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Vaccination with Tumor Peptide in CpG Adjuvant Protects Via IFN-γ-Dependent CD4 Cell Immunity

Britta V. Stern,*† Bernhard O. Boehm, † and Magdalena Tary-Lehmann2*#

The low frequency of tumor Ag-specific T cells in vivo has made it challenging to directly measure their clonal sizes and cytokine signatures. We used a new generation ELISPot approach to study the constitutive immunogenicity of the RMA tumor in syngeneic B6 mice and adjuvant-guided immunity against an MHC class II-restricted RMA peptide, H11.1. The RMA tumor was found to activate cells of the innate immune system and to induce a type 1 polarized, RMA-specific CD4 and CD8 T cell response. With clonal sizes ~10^10, the magnitude of this constitutively induced immune response did not suffice to control the tumor cell growth. In contrast, immunization with H11.1 peptide, using an immunostimulatory CpG oligonucleotide or CFA as adjuvant, engaged ~25- or ~10-fold higher clonal sizes of type 1 polarized CD4 cells, respectively. Therefore, the CpG oligonucleotide functioned as a stronger type 1 adjuvant and, unlike CFA, elicited protective immunity. The protection was IFN-γ dependent, as it was not inducible in IFN-γ knockout mice. Therefore, CpG adjuvant-guided induction of type 1 immunity against tumor Ags might be a promising subunit vaccination approach. The Journal of Immunology, 2002, 168: 6099–6105.

I


t has become a focus of interest to define to what extent infectious non-self signals can be exploited for therapeutic purposes to trigger immune responses against tumors. Protein or peptide Ags are poor immunogens unless injected with an adjuvant (1). This is in part because the Ag itself does not engage the second signal and, additionally, is readily cleared from the organism. Ag injection with adjuvants can circumvent both limitations; however, the formulation of effective adjuvants continues to be a challenge. The half-life of Ags emulsified in oil-based adjuvants is extended to several hundred days vs minutes when the Ags are injected in a soluble form (2). In animal models, a mineral oil emulsion (IFA) has been classically used to generate such Ag depot effects. Ag injections in IFA tend to induce weak, type 2 immunity (3, 4). Immunization with CFA (which consists of IFA supplemented with heat-inactivated mycobacteria) has been the standard in animal models for the induction of cell-mediated (type 1) immunity. However, CFA is not suited for human vaccination because it results in severe granulomatous reactions at the injection site.

As the success of subunit vaccinations against cancer cells can be expected to depend on the induction of type 1 immunity against the tumor Ag/peptide, there is a clear need for an adjuvant capable of preferentially inducing type 1 immunity that is also suited for use in humans. Adjuvant effects exerted by immunostimulatory (ISS) oligodeoxynucleotides (ODN) hold promise for this purpose (5–8). Using Toll-like receptor-9, cells of the innate immune system (including dendritic cells (DC), macrophages, and NK cells) can recognize CpG motifs as a common feature of infectious non-self, and this triggers production of cytokines (such as IL-1, IL-6, IL-12, TNF-α) and up-regulation of costimulatory cell surface molecules (6, 9, 10). Thus, CpG-containing DNA, or synthetic ODN that contain this motif, are potent inducers of the second signal link: admixing of CpG ODN into IFA has been shown to generate a CFA-like adjuvant that can be used to guide the engagement of Th1-type immune responses (11). ODNs cannot bind to MHC molecules and are not recognized as Ags by T cells. Therefore, CpG-based adjuvants do not trigger the severe granulomatous reactions that are inherently caused by the immunogenic mycobacterial proteins contained in CFA. These properties make CpG-containing adjuvants prime candidates for adjuvant-guided type 1 immunity (6, 7, 12). Therefore, it is of considerable interest to what extent the adjuvant properties of CpG ODN can be exploited for cancer therapy.

The experiments presented in this work address the question of whether immunization with an MHC class II-restricted tumor peptide with CpG ODN can generate a CD4 cell response in nontransgenic mice potent enough to control tumor cell growth. We selected the C57BL/6-derived class II-negative T cell lymphoma RMA as a model system because it possesses a well-defined I-Aβ-restricted determinant (peptide H11.1) (13). Our studies evaluate the in vivo clonal sizes and the cytokine lineage of H11.1 peptide-specific CD4 and CD8 cells induced by vaccination with this peptide using CpG, non-CpG (nCpG), CFA, and IFA as adjuvants, using a single-cell resolution cytokine ELISPOT approach. We further evaluate the clinical effects of such immunizations on the otherwise lethal injection of RMA cells into recipient mice (3).

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3 Abbreviations used in this paper: ISS, immunostimulatory; MuLV, murine leukemia virus; RAG, recombination-activating gene; KO, knockout; ODN, oligodeoxynucleotide; DC, dendritic cell; nCpG, non-CpG; WT, wild type.
Materials and Methods

Mouse and RMA tumor

C57BL/6, IFN-γ−/−, IL-2−/−, IL-4−/−, IL-5−/−, IL-6−/−, and recombination-activating gene (RAG)-1−/− mice, all on the C57BL/6 background, were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained at the animal facility of Case Western Reserve University (Cleveland, OH) under specific pathogen-free conditions. Female mice were used at 6–10 wk of age. The tumor cell line RMA is a mutant derivative of RBL-5, a Rauscher murine leukemia virus (MuLV)-induced T lymphoma cell line of C57BL/6 origin (14). For RMA tumor challenge 1000 RMA cells were injected in a volume of 500 μl PBS i.v. into one mouse, a dose that has been established to invariably cause 100% lethality (12 days after injection (13)).

ODN and peptides

The ODN were purchased from Oligos Etc. (Wilsonville, OR). The sequences of ODN that were phosphorothioate modified (SODN) are as follows: CpG ODN 1826, TCCATGACCTTCCTGAGTT; nCpG ODN 1745 (control), TCCATGACCTTCCTGAGTCT, as they have been previously defined (11). ODN were dissolved in sterile PBS, aliquoted, and then stored at −20°C until used. The peptide H11.1 (SLT-RFCNTAWN) is a defined determinant of the envelope protein of Rauscher MuLV that originally caused the transformation of the RMA T cell lymphoma (13). As a control peptide we used OVA323-339 (KISQAVHAA-HAEINNEAG), which is also an I-Ab restricted determinant (15). Both were prepared by mixing heat-inactivated Mycobacterium tuberculosis H37RA (Difco, Detroit, MI) at 5 mg/ml into IFA. The CpG and nCpG adjuvant were prepared by adding 25 μl of the CpG or nCpG ODN as specified above at 12 μg/ml each to the IFA/peptide solution. One milliliter of each adjuvant suspension was emulsified with 500 μl of either peptide (at 2 mM) and 500 μl of sterile PBS; 200 μl of this emulsion was injected i.p. into female 6- to 10-wk-old C57BL/6 mice (thus, the dose of ODN and H11.1 peptide was 100 g/kg per mouse, respectively). Three weeks after the immunization the mice were either sacrificed for ELISPOT analysis or challenged with 1000 RMA tumor cells, i.v. injected in 500 μl PBS per animal. The survival of these mice was monitored daily.

Immunization and tumor challenge

IFA was purchased from Life Technologies (Grand Island, NY). CFA was prepared by mixing heat-inactivated Mycobacterium tuberculosis H37RA (Difco, Detroit, MI) at 5 mg/ml into IFA. The CpG and nCpG adjuvant were prepared by adding 25 μl of the CpG or nCpG ODN as specified above at 12 μg/ml each to the IFA/peptide solution. One milliliter of each adjuvant suspension was emulsified with 500 μl of either peptide (at 2 mM) and 500 μl of sterile PBS; 200 μl of this emulsion was injected i.p. into female 6- to 10-wk-old C57BL/6 mice (thus, the dose of ODN and H11.1 peptide was 100 g/kg per mouse, respectively). Three weeks after the immunization the mice were either sacrificed for ELISPOT analysis or challenged with 1000 RMA tumor cells, i.v. injected in 500 μl PBS per animal. The survival of these mice was monitored daily.

Cytotoxicity assay

The assay was performed as previously described (16). Briefly, spleens were removed 12 days after PBS or tumor injection and single-cell suspensions were prepared. Cells from four animals per group were pooled. Spleen cells (1 × 10^7) were coincubated with 5 × 10^3 irradiated (10,000 rad) RMA cells or RMA cells together with H11.1, or OVA peptide at a concentration of 2 mM, aliquoted in a volume of 500 μl, and stored at −20°C.

Immunization and tumor challenge

Erythrocytes were depleted from spleen cells by Ficoll (Sigma-Aldrich, St. Louis, MO) density gradient separation. CD4+ cells and CD8+ cells were obtained by negative selection, passing erythrocyte-depleted spleen cells through murine CD4+ or CD8+ T cell Enrichment Columns (R&D Systems, Minneapolis, MN). The efficacy of enrichment was controlled by FACS analysis staining with labeled anti-CD4, anti-CD8, and anti-CD3 Abs (all from BD Pharmingen, San Diego, CA). More than 95% enrichment for the desired phenotype was obtained. All cell fractions were plated at 2 × 10^5 irradiated C57BL/6 APCs and the appropriate cytokine knockout (KO) APCs.

Cytokine ELISPOT assays

These assays were performed as previously described (3). Briefly, ImmunoSpot M200 plates (Cellular Technology, Cleveland, OH) were coated overnight at 4°C with the cytokine-specific capture Abs specified below. The plates were washed three times with PBS, then blocked with 1% BSA in PBS for 2 h at room temperature. After washing, freshly isolated splenocytes were plated at 10^5 cells/well in serum-free medium, HL-1 (BioWhitaker, Walkersville, VA), supplemented with l-glutamine and penicillin/streptomycin, in the presence or absence of 10,000-rad irradiated RMA tumor cells, H11.1, or OVA peptide at a final concentration of 70 μg/ml. As a positive control, anti-CD3 (2C11) at 3 μg/ml was used. After 24 h (for IFN-γ, IL-2, IL-6, IL-12, and TNF-αβ) or 48 h (for IL-4 and IL-5) of cell culture in the incubator, the cells were removed by washing three times with three volumes of PBS and four times with PBS containing 0.05% Tween (PBS-T). The supernatants were added and incubated at 4°C overnight. The plates were then washed three times with PBS and subsequently streptavidin-HRP conjugate (DAKO, Carpinteria, CA) was added at 1/2,000 dilution, incubated for 2 h at room temperature, and removed by washing twice with PBS and PBS. The spots were visualized by adding HRP substrate 3-aminio-9-ethylcarbazole (Pierce, Rockford, IL). The plates were then washed with distilled water, air dried, and analyzed the next day with the Series 1 ImmunoSpot Analyzer (Cellular Technology) customized for analyzing ELISPOTs to meet objective criteria for size, chromatic density, shape, and color. We used the following combinations of capture mAbs for the cytokines tested: IFN-γ (R46A2, 4 μg/ml), IL-2 (JES6-1A12, 4 μg/ml), IL-4 (1B11, 4 μg/ml), IL-5 (TRFK5, 2.5 μg/ml), IL-6 (MP5-20F3, 4 μg/ml), IL-12 (10A5, 2.5 μg/ml), and TNF-αβ (G281-2626, 4 μg/ml). For their detection the mAbs IFN-γ (XMGl1.1-biotin, 0.25 μg/ml), IL-2 (JES6-5H4-biotin, 2 μg/ml), IL-4 (BVD6-24G2-biotin, 4 μg/ml), IL-5 (TRFK4-biotin, 4 μg/ml), IL-6 (MP5-32Cl1-biotin, 2 μg/ml), IL-12 (C17.8-biotin, 2 μg/ml), and TNF-αβ (MP6-XT3-biotin, 1 μg/ml) were used in these assays.

Results

In naive C57BL/6 mice, the frequency of RMA- or H11.1-specific memory T lymphocytes capable of producing IFN-γ and IL-2 is <1/10^6.

Taking advantage of the high resolution of ELISPOT analysis (detection limit, 1/10^6), we first addressed the question of the RMA-specific preimmune repertoire. We tested spleen cells of naive C57BL/6 mice for RMA-induced cytokine production (Fig. 1, open bars). The tumor cells triggered the spleen cells to secrete IL-4, IL-6, IL-12, and TNF-αβ, in the absence of detectable IFN-γ and IL-2. The number of spot-forming units was 15/10^5 for IL-4 (medium control, 3/10^5), 197/10^5 for IL-6 (medium control, 92/10^5), 13/10^5 for IL-12 (medium control, 5/10^5), and 159/10^6 for TNF-αβ (medium control, 69/10^5). This IL-4, IL-6, IL-12, and TNF-αβ was produced by cells of the innate immune system; spleen cells of RAG-1 KO mice responded with a similar cytokine secretion profile when exposed to RMA cells (Fig. 1, filled bars). As expected, the frequencies of cells producing these cytokines in the RAG-1 KO mice were higher than that found in the wild type (WT; only ~10% of the spleen cells in a WT mouse are members of the innate immune system). The RMA cells themselves did not produce any of these cytokines (data not shown). The data show, first, that RMA cells activate cells of the innate immune system and, second, that the frequency of RMA- or H11.1-specific T lymphocytes in the preimmune repertoire is <1/10^6.

RMA-injected C57BL/6 mice generate RMA-specific cytokine memory

First, we studied the clonal size and the cytokine signature of RMA-specific T cells under these conditions of apparently uncon-
cells from PBS-injected control mice (Fig. 2) behaved as did the spleen cells of un.injected mice described above (Fig. 1), showing no tumor Ag-triggered IFN-γ and IL-2 production (<1/10^6). Also, vigorous production of IL-4 and IL-6 was seen that occurred over an elevated medium background (Fig. 2, C and D). IL-3 and IL-5 production was not induced over medium background (both <1/10^6, data not shown). Cytokine-producing cells of similar frequencies and cytokine signatures were detected on days 5 and 9 (data not shown). Based on experience gained in allogenic models (16), we tested for cytolytic activity on day 12. Neither RMA- nor H11.1-specific cytotoxicity was detected in standard chromium release assays (Fig. 2).

With the frequency of tumor-induced IL-4- and IL-6-secreting cells being ~10-fold higher than those producing IFN-γ and IL-2, these data obtained by testing bulk spleen cell populations seemed to suggest that the immunity that RMA cells elicit is type 2 biased. Cells from the innate immune system, in addition to T cells, could contribute to this cytokine signature; however, it was critical to define the cell population that actually produces the measured cytokine. Obtaining this information was also critical because only T cells are clonally expandable effector cells with long-term cytokine commitment and are therefore of primary relevance for vaccination purposes.

**RMA-induced T cells produce type 1 cytokines**

We purified CD4 and CD8 cell subsets from spleens of RMA-injected mice and tested these on splenic APCs of congenic naive mice that were gene-disrupted for the cytokine in question. In this setting, all cytokine produced is T cell derived and bystander reactions by APC can be excluded. In response to RMA cells and H11.1 peptide, the CD4 cells from such tumor-experienced mice produced IFN-γ and IL-2 (Fig. 3). This recall response by CD4 cells required the presence of the splenic APCs (data not shown), consistent with indirect pathway recognition of the (MHC class II-negative) RMA tumor. The CD8 cells from the RMA-injected mice also produced IFN-γ and IL-2 in response to the RMA cells (Fig. 3), while CD4 or CD8 cells from PBS-injected mice did not show this cytokine response (<1/10^6, data not shown). Apparently contradicting the measurements done on unseparated spleen cells (Fig. 2), neither the CD4 nor the CD8 cells from the RMA-primed mice produced IL-4 or IL-6 over background when tested on IL-4 or IL-6 KO APC, respectively (data not shown). Because we had no access to TNF-αβ KO APC we could not more closely define the contribution of T cells to this cytokine recall response, which we measured in the bulk spleen cells.

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**FIGURE 2.** Cytokine recall response and cytotoxic activity in RMA-injected mice. C57BL/6 mice were injected with 1000 RMA tumor cells i.v. or with PBS, as indicated. Twelve days later, bulk spleen cells were tested in ELISPOT assays using H11.1 peptide or RMA tumor cells for recall, as specified. A–E, Results for IFN-γ, IL-2, IL-4, IL-6, and TNF-αβ. The results were obtained with pooled cells from four mice per group showing the mean and SD from triplicate wells. The data are from one experiment that is representative of the four experiments performed. F, Lack of specific cytotoxicity in RMA-injected mice. Mice were injected as above and tested on day 12 in a chromium release assay. The inserted legend specifies the treatment of the mice whose effector cells were used and the type of target cells. SD is shown for triplicate wells for each data point; if invisible, SD fell within the size of symbol. The data shown were reproduced in five independent experiments.
24 h, 50 are representative of three independent experiments performed. The presumably Ag-specific induction of IL-4 and IL-6 in bulk spleen cells results from a cytokine-driven bystander reaction

We hypothesized that the lack of IL-4 and IL-6 production by the purified T cells on the respective cytokine KO APC suggests that the Ag-stimulated T cells may trigger an IL-4 and IL-6 bystander response in the splenic APC (17). To test this hypothesis, we purified CD4 or CD8 cells from primed mice and cultured them with and without the H11.1 peptide on naive spleen cells. Twenty-four hours later, the culture supernatants were collected and added to spleen cells of naive mice for 48 h, after which the ELISPOT assays were developed. While no IFN-γ and IL-2 spots were induced, the supernatants of the Ag-stimulated cultures triggered 3- to 5-fold elevated numbers of IL-4, IL-6, and TNF-αβ spots over the medium background (Fig. 4, B-D). The peptide alone did not induce the production of any of these cytokines over medium background in RAG-1 KO or naive WT spleen cells (data not shown; see also Figs. 1 and 2). Therefore, secretory products released by activated T cells induced this Ag-specific IL-4, IL-6, and TNF-αβ bystander reaction in cells of the innate immune system. This bystander reaction might also occur in vivo, as signified by the up to 17-fold elevated IL-4, IL-6, and TNF-αβ medium background of tumor-bearing mice (Fig. 2), and positively or negatively affect immune surveillance of the tumor. However, the T cells that fail to mediate the immune surveillance under these conditions of fulminant tumor growth are essentially of a pure type 1.

**H11.1 peptide immunization with different adjuvants induces cytokine recall responses with distinct frequencies and cytokine profiles**

When H11.1 peptide was injected without adjuvant, in PBS, and the spleen cells of the mice were tested for peptide-induced recall response 21 days later, no significant induction of cytokine production was seen over PBS-injected control mice (Table I). The injection of H11.1 peptide in PBS was apparently not immunogenic. An adjuvant seemed to be required for effective subunit vaccination with this peptide. We selected IFA, because it was found to favor the induction of type 2 immunity (4), CFA, and iSS CpG ODN because the latter two have been implicated in adjuvant-guided type 1 immunity. All three adjuvants had the same mineral oil as the carrier (IFA is the oil alone, CFA contains admixed mycobacteria, and the CpG adjuvant contained the specified ODN emulsified in IFA). To control for the ISS CpG effect, we used as nCpG adjuvant an ODN that lacks the CpG motif (specified in Materials and Methods) and its pronounced stimulatory activity (11). As a specificity control, we also injected OVA peptide 323–339, which, like H11.1, is also restricted by the I-A^k molecule (15) in the four adjuvants. Three weeks after the peptide injection, the mice were sacrificed and their spleen cells were tested for cytokine recall responses in the ELISPOT assays. The data are summarized in Table I. The 2- to 3-fold higher frequencies of CpG-induced T cells vs CFA-induced T cells was also seen on day 12 after immunization (data not shown).

Spleen cells from the CpG:H11.1-injected mice produced a high-frequency IFN-γ recall response to the H11.1 peptide (125/10^6 over a background of 1/10^6). This induction of IFN-γ production was specific because it was not elicited by H11.1 peptide in CpG OVA peptide-immunized mice (Table I, on the right). IL-2, IL-4, IL-5, IL-6, and TNF-αβ were also specifically induced with stimulation indices of 36, 12, 5.8, 2, and 2.6, respectively. The CFA-guided response to H11.1 peptide was of a similar cytokine profile, albeit the specific peptide-induced frequencies of IFN-γ-producing cells were ~50% lower in all three independent experiments performed. Even lower frequencies of IFN-γ-producing cells were engaged following immunization with the nCpG adjuvant (24/10^6). Immunizations with IFN-γ and TNF-αβ-producing cells over background but triggered the production of IL-2, IL-4, IL-5, and IL-6 with stimulation indices of 10, 9.7, 15, and 3.6, respectively.

**H11.1:CpG engages higher clonal sizes of IFN-γ-producing CD4 cells than immunizations with CFA, nCpG, and IFA**

To directly measure the frequency of the specific T cells engaged and their cytokine lineage (3), we isolated CD4 and CD8 cells from the spleens of H11.1:adjuvant-injected mice and tested them on the respective cytokine gene KO APC. While unstimulated CD4 cells did not secrete any of the cytokines tested (<1/10^6 T
cells), they did produce IFN-γ and/or IL-2 upon tumor Ag stimulation (Fig. 5). We found no evidence for the induction of IL-4- and IL-6-producing CD4 cells after H11.1 peptide injection with either of the adjuvants (data not shown). The frequency of IFN-γ-secreting, H11.1 peptide-specific CD4 cells was by far the highest in the CpG-immunized mice, at ~250/10^6. This CD4 cell fraction was also activated in the presence of the RMA tumor with a frequency of ~30/10^6. CD4 cells from nCpG- and CFA-immunized mice each responded with a frequency of ~90/10^6 to the H11.1 peptide and with 10 and 20 to the RMA tumor, respectively. No specific IFN-γ was detected in the IFA-immunized mice, whereas their CD4 cells produced IL-5 in a frequency of 13 SFU/10^6 in response to the peptide (data not shown). CpG also induced the highest frequency of IL-2-secreting, peptide-specific CD4 cells (80/10^6), when compared with the immunizations with nCpG, CFA, and IFA (frequencies of 55/10^6, 35/10^6, and 20/10^6, respectively). The data demonstrate that immunizations with CpG, CFA, and nCpG induced highly type 1-polarized CD4 cell immunity to H11.1 peptide with clonal sizes highest in CpG-injected mice, followed by CFA and nCpG. This hierarchy in magnitudes of frequencies was observed in five independent experiments. The administration of the H11.1 peptide in IFA was also immunogenic, inducing CD4 memory cells that produced IL-2 and IL-5 but not IFN-γ, IL-4, and IL-6.

As seen in Table I, the recall response in the bulk spleen cells from the immunized mice in all groups had a type 2 (IL-4, IL-6) component that was not detected when purified CD4 (-CD8) cells were tested on IL-4 and IL-6 KO APC. Analogous to the data shown for the tumor-primer mice (Fig. 4), we found that the apparently Ag-specific production of IL-4 and IL-6 seen in unseparated spleen cells (Table I) resulted entirely from a bystander reaction by cells of the innate immune system that could be elicited by the supernatants of peptide-activated T cells (data not shown). We also tested on cytokine KO APC the purified CD8 cell fractions of the mice that were immunized with the H11.1 peptide in the four adjuvants and did not detect IFN-γ, IL-2, IL-4, or IL-6 spot formation exceeding 3/10^6 CD8 cells in any of them (data not shown). Therefore, the H11.1 peptide-induced cytokine-producing T cells were, with all four adjuvants, CD4 cells.

**Injection of H11.1 with CpG, but not with the other adjuvants, induces protective immunity**

To assess the efficacy of the induced immune responses, we immunized C57BL/6 mice with H11.1 (or the OVA control) peptide in the four adjuvants, as above, followed 3 wk later by injection of

![FIGURE 5. Type 1 cytokine signature and frequencies of specific CD4 cells induced by immunization with H11.1 in different adjuvants. Shown is the specific cytokine response of purified splenic CD4 cells of mice injected with H11.1 peptide in different adjuvants. C57BL/6 mice were injected i.p. with 100 μg H11.1 peptide, using CFA, IFA, IFA:nCpG ODN, or IFA:CpG-ODN as the adjuvant. Twenty-one days after the mice were sacrificed, the four spleens in each group were pooled, and the CD4 subpopulation was purified by negative selection on an affinity column. The CD4 cells were tested for the H11.1 peptide- or RMA-induced production of IFN-γ (A) and IL-2 (B) using spleen cells from the respective cytokine KO mice as APC. The medium control wells contained less than one spot per 10^6 CD4 cells; SD among triplicate wells was <10%. The data are representative of two experiments performed.]
1000 RMA tumor cells. Control mice injected with the OVA peptide showed the same rate of survival as did the untreated or PBS-injected mice (Fig. 6A). These data demonstrate that the adjuvants themselves had no protective effects. Of the H11.1-injected mice, only immunization with the CpG adjuvant had an impact on survival. Thirty-three percent of the CpG:H11.1-vaccinated mice survived \( > 120 \) days, whereas 100% of the mice in all the other groups died by day 28 (Fig. 6B). Survival was prolonged even in those CpG:H11.1-vaccinated mice that eventually died when compared with controls (33 ± 6 days for CpG H11.1 vs 23 ± 2 days for the control groups, \( p = 0.010 \)).

The protection endowed by H11.1:CpG immunization is IFN-\( \gamma \) dependent

The observation that immunizations with CpG induced protective immunity, while those with CFA (or the other adjuvants) did not, seemed to suggest that the magnitude of the IFN-\( \gamma \) response by CD4 cells might explain the difference in the survival. To test this hypothesis, we immunized IFN-\( \gamma \) KO mice and WT C57BL/6 mice with CpG H11.1 (and nCpG:H11.1 as the control) and challenged them with 1000 RMA tumor cells 3 wk later. The IFN-\( \gamma \) KO mice immunized with CpG:H11.1 died at a comparable rate (23 ± 1 days) to the control mice injected with nCpG:H11.1 or PBS (23 ± 1 and 22 ± 1 days), whereas the WT C57BL/6 mice that were preinjected with CpG:H11.1 reproduced the aforementioned level of protection (33 ± 5 days) of mean survival. The data demonstrate the IFN-\( \gamma \) dependence of the CpG:H11.1-induced protective immunity.

Discussion

Our first set of experiments addressed the question of whether the RMA tumor indeed induces a T cell response even under conditions of obviously uncontrolled tumor growth. To override the “cytokine noise” created by cells of the innate immune system, we purified T cells from tumor-injected mice and tested them on the respective cytokine KO APC. In this way the only possible source of the cytokine measured was the T cell. Low-frequency, but clear-cut IFN-\( \gamma \)-producing, tumor-specific CD4 and CD8 cells were detected in tumor-injected mice (Fig. 3A). These cytokine-producing T cells qualified as in vivo primed memory cells for two reasons. First, they were not detected in naive mice or in mice injected with unrelated tumors. Second, while memory cells can produce these cytokines within 24 h after Ag exposure, it takes naive T cells \( > 3 \) days to differentiate into producing IFN-\( \gamma \)-secreting memory cells (3). The tumor-specific production of IL-2 was also indicative of in vivo clonal expansion/T cell memory, because no tumor-specific, IL-2-producing cells were detected in naive spleen cells or purified T cells. The 24-h duration of the assay precluded in vitro proliferation affecting the frequencies measured. Clearly, the RMA tumor itself induced a specific T cell response, albeit of low frequency.

Our next set of experiments showed that cells of the innate immune system become activated by the RMA tumor as required for eliciting the second signal. When we exposed spleen cells from RAG-1 KO mice (which lack T cells, B cells, and NK.1.1 cells but contain abundant numbers of macrophages, NK cells, and DC) with RMA tumor cells, cytokine production was induced in short-term assays. In particular, IL-4, IL-6, IL-12, and TNF were elicited (Fig. 1). Notably, these cytokines produced by APC are critical for guiding T cell differentiation along the type 1 (IL-12, TNF) and type 2 (IL-4) pathway. Although we did not further narrow down the cell type that produced them, macrophages, NK cells, and DCs have pattern recognition capabilities and can be induced to express these cytokines (18). The RMA tumor originated by transformation with Rauscher MuLV, and it is possible that viral proteins render this tumor stimulatory (infectious non-self). Alternatively, it is also possible that the lowered level of MHC class I molecule expression on the tumor cells is recognized by NK cells (18) or that the altered glycosylation of cell surface molecules (19) is recognized by pattern recognition receptors (20). We favor the notion that the cytokine response induced in the innate immune system is not solely a consequence of the viral origins of the RMA tumor, because other tumors (P815 mastocytoma, L5178Y-R T cell lymphoma, M3 melanoma) that are of nonviral origin also triggered similar responses of the innate immune system and were also immunogenic (M. Tary-Lehmann, unpublished observation).

Immunization with a tumor peptide is a promising approach for the induction of protective antitumor immunity. However, injection of a soluble peptide in saline is generally not immunogenic (1). To the contrary, soluble peptides frequently induce immune tolerance because they provide signal 1 (Ag recognition by the specific T cells) in the absence of signal 2. Consistent with this, we did not find peptide H11.1 to be immunogenic when injected in PBS. H11.1-injected mice did not display significantly elevated frequencies of peptide-specific, cytokine-producing memory cells (Table I) and were not protected when challenged with the RMA tumor (Fig. 6B). In contrast, the injection of peptides mixed with an adjuvant reliably guided the engagement of immunity and of the effector class. We have previously demonstrated that peptide immunizations performed using either CFA (4) or CpG as adjuvants (11) induced type 1 immune responses. This occurred overriding genetic type 1 or type 2 biases of the murine hosts (4), a property that would also be required for a reliable vaccination strategy in the outbred human population. In this report we showed that injection of H11.1 peptide both in CFA and in CpG induced IFN-\( \gamma \)-producing memory cells at frequencies of \( \sim 100/10^6 \) and \( 250/10^6 \) peptide-specific CD8 and CD4 cells, respectively.
CD4 cells, respectively. These clonal sizes were 10–25 times higher after immunization than following tumor injection alone. The cytokine profile of the primed T cell response was purely type 1, in that purified CD4 cells specifically produced IFN-γ and IL-2, but no IL-4 or IL-6, when tested on cytokine KO APC. Therefore, both adjuvants seemed to be equally suited for the type 1 polarization of the H11.1 peptide-specific immune response, but immunization with CpG induced a 2.5-fold higher frequency of H11.1 peptide-specific memory cells than did the injection of the peptide in CFA (Table I).

Unlike the immune response induced by the other adjuvants, injection of H11.1 peptide in CpG induced protective immunity against the RMA tumor. In five repeat experiments, 20–30% of the vaccinated mice survived, and those that succumbed to the tumor showed a prolonged survival rate. This protection was quite striking, because injection of RMA cells led to 100% mortality in all 100 mice that either were untreated or were vaccinated differently.

The superior protective effect of CpG over the other adjuvants may be due to its ability to engage an average 3-fold increase in clonal sizes of H11.1-specific type 1 CD4 cells. It has been well established in autoimmune disease models and graft-vs-host disease that as little as 2-fold differences in the specific T effector cell mass can determine whether or not the animal will develop disease (21), thus providing a precedent for this hypothesis. Accordingly, it is possible that the interindividual variation in clonal sizes of H11.1-specific CD4 cells engaged by CpG immunization accounts for the spectrum of complete protection seen in ~30% of the mice to partial protection observed in others. If this hypothesis is correct, then further modifications of the CpG motif constellation, and/or repeated injections of the adjuvant, may promote an even stronger clonal expansion and result in a fully protective immunization regimen.

The protection afforded by the H11.1 peptide-specific, CpG-induced CD4 cells was IFN-γ dependent, as no protection could be induced in IFN-γ KO mice. IFN-γ is a key effector molecule of type 1 CD4 cells. Its secretion by such T cells at the site of Ag recognition elicits the activation of macrophages and of NK cells at that site; these locally stimulated cells of the innate immune system are thought to be the actual effector cells of CD4-mediated type 1 immunity. This indirect mechanism of T cell-derived IFN-γ action implies that there may be no advantage if the cytokine-producing T cell directly recognizes the tumor vs when the T cell secretes IFN-γ in close vicinity of the tumor, following indirect recognition. Additionally, the local release of IFN-γ has been shown to interfere with tumor growth by inhibiting the vascularization of the tumor (22), which also represents an “indirect pathway” effector mechanism (23). Furthermore, IFN-γ is required for the induction of the “determinant spreading” reaction (24), which also contributes to antitumor immunity. It is conceivable that the higher frequency of H11.1-specific CD4 effector cells induced by CpG immunization provides a stronger trigger for the elicitation of the second wave response to the tumor, providing an additional explanation for why the immunization with CpG was more protective than, for example, H11.1 injection with CFA.

In summary, we showed that RMA tumor activates cells of the innate immune system and induces, on its own, a weak type 1 CD4 and CD8 response that apparently is of insufficient magnitude to control the tumor. Immunization with the class II-restricted tumor peptide H11.1 in CpG, in contrast, induces protective immunity that is CD4 cell mediated and operates via IFN-γ-dependent indirect pathway mechanisms. Therefore, CpG adjuvant-guided induction of type 1 immunity against tumor Ags might be a promising subunit vaccination approach.

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