanded NK cells suppress tumor growth in vivo**

Several reports have suggested a potential antitumor role of NK cells in vivo (1, 16, 36). Thus, in this study, we examined the impact of our HC/IL-15–activated and –expanded NK cells in an experimental pulmonary metastasis model. The choice of this tumor model was based on the finding that the majority of intravenously administered, activated NK cells accumulate in the lung and are retained at the tumor site (36).

As depicted in Fig. 2A and 2B, the mortality rates of BALB/c animals were high and dependent on the numbers of i.v. inoculated CT26 syngeneic tumors. Immunotherapy with both HC/IL-15– and IL-15–induced NK cells was quite effective, as already 10 d after i.v. co-injection with $5 \times 10^5$ CT26 cells, the numbers of lung foci were dramatically reduced (2.5–5.5 versus >200 in untreated mice; $p < 0.0001$) (Fig. 2C). Mice treated with either IL-15 alone or IL-15 plus HC expanded NK cells survived significantly longer (median survival for both treated groups 52 d versus 13.5 d for control mice; $p < 0.001$) (Fig. 2D). Thus, in all further experiments, NK cells activated and expanded for 10–20 d with IL-15 and HC were used, given their proliferative advantage over IL-15–alone expanded cells.

Effects of therapeutic intervention with activated NK cells against CT26 in BALB/c mice after treatment with Ontak

Next, we evaluated the effect of in vitro activated and expanded NK cells adoptively transferred in BALB/c mice in the therapeutic setting. Mice harboring established tumors resemble more closely the human situation and therefore are more suitable for assessing...
BALB/c mice injected with 5 \times 10^3 d versus 22 d, of survival compared with that of control animals (median survival growth, although there was a statistically significant prolongation. Mice treated with Ontak alone remained susceptible to tumor metastases were enumerated, as described in Materials and Methods. Values represent mean \pm SD from five mice per group.

FIGURE 2. Pulmonary metastases induced by the colon carcinoma CT26 cell line in syngeneic BALB/c mice and treatment with NK cells expanded in the presence or absence of HC. A, Pulmonary metastases 14 d after i.v. inoculation of BALB/c mice with various CT26 tumor cell numbers. Representative examples of two CT26 inoculants and the untreated group are shown. B, Survival rates in mice inoculated with various numbers of CT26 cancer cells. Cumulative data from two independent experiments are depicted. C, BALB/c mice were inoculated i.v. with 5 \times 10^5 CT26 cells and NK cells (4 \times 10^6) activated with IL-15 or HC/IL-15 or CT26 cells alone. Two weeks later, mice were sacrificed, and lung metastases were enumerated, as described in Materials and Methods. Values represent mean \pm SD from five mice per group.

The efficacy of certain immunotherapeutic modalities. To this end, we first injected mice i.v. with 5 \times 10^4 CT26 cells followed 10 d later by HC/IL-15–activated NK cell treatment (i.v. injections with 4 \times 10^6 cells followed by 3 weekly injections with 1 \times 10^6 cells each). Two days prior to NK cell treatment, mice were treated with Ontak (5 \mu g i.v.) to reduce the numbers of Tregs or control mice. In some groups, mice were sacrificed 3 and 5 d after the first NK cell injection, and the number of lung-infiltrating NK cells and Treg frequency among the data of Fig. 4 show that the survival of CT26-injected mice was further improved (50% of the mice became long-term survivors) by combining Ontak treatment with transfer of activated NK cells (median survival >123.5 d, p = 0.0117, compared with NK cell treatment alone).

Induction of adaptive responses by activated NK cell adoptive transfer

It is currently established that NK cells have the potential to initiate adaptive immune responses (15, 37). To this end, we performed a series of in vitro experiments to show the induction of anti-CT26 T cell immunity in our model. Lung metastases-bearing mice were treated with NK cells, with or without Ontak pretreatment. On day 60 after tumor cell inoculation, splenocytes from surviving mice activated NK cells were far less susceptible to the growth of CT26 lung tumors and survived up to 130 d (median survival 63.5 d, p < 0.0001, compared with control or Ontak-treated mice). Moreover, the data of Fig. 4 show that the survival of CT26-injected mice was further improved (50% of the mice became long-term survivors) by combining Ontak treatment with transfer of activated NK cells (median survival >123.5 d, p = 0.0117, compared with NK cell treatment alone).

Induction of adaptive responses by activated NK cell adoptive transfer
were examined for reactivity against CT26 cells in vitro. As controls, splenocytes from untreated mice on day 20 after i.v. tumor inoculation, as well as naive animals, were tested in parallel. The data shown in Fig. 5 convincingly demonstrate that splenocytes from CT26 survivors treated with NK or NK plus Ontak, responded with increased IFN-γ (Fig. 5A), cytotoxicity (Fig. 5B), and proliferation (Fig. 5C) upon stimulation with CT26 cells or cell lysate. We could not achieve statistical significance in the proliferation assay against the CT26 lysate between the untreated control and the NK-only–treated groups (Fig. 5C) upon stimulation with CT26 cells or cell lysate. The number of surviving animals tested (three mice in the NK-treated group) and the heterogeneity of the responses. Of interest, as also shown in Fig. 5C, treatment with NK plus Ontak enabled the generation of higher frequencies of CT26-recognizing splenic T cells mounting robust proliferative responses (p < 0.01 compared with control and NK-treated group).

To prove directly the involvement of T lymphocytes as the responding cell population upon stimulation with CT26 tumor cells in vitro, the above assays were repeated with purified splenic CD3+ DX5+ T lymphocytes. The results depicted in Fig. 6A and 6B show significantly higher T cell-mediated antitumor IFN-γ and proliferative responses, respectively, from the groups of animals treated with NK plus Ontak compared with those for NK-only treatment. These data convincingly describe the central role of T cells as mediators of anti-CT26 immunity after NK/Ontak combinatory treatment. Furthermore, we showed that both CD4+ and CD8+ T cell subsets respond with increased proliferation rates in cultures with CT26 cells (Fig. 6C).

To substantiate further this NK cell-mediated bridging between innate and adaptive antitumor immunity, we have tested if BALB/c mice that had been therapeutically treated with NK cells, with or without Ontak pretreatment, could resist a second challenge with CT26 cells administered s.c. in their flanks 1 wk after completion of treatment. Tumor growth was also monitored in control naive mice receiving CT26 cells s.c. on the same day as treated lung-tumor–bearing animals. As shown in Fig. 7, growth of s.c. CT26 tumor in the NK-only–treated group of mice was retarded compared with that in control animals, although a great heterogeneity among the NK-treated group was observed, in accordance with the in vitro experiments. On the contrary, all lung-tumor–bearing mice treated with Ontak and NK cell adoptive transfer exhibited a significantly delayed s.c. CT26 tumor growth compared with that of the group treated with NK cells only, a type of protection indicative of the mediation of immune cells susceptible to the suppressive action of Tregs (e.g., CD4+ and/or CD8+ T cells).

### Discussion

The immune system consists of an integral network, where the function of each component population affects, positively or negatively, the other immune cell populations. As part of this network, nonspecifically acting NK cells, APCs, and cells of the adaptive immune compartment could, by joining their functions, generate persistent memory antitumor immune responses. To this end, in the current study we demonstrate for the first time to our knowledge that adoptive transfer of HC/IL-15–expanded and –activated syngeneic NK cells, combined with the elimination of endogenous CD4+CD25+ Tregs, also elicits effective adaptive immune responses when applied to a therapeutic metastatic pulmonary cancer mouse model.

Although adoptive transfer of appropriately in vitro-activated and -expanded NK cells is a promising strategy to treat cancer (15, 18, 38), the low numbers of NK cells normally present in peripheral blood provide an obstacle in NK cell-based immunotherapy. Hence, obtaining a large number of NK cells is an essential though difficult task toward the development of successful NK cell adoptive transfer protocols. We have previously described

**FIGURE 4.** Significant prolongation of survival in tumor-bearing mice treated with NK cells and Ontak. On day 0, BALB/c mice were inoculated i.v. with 5 × 10⁶ CT26 cells. Eight days later, mice were administered 5 μg Ontak by i.v. injection. Two days later (4 × 10⁶) NK (HC/IL-15) cells were adoptively transferred into the tumor-bearing mice by i.v. injection followed by three consecutive i.v. transfers of (1 × 10⁶) NK (HC/IL-15) cells on days 17, 24, and 31. Survival rates were monitored as shown.

**FIGURE 5.** Splenocytes from CT26 tumor-bearing mice treated with in vitro-expanded NK cells exhibit increased in vitro immunity against CT26 cells. BALB/c mice with CT26 lung metastases were treated with NK cells as described in Fig. 4. Fifty days after the initiation of the experiment, animals were sacrificed, and their splenocytes were tested in vitro for immunity against CT26 tumor cells. Splenocytes from naive animals and control mice with CT26 metastases were used for comparison (on day 20). NK cell treatment alone or combined with Ontak pretreatment resulted in increased number of IFN-γ–producing cells (A), exhibiting significant cytotoxic (B) and proliferative (C) responses against CT26 cells. Results represent mean ± SD of at least three animals tested in quadruplicates in the case of IFN-γ ELISPOT and proliferation assay or duplicates in the case of the cytotoxicity assay.
a method to activate and greatly expand human peripheral blood-derived NK cells with IL-15 in the presence of HC, thus providing the possibility of obtaining high numbers of functional activated NK cells from a limited volume of peripheral blood (19). Moreover, expansion of NK cells in the presence of HC renders them resistant to the in vivo effects of endogenous GCs (39). In this study, we show that murine DX5+ cells are rapidly expanded upon activation with IL-15 and HC, and this expansion is significantly higher compared with that with IL-15 alone. In our previously published study, we reported an almost 10-fold increase in the expansion rates of human NK cells cultured with HC/IL-15 compared with that with IL-15 alone (19). The fact that this expansion rate was lower with murine NK cells (3-fold) may be attributed to the source where NK cells were isolated from [i.e., peripheral blood versus spleen (40)] or to differences among species.

More importantly, NK cells expanded this way better preserve their viability and retain the high expression of their activating receptors NKG2D and NKp46, their ability to lyse tumor cells, and their potential to produce IFN-γ, thus being functionally intact. Furthermore, the HC/IL-15–activated and –expanded NK cells may have better migratory capacity toward the tumor and/or the tumor-draining lymph nodes, as supported by their increased expression of the chemokine receptors CXCR3 and CXCR4 and of CD62L (41, 42).

Activated NK cells are known to infiltrate in lung tissue within minutes of i.v. injection, and their density increases at the tumor sites within 24 h (43–45), thus implying that adoptive transfer of in vitro-activated and -expanded NK cells might be a promising therapeutic approach for the treatment of lung cancer. To investigate whether in vitro HC/IL-15–activated and –expanded NK cells could be effective in the treatment of lung cancer, we applied an NK cell-based adoptive transfer protocol in a BALB/c model of CT26-induced lung metastasis. Our results clearly show that intravenously administered HC/IL-15–activated and –expanded NK cells home to the lungs of these BALB/c mice, where they persist for more than 5 d, as evidenced by the increased number of lung-infiltrating NK cells. In line with our findings, Alici et al. (46), when they injected activated NK cells into non-tumor-bearing mice, observed that they were located mainly in the lungs and

FIGURE 6. NK/Ontak treatment triggers T cell responses against CT26 syngeneic tumor cells. The in vivo treatment is described in the legend of Fig. 5. The in vitro testing is also described in the legend of Fig. 5 with the exception that highly enriched T cells were tested as responders for IFN-γ production (A) and proliferation (B). Data with pooled T cells from three animals per group are shown ± SD. Proliferative responses to CT26 in CFSE-labeled CD4+ and CD8+ T cell subsets from the indicated groups were analyzed by FACS (C). One representative experiment of three performed is shown.
NK/Ontak, and NK versus NK/Ontak) on day 30. Statistically significant between groups (i.e., control versus NK, control versus regular every 4 d. Curves represent individual mice. Differences statistically significant between groups (i.e., control versus NK, control versus NK/Ontak) on day 30, \( p < 0.0001 \).

FIGURE 7. CT26 lung-tumor-bearing mice adoptively transferred with in vitro-expanded NK cells acquire resistance to a distal rechallenge with CT26 cells. Eight days after the last NK cell infusion, as described in Fig. 4, BALB/c mice with preestablished CT26 lung metastases received an s.c. inoculation of \( 10^7 \) CT26 cells on the right flank. Ten days later, once palpable tumors were established, tumor size (area in \( \text{mm}^2 \)) was measured with the use of an external caliper. Tumor growth, thereafter, was observed regularly every 4 d. Curves represent individual mice. Differences statistically significant between groups (i.e., control versus NK, control versus NK/Ontak, and NK versus NK/Ontak) on day 30, \( p < 0.0001 \).

liver for the first week and were subsequently undetectable. Although from the data presented in this study we cannot exclude the possibility that at least a part of NK cells infiltrating in lung tissue may be represented by endogenous NK cells, still this seems to be unlikely because no exogenous cytokine (IL-2 or IL-15) to support their proliferation was administered to the mice.

More importantly, we were able to show that adoptively transferred HC/IL-15–activated and −expanded NK cells are efficient effectors of tumor cell killing thereby significantly prolonging mouse survival. In addition, Treg elimination by Ontak treatment prior to NK cell administration significantly improved the outcome of the adaptive therapy: 50% of mice with preestablished lung metastases treated with the combination regimen were cured. It is currently well documented that Tregs inhibit NK-mediated lysis of RENCA cells by ∼50% at a 1:1 Treg/NK ratio, as opposed to the 1:9 ratio, which had no significant effect. According to our findings, the number of adoptively transferred NK cells 3 d after their i.v. administration significantly exceeded the number of Tregs in the lungs of the tumor-bearing mice, even in the group receiving only NK cells (less than 1:13 and 1:20 Treg/NK ratio in NK and NK plus Ontak treated mice, respectively). Although Lundqvist et al. (49) did not count the number of lung-infiltrating endogenous Tregs and adoptively transferred NK cells in their RENCA lung tumor model, it seems rather unlikely that in both our model presented in this study as well as that described by Lundqvist et al. (49), endogenous Tregs could account for the inability of adoptively infused activated NK cells to eliminate tumors. It has been recently demonstrated that NK cell-mediated killing of target cells triggers robust Ag-specific T cell–mediated and humoral responses (37). Thus, it seems more plausible that elimination of Tregs before NK cell infusion might favor the generation of potent antitumor T cell responses in a cascade of events normally occurring in the integrated innate–adaptive immune network. Indeed, total splenocytes as well as isolated splenic T cells from lung-tumor–bearing mice treated with NK cells alone or in combination with Ontak pretreatment, on day 60 after CT26 tumor cell inoculation (i.e., 29 d after the last NK cell infusion), were found to exhibit enhanced in vitro reactivity against CT26 as evidenced by the increased frequency of IFN-γ–producing cells, cytotoxicity, and proliferation compared with those of untreated littermates bearing 20-d CT26 lung metastases. Furthermore, mice that developed efficient immunity against CT26-induced lung tumors survived a rechallenge with CT26 cells that were implanted s.c. in their flanks. These data strongly support that the adoptively transferred NK cells were responsible for the initiation of adaptive antitumor responses. In fact, 50% of mice depleted of Tregs before NK cell infusion were cured, but mice treated with NK cells alone exhibited only prolonged survival. This finding may suggest that Treg elimination prior to T cell priming against CT26 tumor-associated Ags cross-presentation by APCs after uptake and processing of apoptotic bodies of NK-killed tumor cells (50) is a prerequisite for the induction of long-lasting memory antitumor responses.

To conclude, by applying an adaptive transfer protocol of appropriately activated and expanded NK cells in a lung metastasis mouse model, we were able to induce adaptive immune responses, further augmented upon Ontak-mediated Treg elimination. Overall, our data signify the exploitation of IL-15 plus HC large-scale expanded autologous, or allogeneic, NK cells in eradicating minimal residual disease as an alternative or complement to the currently used approaches in cancer immunotherapy.

Disclosures
The authors have no financial conflicts of interest.

References


