Can the Low-Avidity Self-Specific T Cell Repertoire Be Exploited for Tumor Rejection?1

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Can self-specific T cells that have escaped intrathymic deletion be exploited to generate antitumor immunity? To determine whether antitumor immunity to a self-Ag for which central tolerance exists can be generated, a mouse model is used in which a fragment of the influenza nucleoprotein (NP) is expressed as a transgene under the control of the H-2K promoter in C57BL/10 mice (B10NP mice). In these mice an oligoclonal population of NP-specific T cells escapes thymic and peripheral deletion and can be activated upon immunization. The main hallmark of these self-specific CD8+ T cells is diminished avidity for the pertinent MHC/peptide complex. We show in this study that intranasal infection with influenza virus can stimulate low-avidity NP-specific T cells to recognize and destroy NP-expressing microtumors in the lung, but not NP-expressing tumors growing s.c. Only a memory NP-specific CD8+ T cell response can suppress the growth of an s.c. growing NP-expressing tumor. This delay in tumor growth is associated with a dramatic increase in the number of circulating NP-specific CD8+ T cells. In addition, cultured memory NP-specific T cells require ~100-fold less Ag to induce NP-specific lysis than primary T cells, consistent with the observation that memory T cells have an increased avidity due to affinity maturation. Finally, during an NP-specific memory response, substantial numbers of low-avidity NP-specific T cells can be recovered from s.c. growing tumors. Together, these findings indicate that, when only a low-avidity repertoire is available to generate antitumor immunity, the best strategy may be to enhance memory responses.

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D uring T cell development, immature thymocytes reactive to self-Ags expressed in the thymus are triggered to undergo apoptotic cell death (1–4). However, this process is incomplete, resulting in the escape of potentially autoreactive T cells to the periphery (reviewed in Refs. 5 and 6). This may be due to the fact that not all self-Ags are presented in the thymus, thereby precluding thymic deletion. In addition, some self-reactive T cells may escape this central tolerance induction, because their TCRs have too low an affinity for the self-Ag, even when the Ag is expressed in or is available to the thymus (7–11). Multiple mechanisms exist that can silence autoreactive T cells in the periphery. These extrathymic tolerance induction mechanisms include peripheral clonal deletion (12–14), clonal anergy (15–19), clonal ignorance (20, 21), down-regulation of TCR and CD8 molecules (14, 22), and active suppression or regulation by other cell populations (reviewed in Ref. 23).

Despite these multiple mechanisms of peripheral tolerance induction, immunological self-tolerance is far from absolute, as is underscored by the occurrence of T cell-mediated autoimmune diseases (20, 21, 24). However, self-specific T cells can also have beneficial effects, because many of the Ags identified in human tumors are normal self-Ags inappropriately expressed or overexpressed (25–28). These self-Ags represent potential targets for T cell-based immunotherapy and vaccine design, and it has been demonstrated that self-specific T cells can mediate the rejection of tumors (29–35). Most of these studies (31–33, 35) concerned self-epitopes with a restricted expression pattern.

To define the conditions for self-specific antitumor immunity against a ubiquitously expressed self-epitope, we here investigate whether self-specific T cells that have escaped intrathymic deletion can be exploited to generate antitumor immunity. A mouse model is used in which a fragment of the influenza nucleoprotein (NP)1 is expressed as a transgene under control of the H-2K promoter in C57BL/10 mice (B10NP mice). In these mice, an oligoclonal population of self-specific CD8+ T cells which has undergone neither thymic nor peripheral deletion can be activated upon strong immunization (36). This population of cells can persist for prolonged periods of time, with strikingly low avidity for NP/MHC complexes (36), and has severely impaired induction of IL-2 production and clonal expansion (37). Yet these low-avidity self-specific T cells maintain their ability to differentiate into cytolytic effector cells and memory cells (36). In this study, we investigate the in vivo antitumor reactivity of low-avidity self-specific T cells.

Materials and Methods

Mice

C57BL/10 (H-2b; B10) mice were obtained from the experimental animal department of The Netherlands Cancer Institute (Amsterdam, The Netherlands). To obtain CD8-depleted mice, B10 mice were injected i.p. with 250 µg/ml anti-CD8 mAb (clone 2.43) 3 days in a row, followed by three times a week during the entire experiment. B10 mice transgenic for a fragment of the influenza NP (from which amino acids 3–327 are deleted) under control of the widely expressed class I MHC promoter H-2Kb (B10NP mice) were previously described (38). All mice were kept under specific pathogen-free conditions and used when they were 6–10 wk of age.

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3 Abbreviations used in this paper: NP, nucleoprotein; eGFP, enhanced green fluorescence protein; HAU, hemagglutinin unit.
Cells and tissue culture conditions

All cell lines were cultured in IMDM (Life Technologies, Glasgow, U.K.) supplemented with 5% heat-inactivated FCS (PAA Laboratories, Linz, Austria), 100 IU/ml penicillin, 100 μg/ml streptomycin (both from Roche, Mannheim, Germany) and 5 × 10−3 M 2-ME (Merck, Hohenbrunn, Germany) (complete medium) at 37°C in humidified air containing 5% CO2.

The melanoma cell line B16 (H-2b) and the murine thymoma EL4 (H-2b) were transduced with a CDNA encoding for fragment amino acids 1, 2, and 328–498 of influenza A/NT/60/68 virus NP together with enhanced green fluorescence protein (eGFP; B16-NP and EL4-NP) or only with eGFP (B16 and EL4) (40, 41). In all experiments cells sorted for comparable levels of eGFP expression were used. In some experiments the NK-sensitive murine T cell lymphoma cell line YAC was used (42) to block NK cell activity.

Viruses, peptides, and tetramers

Purified influenza A/NT/60/68 virus was provided by Dr. R. Consalves (National Institute for Medical Research, London, U.K.). Influenza B/Lee/40 virus was acquired from the American Type Culture Collection (Manassas, VA). Virus was grown and titrated at the Department of Virology, Erasmus University (Rotterdam, The Netherlands). Virus was stored at −70°C in 50% sucrose and was thawed immediately before use.

The NP366–374 peptide (sequence: ASNENMDAM) was produced in the peptide synthesis facility at our institute. Tetramers of soluble MHC class I molecules complexed with the NP366–374 peptide were synthesized according to the original protocol developed by Altman et al. (43) and modified as previously described (44) to block NK cell activity.

Virus infection

For live virus infections, anesthetized mice were infected by intranasal administration of 50 μl of HBSS (Life Technologies) containing 25 hemagglutinin units (HAU) of A/NT/60/68 virus or 125 HAU of B/Lee/40 virus. For a secondary infection mice were infected with 250 HAU of A/NT/60/68 virus.

Enriching NP-specific T cells in culture

Fifteen days after primary or secondary influenza infection, spleens were isolated from B10 and B10NP mice, homogenized over a nylon filter (NPBI, Emmer Compascuum, The Netherlands) and treated with NH4CI to remove RBC. Splenocytes were seeded into 24-well plates at 5 × 105 cells/well in 2 ml of complete medium supplemented with 5 × 10−4 μg/ml NP366–374 peptide for B10 mice or 0.05 μg/ml NP366–374 peptide for B10NP mice and 20 μ/ml IL-2 (Cetus, Emeryville, CA). Cultures were tested on day 7 for their levels of NP tetramer positive cells, cytokine production in cell culture supernatant, and cytolytic activity (see below). At this time point the percentage of NP tetramer positive cells of the CD8+ population is 70–80% for B10 mice and 15–30% for B10NP mice.

Flow cytometry

To follow the fate of NP366–374-specific T cells in B10 and B10NP mice over time, analysis by flow cytometry was performed on cells isolated from spleen, lungs, and blood. Spleen and lungs were isolated and homogenized over a nylon filter (NPBI). Blood was drawn from the tail vein. RBC were lysed by NH4Cl treatment. A total of 5 × 106 cells were washed twice with PBS containing 0.5% BSA and 0.02% NaN3 (PBS/BSA) and incubated at 37°C for 20 min with 20 μl of the appropriate dilutions of allophycocyanin-conjugated anti-CD8 (BD PharMingen, San Diego, CA) and PE-conjugated tetramer class I-NP366–374-peptide complexes. Cells were washed twice and resuspended in PBS/BSA. Propidium iodide was used to discriminate between viable and dead cells. Data acquisition and analysis were performed on a FACScalibur using CellQuest software (BD Biosciences, Mountain View, CA).

To determine the percentage of tumor-infiltrating NP-specific T cells, tumors were excised and transferred to a 24-well plate filled with 1 ml of PBS containing 1 mg/ml collagenase (Sigma, Zwijndrecht, The Netherlands) and 10 μg/ml DNase (Sigma). Tumors were cut into pieces using a scalpel and incubated at 37°C for 30 min at 37°C. After the incubation, tumors were homogenized over a nylon filter (NPBI) and treated with NH4CI to remove RBC. The cells were stained as described above.

To determine NP366–374 specific IL-2 and IFN-γ production, intracellular cytokine staining was performed as previously described (45). Splenocytes from vaccinated B10 and B10NP mice were cultured as described above in bulk cultures. On day 7 cells were purified over a Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada) gradient. Such purified splenocytes were then stimulated in 96-well flat-bottom tissue culture plates (Costar, Corning, NY) at a concentration of 1 × 105 cells/well in 200 μl of complete medium supplemented with 1 μl/ml brefeldin A (Golgi-plug; BD PharMingen) and 50 U/ml human IL-2 either with the indicated concentration of NP366–374 or without the peptide. After 5 h of culture cells were washed with FACS buffer and surface-stained with allophycocyanin-conjugated anti-CD8 (BD PharMingen) for 20 min. Cells were washed twice and stained intracellularly with FITC-conjugated anti-mouse IL-2 or IFN-γ and isotype control IgG2b or IgG1, respectively. The staining was performed using the Cytofix/Cytoperm kit according to the manufacturer’s protocol (BD PharMingen).

Tumor protection assay

B16 tumor model. Four days after infection, B10NP mice were injected i.v. via the tail vein with 1 × 105 B16-NP tumor cells. After 4 wk, lungs were removed and the pulmonary nodules were enumerated using a microscope.

EL4 tumor model. Three (memory) or 7 (primary) days after infection (anticipating the upcoming peak of the NP-specific T cells), B10 and B10NP mice were inoculated s.c. with 1 × 105 EL4-NP tumor cells. The tumor diameter was measured three times a week along two perpendicular axes (x and y) in millimeters using calipers. The mean tumor diameter for each mouse was calculated [(diameter x) + (diameter y)/2]. Mice were sacrificed when the tumors reached diameters >20 mm or if the mice became overtly ill or the tumor became ulcerated.

51Cr release assay

For analysis of cytolytic activity, bulk cultures were prepared as described above. After 7 days of culture cells were purified over a Lympholyte-M gradient and used as effectors in a 51Cr release assay. Splenocytes were serially diluted in triplicate in round-bottom tissue culture plates (Costar). As targets wild-type EL4 and EL4 cells pulsed with various concentrations of NP366–374 peptide and EL4-NP were used. Target cells were labeled with 100 μCi of 51Cr (Amersham Pharmacia Biotech, Little Chalfont, U.K.) for 1 h at 37°C. The labeled cells were washed, and 2 × 106 cells were added per well. To block NK cell activity, a 50-fold excess of unlabeled YAC cells was added to the wells. Per target, spontaneous release was measured by incubating the labeled cells in medium alone. Maximum release was measured by incubating the labeled cells in 2% Triton X-100. After a 5-h incubation at 37°C, 25 μl of supernatant from triplicate cultures was harvested in Luma plates (Packard Instrument, Meriden, CT) and counted in a TopCount Microplate Scintillation Counter (Packard Instrument). The percentage of specific 51Cr release was calculated as a ratio of 100 × (cpm experimental release – cpm spontaneous release)/cpm 2% Triton X-100 release – cpm spontaneous release).

Results

NP-expressing tumors can be recognized and rejected by NP-specific low-avidity T cells in vivo

Previous studies from our laboratory have demonstrated that an oligoclonal population of self-specific, NP366–374-specific CD8+ T cells (referred to as NP-specific T cells) persisted in mice expressing NP as a self-Ag on all MHC class I-positive cells (B10NP mice) and could be activated and expanded by a localized infection with an NP-containing influenza virus (36). This NP-specific T cell population, which has escaped central tolerance, had diminished avidity for tetrameric MHC/NP-peptide complexes (36) but could generate Ag-specific IFN-γ and CTL responses. Nevertheless, high Ag doses were required to perform those effector functions. However, these low-avidity NP-specific T cells were severely limited in producing IL-2 and had a dramatic defect in expansion upon Ag exposure (37). This raised the question of whether such a “crippled” T cell population could perform antitumor effector function in vivo, and B10NP mice provided an excellent model to study this issue. Only a low-avidity T cell repertoire will be available for ubiquitously expressed tumor Ags (such as telomerase, p53, and Her-2/neu) for which central tolerance exists, and it is important to define which conditions, if any, must be employed to exploit this repertoire.

As most NP-specific T cells could be detected in the lungs after intranasal infection with an NP-containing influenza virus, we first tested whether tumor growth could be prevented in the lungs of B10NP mice. Therefore, B10NP mice were infected intranasally...
with NP-containing influenza A/NT/60/68 virus or with control influenza B/Lee/40 virus, before i.v. inoculation of NP-expressing B16 melanoma cells. i.v. inoculation of B16 melanoma cells resulted in the formation of numerous pulmonary microtumors in the lungs in control mice (Fig. 1, left). However, mice infected with influenza A/NT/60/68 virus showed a dramatic reduction in the number of NP-expressing pulmonary tumors (Fig. 1, middle), and this was correlated with abundant infiltration of low-avidity NP-specific CD8+ T cells into the lungs (36, 37). This antitumor response was Ag specific, as infection of B10NP mice with a control influenza virus did not result in reduced numbers of pulmonary tumors (Fig. 1, right). In addition, no effect of influenza A/NT/60/68 virus infection could be observed on the number of pulmonary B16 tumors only expressing eGFP and not NP (data not shown). Together, these results demonstrated that an intranasal challenge with influenza virus that expresses NP generated infiltration of NP-specific T cells and clearance of multiple tumors in the lung that expressed that same influenza epitope. These data prove that low-avidity, NP-specific CD8+ T cells in B10NP mice can perform effector functions in vivo.

**B10NP, but not B10, mice fail to reject an s.c. growing NP-expressing EL4 tumor**

Having demonstrated that self-specific T cells, despite their low avidity, can indeed have effector function in vivo in the lung tumor model, we next asked whether these low-avidity, NP-specific T cells could also reject s.c. growing tumors. Importantly, we found that the growth of s.c. growing B16-NP tumors was not affected by an NP-specific response induced by A/NT/60/68 virus infection in B10NP mice (data not shown), while in B10 mice s.c. growth of B16-NP tumors can be dramatically reduced by A/NT/60/80 infection. However, because it was difficult to study the infiltration of T cells into s.c. growing B16 melanoma cells (it grows in a somewhat diffuse pattern and is difficult to process), the EL4 thymoma was used in all subsequent experiments.

An s.c. growing NP-expressing EL4 tumor (EL4-NP) could easily be kept under control in B10 mice (Fig. 2A). This antitumor response was correlated with the activation and expansion of NP-specific CD8+ T cells that could easily be detected in peripheral blood (Fig. 2B) and secondary lymphoid organs (data not shown). The antitumor response was CD8 mediated, since no tumor rejection was observed in CD8-depleted EL4-NP tumor-bearing B10 mice (Fig. 2A). The activated NP-specific T cells also exhibited ex vivo NP-specific cytolytic activity, as EL4 targets pulsed with NP366–374 peptide and NP-transduced EL4 cells could be lysed by such tumor-induced activated NP-specific T cells (data not shown). Therefore, the EL4-NP tumor effectively triggered activation, expansion, and differentiation of NP-specific T cells in B10 mice.

Quite in contrast, the EL4-NP tumor did not trigger a rejection response in B10NP mice (Fig. 2C). Furthermore, the EL4-NP tumor failed to induce a CD8+ T cell response in B10NP mice (Fig. 2D) despite the fact that an NP-specific T cell repertoire did exist in these mice (see below) (36). Thus, a tumor expressing NP as a foreign Ag can induce a CD8+ T cell-mediated rejection response, while a tumor expressing NP as a self-Ag cannot.

To study whether NP-expressing tumors could be rejected in the presence of activated low-avidity NP-specific T cells, B10NP mice were infected with A/NT/60/68 influenza virus. After 7 days, before the peak of the NP-specific T cell response, EL4-NP tumor cells were inoculated s.c. The EL4-NP tumor grew undisturbed in such A/NT/60/68 virus-infected B10NP mice (Fig. 2C) despite the presence of activated NP-specific T cells (Fig. 2D). Thus, while intranasal A/NT/60/68 virus infection in B10NP mice results in reduced growth of NP-expressing tumors locally (in the lung), it is ineffective s.c. Presumably, this is because the lung tumor model represents an ideal setting for tumor rejection by low-avidity T cells: small tumors and massive in situ inflammatory conditions.

**Memory NP-specific low-avidity T cells can delay tumor growth in B10NP mice**

Memory responses can be both qualitatively and quantitatively more robust than primary responses (46–48). Therefore, we investigated whether a memory T cell response might cause tumor rejection in this tumor model. Previous studies from our laboratory have demonstrated that infection of B10NP mice with influenza A/NT/60/68 virus resulted in the induction of an NP-specific T cell memory population, as defined by rapid and massive expansion of NP-specific T cells upon rechallenge (36). To study the in vivo antitumor capacity of NP-specific memory T cells in B10NP mice, a tumor protection assay was performed during this memory response. B10NP mice were infected intranasally with NP-containing influenza virus 5 wk after primary exposure to influenza. Three days later, these mice were inoculated s.c. with EL4-NP tumor cells. At various time points after tumor inoculation, the percentages of NP-specific T cells in lungs, spleen, and blood were determined, and the tumor diameter was measured. In the lungs, spleen, and blood, the NP-specific T cell memory response was ~3- to 4-fold higher than the response in mice undergoing a primary infection (Fig. 3A), and the peak of the response occurred more rapidly after viral infection in memory mice than in naive mice. This NP-specific T cell memory response was correlated with delay of growth of the EL4-NP tumor (Fig. 3B), although it...
clearly had only a partial effect. Nevertheless, such a protective effect was never observed during the primary response (Figs. 2C and 3B). The response was Ag specific, since mice re-exposed to the antigenically unrelated B/Lee/40 virus did not show this retardation in tumor growth (Fig. 3B). Furthermore, the memory NP-specific T cells did maintain their low-avidity phenotype, although selection for cells at the high affinity end of the low affinity spectrum did occur (Fig. 3C). Together, these findings suggested that NP-specific memory T cells in the self-specific T cell repertoire cause a modest delay of tumor growth. Histological analysis revealed no overt signs of autoimmune pathology in these mice despite the presence of activated NP-specific memory T cells (data not shown).

**NP-specific intracellular IL-2 and IFN-γ production, cytolytic activity, and migration behavior of primary and memory NP-specific T cells**

Why is a self-specific memory T cell population more efficient in protection against tumors? First, the observed antitumor immunity may simply be due to higher numbers of memory NP-specific T cells compared to the numbers of primary NP-specific T cells (Fig. 3A). Second, selection for cells at the high affinity end of the low

**FIGURE 2.** B10NP, but not B10, mice fail to reject an s.c. growing NP-expressing EL4 tumor. A and B, B10- and CD8-depleted B10 mice were inoculated s.c. into the right flank with $1 \times 10^5$ EL4-NP tumor cells. C and D, B10NP mice were infected with 25 HAU of influenza A/NT/60/68 virus. Seven days later these mice and uninfected B10NP mice were inoculated s.c. into the right flank with $1 \times 10^6$ EL4-NP tumor cells. A and C, At the indicated time points after tumor inoculation, tumor diameter was measured. Mean tumor diameter is plotted against the days after tumor inoculation. B and D, At the indicated time points, blood was analyzed by flow cytometry using NP tetramers and anti-CD8 mAb. The percentages of tetramer-binding cells of the gated CD8$^+$ T cell population are presented.

**FIGURE 3.** Memory NP-specific low-avidity T cells can delay tumor growth in B10NP mice. B10NP mice were infected with 25 HAU of A/NT/60/68 influenza virus. After 5 wk, these mice were re-exposed to that same virus (memory) or to a control virus (B/Lee/40). In addition, mice infected for the first time (primary) or not infected at all were compared. Three (memory) or 7 (primary) days after the infection (anticipating the upcoming peak of the NP-specific T cells) were inoculated with $1 \times 10^6$ EL4-NP tumor cells. A, At the indicated time points, lungs, spleen, and blood were analyzed by flow cytometry using NP tetramers and anti-CD8 mAb. The percentages of tetramer-binding cells of the CD8$^+$ T cell population are presented. The absolute numbers of NP-tetramer-binding cells also increase, since the numbers of T cells in spleen and lung remain the same (data not shown). B, At the indicated time points, tumor diameter was measured. The mean tumor diameter is plotted against the days after tumor inoculation. C, On day 9 lungs from primary and secondary infected B10 or B10NP mice were removed and stained with NP-tetramers and anti-CD8 mAb. The level of tetramer staining of CD8$^+$ T cells from primary and memory B10 or B10NP mice is shown. The mean fluorescence intensity of the tetramer signal is indicated. Note that TCR levels of NP-tetramer-binding cells from B10 and B10NP mice are identical for both primary and memory responses (36, 37) (data not shown).
affinity spectrum does occur (Fig. 3C). It should be noted that TCR levels of NP-tetramer binding cells from B10 and B10NP mice are equal in both the primary and the memory response (36, 37) (data not shown). In addition, memory cells may produce more or different cytokines, may be more effective in killing NP-expressing targets, or may have a different migration behavior.

To investigate these possibilities, we assessed whether primary and memory T cells differed with respect to their cytokine profile, cytolytic capacity, and migration behavior. Splenocytes of mice undergoing a primary or a secondary infection with A/NT/60/68 influenza virus were cultured with NP366-374 peptide and IL-2, and after 7 days these splenocytes were compared with respect to IL-2 and IFN-γ production and CTL activity. On the average, 70–80% of the CD8+ T cells from primary and secondary B10 cultures stained with NP tetramers. For B10NP primary and secondary cultures 15–30% of the CD8+ T cells were NP tetramer positive. Restimulated splenocytes from memory B10NP mice did maintain a higher avidity phenotype than restimulated splenocytes from mice undergoing a primary infection (data not shown), just as in the primary responses (Fig. 3C).

**FIGURE 4.** NP366-374-specific intracellular IL-2 and IFN-γ production by primary and memory T cells from B10 and B10NP mice. B10 and B10NP mice were infected with 25 HAU of influenza A/NT/60/68 virus, and 5 wk later these mice were re-exposed to that same virus (memory) or mice were infected for the first time (primary). After 15 days, spleens were isolated and cultured (5 x 10⁶ cells/well) with 0.0005 µg/ml NP366-374 peptide (B10) or 0.05 µg/ml NP366-374 peptide (B10NP) and 20 U/ml IL-2 for 7 days. A, NP-specific intracellular IL-2 (left panel) and IFN-γ (right panel) production of the splenocytes was analyzed. Splenocytes were harvested after 7 days and stimulated with the indicated concentrations of NP366-374 Peptide for 5 h. Cells were stained with allophycocyanin-anti-CD8, fixed, permeabilized, and intracellularly stained with FITC-anti-mouse IL-2 or IFN-γ and isotype control FITC-anti-mouse IgG2b or IgG1, respectively. Isotype control staining did not exceed background levels. The percentages of IL-2- and IFN-γ-positive cells of the CD8 population are shown. On average, 70–80% of the CD8+ T cells from primary and secondary B10 cultures stained with NP tetramers. For B10NP primary and secondary cultures 15–30% of the CD8+ T cells are NP tetramer positive. B, IL-2 and IFN-γ data from B10NP mice (A) represented as histograms. Histograms show levels of intracellular IL-2 (solid lines, upper panels) and IFN-γ (solid lines, lower panels) staining or isotype control staining (dashed lines) of gated CD8+ T cells from primary and memory spleen bulk cultures.
As shown in Fig. 4A, primary and memory NP-specific T cells from B10NP mice both produced IL-2 and IFN-γ. However, compared with IFN-γ production, the production of IL-2 was very limited. In addition, high Ag doses were required to perform those effector functions. To determine whether the Ag sensitivity for cytokine production of memory T cells differed from that of primary T cells, in vitro cultured primary and memory splenocytes were stimulated with decreasing concentrations of NP_366–374 peptide. No difference in the IL-2 response could be observed after stimulation of primary or memory T cells from B10NP mice with decreasing concentrations of NP_366–374 peptide (Fig. 4A). Thus, memory T cells from B10NP mice in this tumor model were not more sensitive than primary T cells with respect to IL-2 production. However, memory T cells did display a 10-fold higher Ag sensitivity for IFN-γ production compared with primary T cells. Nevertheless, the level of intracellular IL-2 (Fig. 4B, upper panel) and IFN-γ (Fig. 4B, lower panel) produced per cell did not differ between primary and memory T cells from B10NP mice.

Finally, we compared the cytolytic responses of primary and memory NP-specific cells from B10NP mice. It is important to note that both can lyse EL4 tumor cells expressing NP endogenously (Fig. 5A), providing an explanation for their ability to perform antitumor effector function in vivo (see Figs. 1 and 3B). We then compared the Ag sensitivity of cultured primary and memory cells at a fixed E:T cell ratio. It should be emphasized that the percentage of NP-tetramer binding cells in cultures from spleens of primary and memory B10NP mice is overall comparable (see Fig. 5). Nevertheless, memory T cells from B10NP mice were ~100-fold more potent than primary T cells in executing NP-peptide specific lysis (Fig. 5B). However, it is worth noting that the memory cells from B10NP cells still exhibit somewhat reduced Ag sensitivity compared with primary and memory cells from B10 mice (Fig. 5B).

To investigate whether the failure of primary NP-specific T cells to reject EL4-NP tumors was a consequence of a failure to infiltrate the tumor bed, tumors of mice undergoing a primary or a secondary response were excised at different time points after tumor inoculation and analyzed by flow cytometry for the presence of NP-specific T cells. Higher numbers of NP-specific T cells could infiltrate EL4-NP tumors from memory mice (Fig. 6). No differences among the expression patterns of CD44, CD62L, CD11b, and CD49d, all molecules involved in migration, could be observed in mice undergoing a primary or a secondary infection (data not shown).

Why can higher numbers of NP-specific T cells be retrieved from tumors excised from memory mice? Is it due to higher numbers of T cells present in memory mice because of a higher precursor frequency, or is there a difference in migration capacity between primary and memory NP-specific T cells? To study this issue, equal numbers of restimulated splenocytes from primary or secondary infected B10NP mice were adoptively transferred into EL4-NP tumor-bearing RAG-1 KO mice (41). At various time points after transfer, the percentage of NP-specific T cells was determined in the tumors of these mice. Equal numbers of primary and memory NP-specific T cells could be retrieved from tumors excised from EL4-NP tumor-bearing RAG-1 KO mice (data not shown). Therefore, we concluded that the observed delay in tumor growth of the memory mice was not caused by a difference in migration behavior between primary and memory NP-specific T cells from B10NP mice.

Taken together, these data imply that the enhanced antitumor effect of the memory response is caused by the higher numbers of NP-specific T cells present in B10NP memory mice (Fig. 3A). Overall, the numbers of NP-specific T cells present in B10NP memory mice are ~3- to 4-fold higher than those in B10NP mice undergoing a primary infection. In addition, the observed higher affinity of the TCRs of memory T cells for MHC/peptide complexes (Fig. 3C) translates into higher functional sensitivity (Figs. 4A and 5B). Collectively, these findings may explain the tumor growth delay caused by memory self-specific T cells in mice ubiquitously expressing a self-Ag.

Discussion

In the present study we investigated whether the low-avidity self-specific T cell repertoire could be exploited for tumor rejection without causing autoimmune pathology. We showed in a separate study that low-avidity T cells specific for a ubiquitously expressed self-Ag maintained their ability to differentiate into cytolytic effector cells and memory cells (36). However, induction of IL-2 production and clonal expansion were severely impaired (37). Despite these shortcomings, we found that low-avidity NP-specific T cells in NP-expressing mice could recognize and kill NP-expressing B16 melanoma cells in vivo when tumors grew as micrometastases in the lung. However, these low-avidity NP-specific T cells did not
circulation pathways, with naive T cells selectively trafficking from blood to lymph nodes while memory T cells selectively traffic from blood to peripheral tissues (46). This broader immune surveillance by memory cells could contribute to more rapid responses. Another study showed that memory T cells enter cell division more rapidly than naive cells and have a shortened lag phase (48). In addition, memory T cells often exhibit cytotoxic activity directly ex vivo and disappear more slowly after expansion. In contrast to these studies, we are studying a memory population of low-avidity self-specific T cells. We could not detect differences in Ag sensitivity for production of IL-2 between NP-specific CD8+ T cells during the primary and the memory response. Yet memory NP-specific T cells were ~10-fold more sensitive with respect to IFN-γ production. In addition, memory NP-specific T cells exhibited increased ligand sensitivity in the cytolytic response and also displayed a higher avidity phenotype than the primary T cells. In addition, we found that only during an NP-specific memory response, substantial numbers of low-avidity NP-specific T cells could be recovered from an s.c. growing tumor. This does not appear to be a consequence of altered migratory patterns. Therefore, the greater efficacy of the memory response is a consequence of the fact that memory T cells are somewhat more efficient in effector functions than primary cells and of the magnitude of the response. Nevertheless, the potential of the memory response is limited, because only a delay in tumor growth can be achieved. Therefore, efforts directed at exploiting the low-avidity, tumor-specific T cell response should be focused at increasing the magnitude of a memory response.

An efficient immune response against tumor Ags that also represent self-Ags can potentially be harmful, because immunity against self-Ags may cause autoimmunity. Efficient tumor control in patients may be associated with autoimmune phenomena, and anti-melanoma immunity sometimes correlates with vitiligo (49, 50). Despite the presence of activated NP-specific T cells in the B10NP mice, no overt signs of autoimmune pathology were observed in the present model. Long-term follow-up did not reveal any abnormalities, and immunohistologic analysis of several organs did not show any tissue destruction or abnormal T cell infiltration. Nevertheless, this same low-avidity NP-specific T cell population could kill tumor cells expressing NP endogenously. From these data, it can be concluded that it is possible to exploit the low-avidity T cell repertoire for tumor rejection without causing autoimmune pathology, even for a ubiquitously expressed self-Ag. This window of opportunity for tumor rejection most likely depends on quantitative differences in the expression of Ag between tumor cells and normal cells. Earlier studies already showed that self-specific T cells directed against self-Ags with restricted Ag expression could be exploited for tumor rejection without causing autoimmune pathology (31–33, 35).

Induction of anti-self-immunity to cure cancer cannot always be achieved without causing autoimmune pathology. Antitumor treatment with tumor Ag-loaded DCs causes severe autoimmune disease when the tumor Ags are not tumor specific, but are also expressed in peripheral nonlymphoid organs (51). In certain transgenic mouse models where Ags are exclusively expressed in pancreatic β islets or solely in cells of the cardiovascular system, such Ags may be completely ignored (51). In these mice tumors that also express these Ags can be successfully controlled by specific DC vaccination. However, this antitumor vaccination was accompanied by fatal autoimmune disease, i.e., autoimmune diabetes, or by severe arthritis, myocarditis, and eventually dilated cardiomyopathy (51). Whether self-specific tumor rejection can occur without undesirable autoimmune disease is thus also dependent on the type of tissue in which these self-Ags are expressed.

affect the growth of s.c. growing NP-expressing B16- and EL4 tumors. Only a delay in tumor growth could be accomplished when an NP-specific memory response was ongoing. This delay was associated with a dramatic increase in the number of circulating NP-specific CD8+ T cells, with increased cytolytic activity and with increased tumor infiltration.

Why might a memory T cell response be more efficient in tumor rejection? Are the greater speed and efficacy of a memory response simply due to increased precursor frequency or are they influenced by qualitative differences between naive and memory T cells? In a different model, Zimmermann and colleagues (47) also found that memory cells were far more efficient in tumor eradication. The kinetics of the responses of naive and memory cells were compared in that model, and the main difference between CD8+ memory T cells and naive T cells was the ability of the former to rapidly acquire effector cell functions. Memory T cells could be more rapidly induced to become cytolytic and to secrete high levels of IL-2 and IFN-γ than naive cells, enabling them to perform more efficiently in the early phase of an immune response (47). It has also been shown that naive and memory T cells have distinct re-circulation pathways, with naive T cells selectively trafficking

![FIGURE 6. Both primary and memory self-specific low-avidity T cells can infiltrate the tumor bed, but more NP-specific T cells infiltrate during the memory response. B10NP mice primed 40 days previously with 25 HAU of influenza A/NT/60/68 virus were reinfected (day 0) with 250 HAU of influenza A/NT/60/68 virus or with 250 HAU of B/Lee/40 virus. As a control, B10NP mice received only the infection with 25 HAU of influenza A/NT/60/68 virus on day 0, or B10NP mice were not infected at all. Three days after infection mice were inoculated s.c. with EL4-NP tumor cells. At the indicated time points tumors were excised and analyzed by flow cytometry for the presence of infiltrated NP-specific T cells using NP tetramers and anti-CD8 mAb. The percentages of NP-specific T cells of the CD8 population in tumors are plotted against the days after tumor inoculation. Absolute numbers of NP tetramer binding cells also increase, since the number of T cells infiltrating remains the same during the memory response.](image-url)
Tumor immunotherapy directed against Ags that are also self-Ags might be most promising when Ag-specific T cells of high avidity are recruited. Usually, when the self-Ag is presented in thymus, these high-avidity Ag-specific T cells are deleted in the thymus. Therefore, it has been suggested that it might be preferred to direct immunotherapy against Ags that do not reach the thymus and for which high-avidity T cells persist. However, such high-avidity self- and tumor-specific T cells are recruited, the risk of autoimmune diseases also increases. Our study shows that even low-avidity self-specific T cells can be activated upon vaccination to generate memory responses and antitumor immunity. The low-affinity interactions between TCR and peptide are associated with selective loss of critical T cell functions. Still, we show in this study that this crippled population can delay tumor growth without causing autoimmune pathology. The magnitude of the response is an important parameter for accomplishing this antitumor effect, as is increased Ag sensitivity, and these are most easily achieved in the memory response. Therefore, future efforts directed at exploiting the low-avidity tumor-specific response should be focused at increasing the magnitude of the memory response.

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