Immunization with Wild-Type p53 Gene Sequences Coadministered with Flt3 Ligand Induces an Antigen-specific Type 1 T-Cell Response

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INTRODUCTION

The induction of Ag2-specific, cell-mediated immune responses to tumors is a promising approach for adjuvant cancer therapy. The tumor suppressor gene, p53, is mutated and subsequently overexpressed in >50% of human malignancies, making it an attractive target for tumor immunotherapy. Lung, breast, and colon cancers are among the solid tumors that frequently have p53 mutations (3), further limiting mutation-specific binding epitopes, and a patient’s primary tumor and metastasis can express different p53 mutations (4, 5). Thus, wt p53 sequences can potentially be used in immunotherapy by making the entire p53 sequence available as an Ag. This results in wt p53 peptide-specific CTLs and antibodies that can recognize p53-overexpressing tumors (4–7).

The cellular arm of the immune system is controlled in part by type 1 T cells, which secrete IL-2 and IFN-γ and represent the helper and effector arm of the T-cell immune response. Conversely, type 2 T cells mediate humoral immunity and express a cytokine profile, including IL-4 and IL-10, that can inhibit T-cell response. Professional Ag-presenting cells, or DCs, also play a key role in regulating the type and extent of immune response (8, 9). One mechanism of immune impairment in tumor-bearing hosts has been linked to a defect in Ag processing and presentation (10, 11). Thus, one approach toward successful immunotherapy is to identify an antigen to enhance Ag presentation and selectively induce a type 1 immune response. Recent interest has focused on DCs as cellular adjuvants in tumor immunotherapy because of their capacity to process and present Ag to T cells (11–17). It has been shown that DCs can be significantly expanded in vivo by repeated injections of Flt3L (18–20). It has also been suggested that the injection of Flt3L results in an Ag-specific, type 1 cell cytokine response in mice (21). However, it is unclear whether expansion of DCs in vivo could increase an antitumor immune response.

Viral vectors have been used to efficiently deliver wt p53 in vivo (22, 23). However, the presence of neutralizing antibodies precludes boosting using the same viral vector (11, 15, 24, 25). Furthermore, there is an associated potential for nonspecific stimulation of the immune system with viral vectors (11, 24, 26). Immunization with naked DNA (nonviral) is also used to induce a specific immune response against tumor Ags. However, compared with viral vectors, plasmid vectors induce lower Ag levels and result in a decreased T-cell response. Conversely, naked DNA may generate a smaller inflammatory response than live viral vectors (11, 16, 26). Therefore, to overcome these drawbacks, a heterologous prime-boost strategy with Adv-p53 and plasmid-p53 constructs was developed.

We report herein that a priming immunization with Adv-p53 and boosting with a p53 plasmid DNA induced a p53-specific, type 1 T-cell response in vivo, greater than that associated with either vector alone. Furthermore, expansion of DCs using the injection of Flt3L before immunization with p53 gene sequences enhanced the tumor-specific type 1 response resulting in protective immunity to challenge with a metastatic mammary adenocarcinoma with high p53 (murine) expression. This strategy should be useful for the treatment of tumors with mutated p53 regardless of the mutation.

MATERIALS AND METHODS

Mice and Immunization Protocol. Female BALB/cJ (H2d) mice, 6–8 weeks of age, were purchased from Charles River (Wilmington, MA) and allowed to acclimate for 2 weeks before use. Mice were immunized i.m. with wt human Adv-p53 (107 viral particles/mouse) on day 0 and boosted with p53-based immunotherapy. However, the mutations result in increased levels of p53 protein processing and presentation of wild-type p53 epitopes not normally seen with nonmutated protein (4, 5). Thus, wt p53 sequences can potentially be used in immunotherapy by making the entire p53 sequence available as an Ag. This results in wt p53 peptide-specific CTLs and antibodies that can recognize p53-overexpressing tumors (4–7).

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2 The abbreviations used are: Ag, antigen; wt, wild type; IL, interleukin; DC, dendritic cell; Flt3L, Flt3 ligand; Adv, adenovirus; Ab, antibody; cI66, clone 66; DTH, delayed-type hypersensitivity; IVS, in vitro stimulation; ELISPOT, enzyme-linked immunospot; SI, stimulation index; GM-CSF, granulocyte macrophage colony-stimulating factor; NK, natural killer.
human p53 plasmid (100 μg/mouse, i.m.) on days 14 and 28. The human p53 plasmid, with a mutation at codon 175 resulting in an arginine to histidine amino acid substitution, under the control of the cytomegalovirus immediate early gene promoter (27), was provided by Dr. B. Vogelstein of Johns Hopkins Oncology Center, Baltimore, MD. The mutated p53 sequence was used to stabilize the protein and increase the immunogenicity of the protein as an Ag. The Adv-p53 vector, expressing wt human p53 and driven by the cytomegalovirus immediate early gene promoter (28), was kindly provided by Canji, Inc., San Diego, CA. Recombinant human Flt3L, a kind gift from Immunex (Seattle, WA), was administered daily (i.m.) for 10 days at 10 μg/animal (A) 10 days before immunization (day –10 to day 1) and again before the first boost (day 4 to day 13). Our preliminary investigations revealed that this protocol of Flt3L administration was optimal for DC expansion before immunization.

Preparation of Tumor Cells. 4T1 cells, a murine metastatic breast cancer cell line that is p53 null (29), were provided by Dr. Fred Miller, Michigan Cancer Foundation, Detroit, MI, and transfected with human p53 plasmid DNA using LipofectACE reagent (Life Technologies, Inc., Gaithersburg, MD) following the manufacturer’s instructions. Transfected cells were selected in 500 μg/ml G418-sulfate (Geneticin, Life Technologies, Inc., Gaithersburg, MD) containing media and were subsequently continuously maintained in 300 μg/ml G418-sulfate containing media. The expression of p53 in 4T1/p53 transfectant (clone 1) was confirmed by a Western blot analysis using Ab-7 (Oncogene, Cambridge, MA; Fig. 1). The p53-transfected and nontransfected 4T1 cells are henceforth referred to as 4T1/p53 and 4T1 cells, respectively. The challenge study used cl66, which expresses high levels of murine p53 (30, 31). 4T1 and cl66 are separate cell lines from the same parent tumor (32).

Synthesis of p53 Peptides. p53 nonamers, based on Parker’s algorithms (33) which fit into the Class I groove of H2α mice, P15 (MFRNELNAI) and P16 (TFSDLWKLL), were synthesized and purified by Quality Controlled Biochemicals, Inc. (Hopkinton, MA) using 9-fluorenylmethoxycarbonyl solid phase chemistry and purified using a Gilsen automated high-performance liquid chromatography system.

DTH Response. Two weeks after the last immunization, 4 μg of p53 peptides (2 μg each of P15 and P16) in a volume of 30 μl were injected into the pina. Twenty-four h after challenge, the edema of the test and control (PBS) contralateral ears were measured using a Mitutoyo (South Plainfield, NJ) No. 7300-gauge caliper. To determine the edema level, the thickness of the saline-injected control ear was subtracted from the test ear thickness.

IVS of Splenocytes. Splenocytes (3–4 × 10^7 cells/ml) from each immunized or control mouse were separately stimulated for 5 days in six-well plates with 3–4 × 10^5 irradiated normal splenocytes, preincubated for 2 h with p53 nonamer peptides (10 μg/ml) in complete media composed of Click’s media (Life Technologies, Inc.) supplemented with 0.5% normal mouse serum, 20 μg/ml gentamicin, 4 mM L-glutamine, and 5% rat T-Stim without Con A (Collaborative Biomedical Products, Bedford, MA). After a 5-day IVS, the cells were transferred into 96-well plates, with or without Ag, for lymphocyte proliferation assays.

Lymphocyte Proliferation. Splenocytes from the immunized or control mice, before or after IVS, were plated at 0.2 × 10^6 cells/well into 96-well microtiter plates in complete Click’s media. Ten percent-irradiated 4T1 or 4T1/p53 cells were added into the plates as stimulators. The cocultures were incubated in 5% CO₂ at 37°C for 5 days. [3H]thymidine was added, 1 μCi/well 16–18 h before cell harvest. Cultures were harvested onto fiberglass plates with an automatic cell harvester (Micromat 196, Packard, Downers Grove, IL). [3H]thymidine was quantified by scintillation counting. To represent p53 Ag-specific proliferation, data are expressed as a mean SI calculated as follows:

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\text{stimulation index} = \frac{\text{cpm test splenocytes} + 4T1/p53 cells} {\text{cpm test splenocytes} + 4T1 cells} \]

ELISPOT Assay. Splenocytes from each immunized or control mouse were cocultured at 1 × 10^7 cells/well in 24-well culture plates and stimulated by the presence of 10% irradiated normal splenocytes with or without p53 nonapeptides (10 μg/ml each) or 10% irradiated 4T1 or 4T1/p53 as stimulators. The cultures were incubated in complete Click’s media for 48 h. In some experiments, splenocytes were harvested from mice treated with Flt3L (10 μg/day) or HBSS for 10 days, and ELISPOT assay was performed without Ag-stimulation. The ELISPOT assay was performed as reported previously (34, 35) with some modifications. Antibodies used in these studies included the following: anti-IFN-γ (R4–6A2), biotinylated anti-IFN-γ (XM1G12); anti-IL-4, (11B11), biotinylated anti-IL-4 (BVD6–24G2); anti-IL-10 (JES5–2A5), biotinylated anti-IL-10 (SXC–1); and anti-IL-12 (C17.8), all from Pharmingen, San Diego, CA; and goat polyclonal antimouse IL-12 (R&D Systems, Minneapolis, MN) and biotinylated donkey antigoat IgG (Jackson Immunoresearch Labs, West Grove, PA). Briefly, ELISPOT plates (Millititer HA, Bedford, MA) were coated with cytokine capture antibodies (4 μg/ml), washed, and nonspecific sites blocked with media containing 10% PBS. Serial dilutions of splenocytes were plated into duplicate Ab-coated wells starting from 5 × 10^5 cells/well, and each well received irradiated (5000 rads) feeder splenocytes from normal mice equivalent to 10% of the input cell number. The cells were incubated at 37°C in 5% CO₂ for 48 h, and the plates were washed four times with PBS containing 0.05% Tween 20. Cytokine-secreting lymphocytes were visualized by the sequential addition of 100 μl biotinylated detection antibodies (1 μg/ml); in the case of IL-12, goat polyclonal antimouse IL-12, and then biotinylated donkey antigoat IgG; 100 μl of a 1:1000 dilution of alkaline phosphatase-conjugated streptavidin (Extravidin, Sigma Chemical Co., St. Louis, MO); and 50 μl of a substrate mixture containing stabilizing buffer, 5-bromo-4-chloro-3-idolyl-1-phosphate and nitro blue tetrazolium (Western Blue; Promega, Madison, WI). Numbers of spot-forming cells were enumerated in a blinded fashion under 40 magnification and calculated as the difference between numbers of stained cells from test wells and wells containing only irradiated feeder cells.

In Vivo Tumor Growth and Survival Assay. Mice were immunized with Flt3L and p53 gene sequences as described above. One week after the final injection of p53 DNA, mice were challenged (50,000 cells/mouse, s.c.) with the murine breast cancer cell line, cl66, which has a high expression of mutated p53 (29–31). Tumor growth was measured once a week, and survival was scored. For the Winn assay (36), unfractonated splenocytes or isolated CD3⁺CD4⁺ and CD3⁺CD8⁺ cells from immunized or control mice were admixed with 20,000 cl66 tumor cells at a ratio of 100:1. The admixture was then injected into the mammary fat pads of naive syngeneic Balb-C mice and survival monitored.

Statistical Analysis. SPSS for Windows, release 9.00 (SPSS, Inc., Chicago, IL), was used with the one-way independent sample, Student’s t test to compare means and Mann-Whitney U test to compare the survival data. Each study was repeated at least two times with a n of 4. A n of 5 was used in the survival study.

RESULTS

DTH Response in Mice Immunized with p53 and Flt3L. In these studies, the mice were primed with 10⁹ Adv-p53 particles and boosted
on days 14 and 28 with 100 μg of p53 plasmid DNA injected i.m. The Flt3L cohorts received 10 daily injections of Flt3L (10 μg) before immunization. Cohorts of mice immunized with p53 alone and p53 and Flt3L showed a significantly higher DTH response relative to the Flt3L alone or HBSS-treated cohorts (Fig. 2). However, there was no difference in the DTH induced by p53 treatment alone as compared with p53 and Flt3L administration.

**In Vitro Proliferation of Lymphocytes in Response to p53 Ag with or without IVS.** Splenocytes from animals immunized with a combination of Adv-p53 and plasmid p53, with or without Flt3L, were tested for their lymphoproliferative response before or after an IVS with p53 nonapeptides. The splenocytes from mice injected with p53 gene sequences showed significantly higher lymphoproliferation SI [SI = 3.4 ± 0.1] compared with the lymphoproliferative response from mice injected with HBSS (SI = 1.0 ± 0.1; Fig. 3). The proliferative response was augmented further in groups treated with both p53 and Flt3L (SI = 6.7 ± 2.4) relative to the control groups and the p53-alone group or Flt3L-alone group (SI = 3.5 ± 1.1). In other experiments, the splenocytes were stimulated in vitro for 5 days in the presence of p53 peptides before being assayed for lymphoproliferation. The lymphoproliferative response to 4T1 p53 cells after IVS was significantly higher in the p53 and Flt3L-treated group (SI = 29 ± 2.4) compared with mice immunized with p53 alone (SI = 10.5 ± 4.5; Fig. 3).

In other studies, we compared the lymphoproliferative response of splenocytes from mice receiving Adv-p53 (prime) plus plasmid p53 (boost) with those that received either immunostimulation alone. As shown in Fig. 4, splenocytes from mice treated with either Adv-p53 or plasmid p53 with Flt3L had a significantly enhanced lymphoproliferative response (SI > 2.5) against p53 compared with the HBSS- or Flt3L-treated controls (SI < 1.2). The splenocytes from mice treated with a combination of Adv-p53 and plasmid p53, on the other hand, had a significantly enhanced proliferative response (SI > 4.5) compared with the groups treated with either alone (Fig. 4).

**Frequency of Ag-specific IFN-γ or IL-4-secreting Cells as Determined by ELISPOT Assay after Immunization with p53 and Flt3L.** To ascertain the nature (either type 1 or type 2) of the p53 Ag-specific T-cell immune responses in the above immunized mice,
we performed an ELISPOT assay for IFN-γ- and IL-4-secreting cells. As shown in Fig. 5, there was a significantly higher frequency of IFN-γ-secreting cells in the splenocytes of mice immunized with p53 and Flt3L compared with all other groups, including mice immunized with p53 alone. Moreover, the frequency of IFN-γ-secreting cells was enhanced only in groups stimulated with 4T1/p53 cells, as opposed to stimulation with 4T1 cells. This provides a control indicating that this response was p53 Ag-specific. In contrast, there was no significant difference in the frequency of IL-4-secreting cells among p53- and Flt3L-treated or p53-immunized groups of mice (Fig. 5). This result confirmed that the T-cell response observed after p53 and Flt3L immunization was a type 1 response and p53 Ag-specific.

**Effect of Flt3L Administration on the Frequency of IL-12-, IL-10-, IFN-γ-, and IL-4-secreting Cells in Splenocytes.** To examine the mechanism of Flt3L adjuvant activity, the frequency of IL-12-, IL-10-, IFN-γ-, and IL-4-secreting cells was determined by ELISPOT assay 24 h after 10 daily injections of Flt3L (10 μg/day, i.m.). The splenocytes were harvested on day 11 and ELISPOT assays performed in the presence of irradiated splenocytes (feeder cells) without stimulation by any mitogen or specific Ag. As shown in Fig. 6, the frequencies of IL-12- and IFN-γ-secreting cells were significantly higher in mice treated with Flt3L (10-fold and 8-fold increase, respectively) compared with those in HBSS-treated mice. In contrast, the frequencies of IL-10- or IL-4-secreting cells were not changed by administration of Flt3L (Fig. 6).
though immunization was against human p53, demonstrating an immune cross-reaction between human and murine p53.

**Effectors Cells Induced after Vaccination with p53 and Flt3L Using ELISPOT and Winn Assays.** Studies were initiated to examine the effector cell(s) responsible for protection to tumor challenge and the cytokine-producing cells in the ELISPOT assay. A vaccination protocol similar to that used in the tumor-challenge study (Fig. 7) was undertaken (prime with Adv-p53 and two p53 plasmid boosts with Flt3L administration before the prime and first boost). Three weeks after the last boost, CD3⁺CD4⁺ and CD3⁺CD8⁺ cells (CD8⁺) were fractionated using the Miltenyi MiniMacs system and the cell function examined by ELISPOT (without IVS) and Winn assays (36). The CD8⁺ cells were isolated by negative selection to prevent inclusion of CD8⁻ NK cells, and purity was determined by flow cyometric analysis, which was found to be ≥95%. The unfractionated spleen cells from the vaccine alone and the vaccine and Flt3L cohorts had a significant increase in the frequency of IFN-γ-secreting cells compared with both the control (HBSS) and Flt3L-treated cohorts (Fig. 8). The CD4⁺ cells obtained from the vaccine and Flt3L cohort also had a significant increase in IFN-γ-secreting cells, although CD4⁺ cells from the vaccine alone-treated mice did not. In contrast to the unfractionated cells, IFN-γ-secreting CD4⁺ cells from the Flt3L-treated mice were significantly increased, suggesting a nonspecific increase caused by Flt3L administration. Isolated CD8⁺ cells had a significant increase in IFN-γ-secreting cells in both vaccine-treated groups (with or without Flt3L) compared with the control (HBSS) and Flt3L cohorts. In a concomitant Winn assay, results shown as a Kaplan-Meyer plot (Fig. 9), unfractionated spleen cells admixed with c66 cells had no impact on the survival of the injected mice, resulting in median survival times ranging from 49 to 54 days. Similarly, coadministration of c66 cells with CD4⁺ cells from mice injected with Flt3L before p53 vaccination did not significantly prolong survival compared with control animals (median survival time, 57 days). In contrast, the admixture of c66 cells with CD8⁺ cells from mice immunized with p53 and Flt3L cured 34% of mice, with a significant prolongation of survival resulting in a median survival time of 71 days compared with the HBSS control (59 days). There was also a significant increase in survival relative to the mice treated with Flt3L alone, which had a median survival time of 53 days. Both the CD4⁺ and CD8⁺ cells from the Flt3L-alone-treated mice had a trend toward a decreased median survival time relative to the mice injected with spleen cells from HBSS-treated mice. This achieved significance with the CD4⁺ cells from Flt3L-treated mice, which, after injection, resulted in a median survival time of 27 days as compared with 57 days for mice receiving CD4⁺ cells from HBSS-injected mice.

**DISCUSSION**

The strategies of in vivo expansion of DC using Flt3L and immunization with wt human p53 using a sequence of Adv and plasmid DNA were examined in this study. We demonstrated the induction of an Ag-specific type 1 T-cell immune response in vitro as well as protection to tumor challenge with a murine tumor overexpressing p53. We observed a high DTH response to p53 peptides in mice immunized with sequential p53 genetic sequences (Adv-p53 and p53 plasmid DNA) and Flt3L; however, the DTH response did not differ from the group immunized with p53 genetic sequences alone. This suggests that the maximum DTH response might have been induced by p53 genetic priming alone and could not be augmented further by Flt3L. In contrast, we observed significant T cell proliferation in p53-immunized cohorts in response to 4T1/p53 cells. This T-cell
proliferation was significantly augmented in cohorts immunized with p53 and Flt3L compared with the HBSS controls. The specificity of the T-cell proliferative response was confirmed by the demonstration that the response increased almost 4-fold after 5 days of IVS in the presence of p53 peptides. As the proliferative response allowed discrimination between immunization strategies, we compared cohorts of mice receiving either Adv, plasmid, or sequential Adv and plasmid injections. There was a 14-fold increase in splenic DCs, with a 16-fold increase in cells expressing CD11c<sup>high</sup>CD11b<sup>low</sup> (type 1) phenotype, which was significantly greater than the 11-fold increase in the CD11c<sup>high</sup>CD11b<sup>high</sup> (type 2) phenotype. There was also a significant increase in the frequency of IL-12-secreting cells in the spleens of mice treated with Flt3L compared with those treated with HBSS alone. This was accompanied by a higher frequency of IFN-γ-producing type 1 cells in the spleens. However, the frequency of type 2 (IL-10 or IL-4) cytokine-secreting cells remained unaltered by Flt3L administration. This alteration of type 1 cytokine response was observed immediately after Flt3L administration and gradually disappeared over a period of 2 weeks (data not shown). Therefore, the significant augmentation in an Ag-specific, type 1 T-cell response in the present report was likely, at least in part, because of the induction of a “type 1 bias” by administration of Flt3L before p53 priming. Codelivery of type 1-biasing cytokines, IL-12 and IFN-γ, have also been demonstrated to enhance the efficacy of immunization with p53 peptides (45, 46) or peptide-pulsed DC (47).

During these studies, we detected a low CTL response (around 15% at an effectortarget ratio of 25:1) against p53–4T1 cells or P815 cells pulsed with p53 peptides, after IVS of immunized splenocytes for 5 days (data not shown). However, we were not confident that the immunity induced was at a biologically relevant level. Furthermore,
the Ag used was derived from the human p53 gene sequence, and although the sequences are conserved, we questioned whether protection to murine p53 expression could be obtained. We, therefore, investigated whether the induction of a type 1 cytokine response and lymphoproliferation could translate into protection from tumor challenge. In these studies, mice immunized with human p53 genetic sequences, with or without Flt3L, were challenged with murine breast carcinoma cells, c66, expressing mutated p53. The cohort of mice that received p53 and Flt3L survived significantly longer, with 40% tumor-free survival, compared with HBSS, Flt3L, or p53 alone controls. Furthermore, the surviving mice completely rejected tumor cells upon rechallenge with c66, injected on day 120 (data not shown), which suggests that Ag-specific memory was induced. Thus, the poor CTLs observed in vitro may be attributable to an IVS with only p53 nonapeptides designed to fit into the groove of MHC class I molecules, and thus inadequate class II Ag expression. This would result in inadequate CD4+ T cell help, which is necessary for the stimulation of CD8+ CTLs (48). In other studies of tumor challenge, we observed significant survival prolongation, but a lower cure rate. This suggests that additional optimization of the protocol is needed before entering into therapeutic studies.

Preliminary studies into the effector cells responsible for the tumor-protection activity and the responding cells suggested that Ag-specific Tc1 cells were the primary responders in the ELISPOT assay. In addition, Tc1 cells seemed to be responsible for the tumor-protective activity as isolated CD8 cells, with their significant increase in Ag-specific IFN-γ-secreting activity, provided protection to tumor challenge in 34% of mice using a Winn assay. Although Ag-specific CD8+ cells were seen in the unfractonated cells, these cells demonstrated no protective activity in a Winn assay, perhaps because of their lower frequency. Thus, the apparent low survival in mice with the unfractonated cells from mice immunized with p53 in the presence of Flt3L, as compared with the significant increase in “cured” mice after the injection of isolated CD8+ cells from the cohort immunized with p53 with Flt3L, may be attributable to an enrichment in the number of CD8+ cells capable of responding to the tumor. There was also the suggestion of an increased frequency of Ag-specific IL-4-secreting cells in the isolated CD4+ T cell population in response to Flt3L (data not shown), which may contribute to the lack of protection with unfractonated cells. Although, there was also an increase in Tc2 cells, the frequency was much lower than that observed with Th2 cells, potentially allowing Tc1 effector activity. The Winn assay confirms the participation of Tc1 cells in the antitumor response in our study. However, the potential role of additional effector cell types, like NK cells, is not ruled out.

In summary, we report the stimulation of a p53-specific, type 1 immune response in mice after immunization with p53 and Flt3L. The immunization protocol was also effective in preventing the growth of murine tumors and prolonging the survival of hosts upon challenge with a p53-expressing tumor. These results demonstrate that immunization with p53 following in vivo expansion of DCs with Flt3L can be an effective strategy to generate p53-specific, type 1 T-cell responses to tumors. Current studies include a focus on a comparison of the efficacy of different injection routes with Adv-p53 (i.v. versus i.m.). We are also comparing different plasmid vectors for Flt3L DNA and the feasibility of one or two injections of Flt3L-plasmid DNA as an alternative to 10 daily injections of Flt3L for the expansion of DC.

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Immunization with Wild-Type \( p53 \) Gene Sequences Coadministered with Flt3 Ligand Induces an Antigen-specific Type 1 T-Cell Response

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