

**Figure 6.** Vaccination with OK-432 activates high-avidity NY-ESO-1-specific CD4<sup>+</sup> T cells. NY-ESO-1-specific CD4<sup>+</sup> T-cell clones were generated from PBMCs of patients harboring NY-ESO-1-specific CD4<sup>+</sup> T cells. CD4<sup>+</sup> T-cell clones from (A) Pt. #1 and (B) Pt. #2 were stimulated and expanded by 30 ng/mL anti-CD3 Ab in the presence of 20 U/mL IL-2 and irradiated PBMCs and EBV-transformed human B lymphocytes as feeder cells. A couple of weeks later, these NY-ESO-1-specific CD4<sup>+</sup> T cell clones ( $1 \times 10^4$ /well) were cultured with  $1 \times 10^4$  NY-ESO-1 protein-pulsed DCs or  $2 \times 10^4$  EBV-B cells pulsed with graded amounts of peptides and NY-ESO-1-specific IFN- $\gamma$  production was analyzed by ELISPOT. Data are shown as mean  $\pm$  SD of two replicates and are from one experiment representative of at least two experiments performed.

exudate fluids was reduced after OK-432 treatment in accordance with decreased expression of Foxp3 [43]. Considering the fact that IL-12, a main effector molecule induced by OK-432, renders effector cells resistant to Treg-cell suppression, direct administration of OK-432 may change the immunological balance in the local microenvironment from suppression by Treg cells to activation of helper T cells by augmenting helper T-cell activity. However, the sample size of patients analyzed in this study was relatively small and warrants cautious interpretation.

We have previously shown that while naive NY-ESO-1-specific CD4<sup>+</sup> T-cell precursors are present in wide range of healthy individuals and cancer patients, their activation is kept under stringent CD4<sup>+</sup>CD25<sup>+</sup> Treg-cell control [20, 21, 28]. Using OK-432 as an adjuvant, we detected high-affinity NY-ESO-1-specific CD4<sup>+</sup> T cells in effector/memory population after vaccination in two esophageal cancer patients. In Pt #1, we found two responses; spontaneous and vaccine-induced NY-ESO-1-specific CD4<sup>+</sup> T cells. Both of them exhibited a similar efficiency to recognize titrated peptide, indicating that these NY-ESO-1-specific CD4<sup>+</sup> T cells had TCRs with similar affinity and were likely activated from naive high-affinity NY-ESO-1-specific CD4<sup>+</sup> T-cell precursors. Vaccination with minimal peptide in incomplete Freund's adjuvant fails to activate high-affinity NY-ESO-1-specific CD4<sup>+</sup> T-cell precursors, rather it dominantly expands low-avidity effector/memory CD4<sup>+</sup> T cells that cannot recognize naturally pro-

cessed antigens [21]. In addition, following DNA vaccination covering the entire sequence of NY-ESO-1, high-avidity NY-ESO-1-specific CD4<sup>+</sup> T cells were not detected persistently because of rapid suppression by Treg cells [44]. While these data suggest a critical role for the inhibition of Treg-cell suppression by OK-432 in the activation of high-affinity NY-ESO-1-specific CD4<sup>+</sup> T-cell precursors, it is still difficult to obtain conclusive evidence without direct *in vivo* Treg-cell inhibition/depletion. To formally address this issue, clinical trials using Treg-cell depletion reagents and another clinical trial having two arms of patients receiving NY-ESO-1 with/without OK-432 would be required.

Certain types of immunization methods or DC stimulations elicit/augment CD4<sup>+</sup>CD25<sup>+</sup> Treg cells *in vivo* [10–12, 45]. As many tumor-associated antigens recognized by autologous tumor-reactive lymphocytes are antigenically normal self-constituents [1–3], they also could be recognized with CD4<sup>+</sup>CD25<sup>+</sup> Treg cells. Given that a proportion of cancer/testis antigens are targets of Treg cells [46], it is necessary to avoid unwanted activation of cancer/testis antigen-specific CD4<sup>+</sup>CD25<sup>+</sup> Treg cells. Though the sample size of patients analyzed in this study was small and warrants cautious interpretation, including OK-432 in vaccine components as an adjuvant would be a promising strategy to establish favorable circumstances for stimulating effector T cells by inhibiting Treg-cell activation. Furthermore, since this agent has a long history and is widely applied as an anticancer drug, particularly

in Japan, its clinical safety profile has been already established. Our data provide a critical cue for effective cancer vaccines and immunotherapy during antigen priming through modulation of CD4<sup>+</sup>CD25<sup>+</sup> Treg-cell function.

## Materials and methods

### Blood samples

All healthy donors were subjects with no history of autoimmune disease. PBMCs, pleural effusions, or ascites from cancer patients were collected before and after local administration of OK-432 based on the protocol approved by the Human Ethics Committees of Mie University Graduate School of Medicine and Nagasaki University Graduate School of Medicine. PBMCs from esophageal cancer patients enrolled in a clinical trial of CHP-NY-ESO-1 and CHP-HER2 vaccination with OK-432 [47] (Supporting Information Fig. 1) were collected based on the protocol approved by the Human Ethics Committees of Mie University Graduate School of Medicine and Kitano Hospital. The clinical trial was conducted in full conformity with the current version of the Declaration of Helsinki and was registered as NCT00291473 of Clinical Trial.gov, and 000001081 of UMIN Clinical Trial Registry. All samples were collected after written informed consent.

### Abs and reagents

Synthetic peptides of NY-ESO-1<sub>1–20</sub> (MQAEGRTGGSTG-DADGPGG), NY-ESO-1<sub>11–30</sub> (STGDADGPGGPGIPDGGGN), NY-ESO-1<sub>21–40</sub> (PGIPDGGGNAGGPGGAGAT), NY-ESO-1<sub>31–50</sub> (AGGPGGAGATGGRGPRGAGA), NY-ESO-1<sub>41–60</sub> (GGRG-PRGAGAARASGPGGGA), NY-ESO-1<sub>51–70</sub> (ARASGPGGAPRG-PHGGAAS), NY-ESO-1<sub>61–80</sub> (PRGPHGGAASGLNGCCRCGA), NY-ESO-1<sub>71–90</sub> (GLNGCCRCGARGPESRLLEF), NY-ESO-1<sub>81–100</sub> (RGPEPESRLLEFYLPMPFATPM), NY-ESO-1<sub>91–110</sub> (YLAMPFATP-MEAELARRSLA), NY-ESO-1<sub>101–120</sub> (EAELARRSLAQDAPPLPVPV), NY-ESO-1<sub>111–130</sub> (QDAPPLPVPVGLLKEFTVSG), NY-ESO-1<sub>119–143</sub> (PGVLLKEFTVSGNILTIRLTAADHR), NY-ESO-1<sub>131–150</sub> (NILTIRL-TAADHRQLQLSIS), NY-ESO-1<sub>139–160</sub> (AADHRQLQLSISCLQLL-SLLM), NY-ESO-1<sub>151–170</sub> (SCLQLSLLMWITQCFLPVF), NY-ESO-1<sub>161–180</sub> (WITQCFLPVFLAQPPSQRR), and HIV P17<sub>37–51</sub> (ASRELERFAVNPGLL) [48] were obtained from Invitrogen (Carlsbad, CA, USA). Recombinant NY-ESO-1 protein was prepared using similar procedures as described previously [49]. OK-432 was purchased from Chugai Pharmaceutical (Tokyo, Japan). LPS (*Escherichia coli* 055:B5) was obtained from Sigma (St. Louis, MO, USA). Purified and FITC-conjugated anti-IL-12 (C8.6; mouse IgG1), purified anti-IL-6 (MQ2–13A5; rat IgG1), purified anti-IFN- $\gamma$  (NIB42; mouse IgG1), purified anti-IL-23 (HNU2319; mouse IgG1), PE-conjugated anti-CD20 (2H7; mouse IgG2b) and PE-conjugated anti-CD56 (MEM188; mouse IgG2a) Abs were purchased from eBioscience (San Diego, CA, USA). Purified anti-IL-1 $\beta$  Ab (8516; mouse IgG1) was purchased from

R&D Systems (Minneapolis, MN, USA). PE-conjugated anti-CD14 (M $\phi$ P9; mouse IgG2b), PE-conjugated anti-CD45RA (HI100; mouse IgG2b), PerCP-conjugated anti-CD4 (RPA-T4; mouse IgG1), and FITC-conjugated anti-CD4 (RPA-T4; mouse IgG1), Foxp3 (259D; mouse IgG1), and CD45RO (UCHL1; mouse IgG2a) Abs were purchased from BD Biosciences (Franklin Lakes, NJ, USA). PerCP-Cy5.5-conjugated anti-CD11c Ab (3.9; mouse IgG1) was obtained from Biolegend (San Diego CA, USA). PE-conjugated anti-CD25 Ab (4E3; mouse IgG2b) was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Recombinant IL-6, IL-12, and TNF- $\alpha$  were purchased from PeproTech (Rocky Hill, NJ, USA).

### Intracellular cytokine staining

PBMCs were cultured with/without OK-432 and GolgiStop reagent (BD Biosciences) for 20 h. Cells were stained for cell surface markers and then for intracellular cytokine (IL-12) after permeabilization. Results were analyzed by flow cytometry (FACSCanto; BD Biosciences).

### Generation of NY-ESO-1-specific CD4<sup>+</sup> T cells

NY-ESO-1-specific CD4<sup>+</sup> T cells were elicited as described previously [20]. Briefly, CD4<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>-</sup> T cells were isolated from PBMCs using a CD4<sup>+</sup>CD25<sup>+</sup> Treg Isolation Kit (Miltenyi Biotec). CD4<sup>+</sup>CD25<sup>-</sup> T cells were further separated into CD45RO<sup>+</sup> T cells or CD45RA<sup>+</sup> T cells by FACSaria (BD Bioscience) after staining with anti-CD45RO and CD45RA Abs. CD4<sup>-</sup> PBMCs pulsed with 10  $\mu$ M of peptide overnight were used as APCs. After irradiation, 5  $\times$  10<sup>5</sup> APCs were added to round-bottom 96-well plates (Nunc, Roskilde, Denmark) containing 1–5  $\times$  10<sup>5</sup> unfractionated CD4<sup>+</sup> or CD4<sup>+</sup>CD25<sup>-</sup>CD45RO<sup>+</sup> T cells and were fed with 10 U/ml IL-2 (Kindly provided by Takeda Pharmaceutical, Osaka, Japan) and 20 ng/mL IL-7 (R&D Systems). Subsequently, one-half of medium was replaced by fresh medium containing IL-2 (20 U/ml) and IL-7 (40 ng/mL) twice per week.

### Generation of NY-ESO-1-specific CD4<sup>+</sup> T-cell clones

Cloning was performed by limited dilution as described previously [50]. Briefly, NY-ESO-1-specific CD4<sup>+</sup> T cell lines (0.3 cells/well) were stimulated and expanded in the presence of irradiated 5  $\times$  10<sup>4</sup> cells/well PBMCs and 1  $\times$  10<sup>4</sup> cells/well irradiated EBV-transformed human B lymphocytes with 10% AB serum, 20 U/ml IL-2, and 30 ng/mL anti-CD3 Ab (OKT3; eBioscience) in 96-well round-bottom plates.

### Proliferation assay

CD4<sup>+</sup>CD25<sup>-</sup> T cells were cultured with 1  $\times$  10<sup>5</sup> irradiated CD4-depleted PBMCs and stimulated with 0.5  $\mu$ g/mL anti-CD3 Ab

(OKT3, eBioscience) in round-bottom 96-well plates. CD4<sup>+</sup>CD25<sup>high</sup> Treg cells (highest 3% of CD4<sup>+</sup>CD25<sup>+</sup> cells) were purified with FACSAria (BD Biosciences), and graded numbers of them added in the culture as indicated in figure legends. Proliferation was evaluated by <sup>3</sup>H-thymidine with 1 μCi/well for the last 18 h of 6-day culture. <sup>3</sup>H-thymidine incorporation was measured by a scintillation counter.

### ELISPOT (enzyme-linked immunospot) assay

The number of IFN-γ secreting antigen-specific CD4<sup>+</sup> T cells was assessed by ELISPOT assays as described [20, 21]. Briefly, flat-bottomed, 96-well nitrocellulose-coated microtiter plates (Millipore, Bedford, MA, USA) were coated with anti-IFN-γ Ab (1-D1K; MABTECH, Stockholm, Sweden). The presensitized T cells and phytohaemagglutinin (PHA HA15; Murex Diagnostics, Dartford, UK) activated CD4<sup>+</sup> T cells, EBV-transformed human B lymphocytes or DCs pulsed with 10 μM of peptides or 25 μg/mL protein overnight were added to each well and incubated for 24 h. Spots were developed using biotinylated anti-IFN-γ Ab (7-B6-1-biotin; MABTECH), alkaline phosphatase conjugated streptavidin (Roche, Mannheim, Germany) and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma) and counted with C.T.L. Immunospot analyzer and software (Cellular Technologies, Cleveland, OH, USA).

### Preparation of monocyte-derived DCs

Monocyte-derived DCs were generated from PBMCs as previously described with some modifications [51]. Briefly, CD14<sup>+</sup> monocytes were enriched by positive selection using CD14 Microbeads (Miltenyi Biotec). Monocytes were cultured in the presence of 20 ng/mL GM-CSF (Immunex, Seattle, WA, USA) and 20 ng/mL IL-4 (R&D systems) in RPMI1640 supplemented with 2.5% fetal calf serum. Medium was replaced by fresh medium containing cytokines 3 days later. On day 6, cells were harvested and used for subsequent experiments.

### ELISA

The concentration of IL-12p70 and IL-10 was measured by ELISA Kit (eBioscience) according to the instruction provided by the manufacturer.

### Statistical analysis

Statistical significance was evaluated by Student's *t*-test; *p* values less than 0.05 are considered significant.



**Acknowledgments:** This article is dedicated to the memory of Lloyd J. Old, M.D. We thank Drs. T. Takahashi and J. B. Wing for critical reading of the manuscript, and L. Wang, C. Brooks, E. Krapavinsky, E. Ritter, and D. Santiago for technical support.

This study was supported by Grant-in-Aid for Scientific Research on Priority Areas (No. 17016031, H. Shiku, and No. 20015019, H. Nishikawa) and Grants-in-Aid for Scientific Research (B) (No. 23300354, H. Nishikawa), the Cancer Research Institute Investigator Award (H. Nishikawa) and Cancer Vaccine Collaborative Grant for Immunological Monitoring (S. Gnjjatic, G. Ritter and L.J. Old), Cancer Research Grant from Foundation of Cancer Research Promotion (H. Nishikawa), Takeda Science Foundation (H. Nishikawa), Kato Memorial Bioscience Foundation (H. Nishikawa), the Sagawa Foundation for Promotion of Cancer Research (H. Nishikawa), and Senri Life Science Foundation (H. Nishikawa). MH is a research fellow of the Japan Society for the Promotion of Science.

**Conflicts of interest:** The authors declare no financial or commercial conflict of interest.

### References

- 1 Kawakami, Y. and Rosenberg, S. A., Human tumor antigens recognized by T-cells. *Immunol. Res.* 1997. 16: 313–339.
- 2 Scanlan, M. J., Gure, A. O., Jungbluth, A. A., Old, L. J. and Chen, Y.-T., Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. *Immunol. Rev.* 2002. 188: 22–32.
- 3 Boon, T., Coulie, P. G., Van den Eynde, B. J. and van der Bruggen, P., Human T cell responses against melanoma. *Annu. Rev. Immunol.* 2006. 24: 175–208.
- 4 Shevach, E. M., CD4<sup>+</sup> CD25<sup>+</sup> suppressor T cells: more questions than answers. *Nat. Rev. Immunol.* 2002. 2: 389–400.
- 5 Sakaguchi, S., Naturally arising CD4<sup>+</sup> regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu. Rev. Immunol.* 2004. 22: 531–562.
- 6 Nishikawa, H. and Sakaguchi, S., Regulatory T cells in tumor immunity. *Int. J. Cancer* 2010. 127: 759–767.
- 7 Sakaguchi, S., Miyara, M., Costantino, C. M. and Hafler, D. A., FOXP3<sup>+</sup> regulatory T cells in the human immune system. *Nat. Rev. Immunol.* 2010. 10: 490–500.
- 8 Onizuka, S., Tawara, I., Shimizu, J., Sakaguchi, S., Fujita, T. and Nakayama, E., Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor α) monoclonal antibody. *Cancer Res.* 1999. 59: 3128–3133.
- 9 Shimizu, J., Yamazaki, S. and Sakaguchi, S., Induction of tumor immunity by removing CD25<sup>+</sup>CD4<sup>+</sup> T cells: a common basis between tumor immunity and autoimmunity. *J. Immunol.* 1999. 163: 5211–5218.
- 10 Nishikawa, H., Kato, T., Tanida, K., Hiasa, A., Tawara, I., Ikeda, H., Ikarashi, Y. et al., CD4<sup>+</sup> CD25<sup>+</sup> T cells responding to serologically defined

- autoantigens suppress antitumor immune responses. *Proc. Natl. Acad. Sci. USA*. 2003. 100: 10902–10906.
- 11 Nishikawa, H., Kato, T., Tawara, I., Saito, K., Ikeda, H., Kuribayashi, K., Allen, P. M. et al., Definition of target antigens for naturally occurring CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells. *J. Exp. Med.* 2005. 201: 681–686.
  - 12 Nishikawa, H., Kato, T., Tawara, I., Takemitsu, T., Saito, K., Wang, L., Ikarashi, Y. et al., Accelerated chemically induced tumor development mediated by CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells in wild-type hosts. *Proc. Natl. Acad. Sci. USA*. 2005. 102: 9253–9257.
  - 13 Curiel, T. J., Coukos, G., Zou, L., Alvarez, X., Cheng, P., Mottram, P., Evdemon-Hogan, M. et al., Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat. Med.* 2004. 10: 942–949.
  - 14 Sato, E., Olson, S. H., Ahn, J., Bundy, B., Nishikawa, H., Qian, F., Jungbluth, A. A. et al., Intraepithelial CD8<sup>+</sup> tumor-infiltrating lymphocytes and a high CD8<sup>+</sup>/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc. Natl. Acad. Sci. USA*. 2005. 102: 18538–18543.
  - 15 Dannull, J., Su, Z., Rizzieri, D., Yang, B. K., Coleman, D., Yancey, D., Zhang, A. et al., Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. *J. Clin. Invest.* 2005. 115: 3623–3633.
  - 16 Chen, Y.-T., Scanlan, M. J., Sahin, U., Tureci, O., Gure, A. O., Tsang, S., Williamson, B. et al., A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc. Natl. Acad. Sci. U S A*. 1997. 94: 1914–1918.
  - 17 Gnjatich, S., Nishikawa, H., Jungbluth, A. A., Gure, A. O., Ritter, G., Jager, E., Knuth, A. et al., NY-ESO-1: review of an immunogenic tumor antigen. *Adv. Cancer Res.* 2006. 95: 1–30.
  - 18 Gnjatich, S., Nagata, Y., Jager, E., Stockert, E., Shankara, S., Roberts, B. L., Mazzara, G. P. et al., Strategy for monitoring T cell responses to NY-ESO-1 in patients with any HLA class I allele. *Proc. Natl. Acad. Sci. U S A*. 2000. 97: 10917–10922.
  - 19 Danke, N. A., Koelle, D. M., Yee, C., Beheray, S. and Kwok, W. W., Autoreactive T cells in healthy individuals. *J. Immunol.* 2004. 172: 5967–5972.
  - 20 Nishikawa, H., Jager, E., Ritter, G., Old, L. J. and Gnjatich, S., CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells control the induction of antigen-specific CD4<sup>+</sup> helper T cell responses in cancer patients. *Blood* 2005. 106: 1008–1011.
  - 21 Nishikawa, H., Qian, F., Tsuji, T., Ritter, G., Old, L. J., Gnjatich, S. and Odunsi, K., Influence of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells on low/high-avidity CD4<sup>+</sup> T cells following peptide vaccination. *J. Immunol.* 2006. 176: 6340–6346.
  - 22 Iwasaki, A. and Medzhitov, R., Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* 2004. 5: 987–995.
  - 23 Pasare, C. and Medzhitov, R., Toll pathway-dependent blockade of CD4<sup>+</sup>CD25<sup>+</sup> T cell-mediated suppression by dendritic cells. *Science* 2003. 299: 1033–1036.
  - 24 Suttmuller, R. P., den Brok, M. H., Kramer, M., Bennink, E. J., Toonen, L. W., Kullberg, B. J., Joosten, L. A. et al., Toll-like receptor 2 controls expansion and function of regulatory T cells. *J. Clin. Invest.* 2006. 116: 485–494.
  - 25 Liu, H. Y., Komai-Koma, M., Xu, D. and Liew, F. Y., Toll-like receptor 2 signaling modulates the functions of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. *Proc. Natl. Acad. Sci. USA*. 2006. 103: 7048–7053.
  - 26 Yang, Y., Huang, C. T., Huang, X. and Pardoll, D. M., Persistent Toll-like receptor signals are required for reversal of regulatory T cell-mediated CD8 tolerance. *Nat. Immunol.* 2004. 5: 508–515.
  - 27 Nishikawa, H., Sato, E., Briones, G., Chen, L. M., Matsuo, M., Nagata, Y., Ritter, G. et al., In vivo antigen delivery by a *Salmonella typhimurium* type III secretion system for therapeutic cancer vaccines. *J. Clin. Invest.* 2006. 116: 1946–1954.
  - 28 Nishikawa, H., Tsuji, T., Jager, E., Briones, G., Ritter, G., Old, L. J., Galan, J. E. et al., Induction of regulatory T cell-resistant helper CD4<sup>+</sup> T cells by bacterial vector. *Blood* 2008. 111: 1404–1412.
  - 29 Nakahara, S., Tsunoda, T., Baba, T., Asabe, S. and Tahara, H., Dendritic cells stimulated with a bacterial product, OK-432, efficiently induce cytotoxic T lymphocytes specific to tumor rejection peptide. *Cancer Res.* 2003. 63: 4112–4118.
  - 30 Okamoto, M., Oshikawa, T., Tano, T., Ohe, G., Furuichi, S., Nishikawa, H., Ahmed, S. U. et al., Involvement of toll-like receptor 4 signaling in interferon- $\gamma$  production and antitumor effect by streptococcal agent OK-432. *J. Natl. Cancer Inst.* 2003. 95: 316–326.
  - 31 Okamoto, M., Oshikawa, T., Tano, T., Ahmed, S. U., Kan, S., Sasai, A., Akashi, S. et al., Mechanism of anticancer host response induced by OK-432, a streptococcal preparation, mediated by phagocytosis and Toll-like receptor 4 signaling. *J. Immunother.* 2006. 29: 78–86.
  - 32 Ono, T., Harada, M., Yamada, A., Tanaka, M., Takao, Y., Tanaka, Y., Mine, T. et al., Antitumor effects of systemic and local immunization with a CTL-directed peptide in combination with a local injection of OK-432. *Clin. Cancer Res.* 2006. 12: 1325–1332.
  - 33 Oshikawa, T., Okamoto, M., Tano, T., Sasai, A., Kan, S., Moriya, Y., Ryoma, Y. et al., Antitumor effect of OK-432-derived DNA: one of the active constituents of OK-432, a streptococcal immunotherapeutic agent. *J. Immunother.* 2006. 29: 143–150.
  - 34 Okamoto, M., Furuichi, S., Nishioka, Y., Oshikawa, T., Tano, T., Ahmed, S. U., Takeda, K. et al., Expression of toll-like receptor 4 on dendritic cells is significant for anticancer effect of dendritic cell-based immunotherapy in combination with an active component of OK-432, a streptococcal preparation. *Cancer Res.* 2004. 64: 5461–5470.
  - 35 Rosenberg, S. A., Yang, J. C. and Restifo, N. P., Cancer immunotherapy: moving beyond current vaccines. *Nat. Med.* 2004. 10: 909–915.
  - 36 Dougan, M. and Dranoff, G., Immune therapy for cancer. *Annu. Rev. Immunol.* 2009. 27: 83–117.
  - 37 Napolitani, G., Rinaldi, A., Bertoni, F., Sallusto, F. and Lanzavecchia, A., Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat. Immunol.* 2005. 6: 769–776.
  - 38 Warger, T., Osterloh, P., Rechtsteiner, G., Fassbender, M., Heib, V., Schmid, B., Schmitt, E. et al., Synergistic activation of dendritic cells by combined toll-like receptor ligation induces superior CTL responses in vivo. *Blood* 2006. 108: 544–550.
  - 39 King, I. L. and Segal, B. M., Cutting edge: IL-12 induces CD4<sup>+</sup>CD25<sup>-</sup> T cell activation in the presence of T regulatory cells. *J. Immunol.* 2005. 175: 641–645.
  - 40 Zhao, J. X., Zhao, J. C. and Perlman, S., Differential effects of IL-12 on Tregs and non-Treg T cells: roles of IFN- $\gamma$ , IL-2 and IL-2R. *Plos One* 2012. 7: e46241.
  - 41 Setoguchi, R., Hori, S., Takahashi, T. and Sakaguchi, S., Homeostatic maintenance of natural Foxp3<sup>+</sup> CD25<sup>+</sup> CD4<sup>+</sup> regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J. Exp. Med.* 2005. 201: 723–735.
  - 42 Oldenhove, G., Bouladoux, N., Wohlfert, E. A., Hall, J. A., Chou, D., Dos Santos, L., O'Brien, S. et al., Decrease of Foxp3<sup>+</sup> Treg cell number and

- acquisition of effector cell phenotype during lethal infection. *Immunity* 2009. 31: 772–786.
- 43 Wan, Y. S. Y. and Flavell, R. A., Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature* 2007. 445: 766–770.
- 44 Gnjatic, S., Altorki, N. K., Tang, D. N., Tu, S. M., Kundra, V., Ritter, G., Old, L. J. et al., NY-ESO-1 DNA vaccine induces T-cell responses that are suppressed by regulatory T cells. *Clin. Cancer Res.* 2009. 15: 2130–2139.
- 45 Zhou, G., Drake, C. G. and Levitsky, H. I., Amplification of tumor-specific regulatory T cells following therapeutic cancer vaccines. *Blood* 2006. 107: 628–636.
- 46 Bonertz, A., Weitz, J., Pietsch, D. H. K., Rahbari, N. N., Schlude, C., Ge, Y. Z., Juenger, S. et al., Antigen-specific Tregs control T cell responses against a limited repertoire of tumor antigens in patients with colorectal carcinoma. *J. Clin. Invest.* 2009. 119: 3311–3321.
- 47 Aoki, M., Ueda, S., Nishikawa, H., Kitano, S., Hirayama, M., Ikeda, H., Toyoda, H. et al., Antibody responses against NY-ESO-1 and HER2 antigens in patients vaccinated with combinations of cholesteryl pullulan (CHP)-NY-ESO-1 and CHP-HER2 with OK-432. *Vaccine* 2009. 27: 6854–6861.
- 48 Gnjatic, S., Atanackovic, D., Jager, E., Matsuo, M., Selvakumar, A., Altorki, N. K., Maki, R. G. et al., Survey of naturally occurring CD4<sup>+</sup> T cell responses against NY-ESO-1 in cancer patients: correlation with antibody responses. *Proc. Natl. Acad. Sci. U S A.* 2003. 100: 8862–8867.
- 49 Stockert, E., Jager, E., Chen, Y.-T., Scanlan, M. J., Gout, I., Karbach, J., Arand, M. et al., A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. *J. Exp. Med.* 1998. 187: 1349–1354.
- 50 Riddell, S. R. and Greenberg, P. D., The use of anti-CD3 and anti-CD28 monoclonal-antibodies to clone and expand human antigen-specific T-cells. *J. Immunol. Methods* 1990. 128: 189–201.
- 51 Nagata, Y., Ono, S., Matsuo, M., Gnjatic, S., Valmori, D., Ritter, G., Garrett, W. et al., Differential presentation of a soluble exogenous tumor antigen, NY-ESO-1, by distinct human dendritic cell populations. *Proc. Natl. Acad. Sci. U S A.* 2002. 99: 10629–10634.

*Abbreviation:* CHP: cholesteryl hydrophobized pullulan

*Full correspondence:* Dr. Hiroyoshi Nishikawa, Experimental Immunology, Immunology Frontier Research Center, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan  
 Fax: +81-6-6879-4464  
 e-mail: nisihiro@ifrec.osaka-u.ac.jp

*Additional correspondence:* Sacha Gnjatic, Ludwig Institute for Cancer Research, New York Branch, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10065, USA.  
 e-mail: gnjatics@mskcc.org

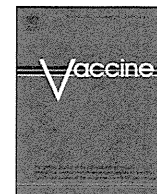
*Additional correspondence:* Hiroshi Shiku, Departments of Cancer Vaccine and Immuno-Gene Therapy, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan.  
 e-mail: shiku@clin.medic.mie-u.ac.jp

Received: 6/7/2012

Revised: 7/12/2012

Accepted: 28/1/2013

Accepted article online: 4/2/2013



## Establishment of animal models to analyze the kinetics and distribution of human tumor antigen-specific CD8<sup>+</sup> T cells

Daisuke Muraoka<sup>a,d</sup>, Hiroyoshi Nishikawa<sup>a,e,\*</sup>, Takuro Noguchi<sup>a,f</sup>, Linan Wang<sup>b</sup>, Naozumi Harada<sup>a,d</sup>, Eiichi Sato<sup>g</sup>, Immanuel Luescher<sup>h</sup>, Eiichi Nakayama<sup>i</sup>, Takuma Kato<sup>c</sup>, Hiroshi Shiku<sup>a,b,1</sup>

<sup>a</sup> Departments of Cancer Vaccine, Mie University Graduate School of Medicine, Mie 514-8507, Japan

<sup>b</sup> Departments of Immuno-Gene Therapy, Mie University Graduate School of Medicine, Mie 514-8507, Japan

<sup>c</sup> Departments of Cellular and Molecular Immunology, Mie University Graduate School of Medicine, Mie 514-8507, Japan

<sup>d</sup> Immunofrontier, Inc., Tokyo, 143-0023, Japan

<sup>e</sup> Experimental Immunology, Immunology Frontier Research Center, Osaka University, Osaka, 565-0871, Japan

<sup>f</sup> Ludwig Institute for Cancer Research, New York Branch, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, United States

<sup>g</sup> Department of Anatomic Pathology, Tokyo Medical University, Tokyo, 160-8402, Japan

<sup>h</sup> Ludwig Institute for Cancer Research, Lausanne Branch, University of Lausanne, Epalinges, 1066, Switzerland

<sup>i</sup> Faculty of Health and Welfare, Kawasaki University of Medical Welfare, Okayama, 701-0193, Japan

### ARTICLE INFO

#### Article history:

Received 21 January 2013

Accepted 27 February 2013

Available online 13 March 2013

#### Keywords:

Translational research

Animal model

Cancer vaccine

Immune responses

CD8<sup>+</sup> T cells

Cancer/testis antigens

### ABSTRACT

Many patients develop tumor antigen-specific T cell responses detectable in peripheral blood mononuclear cells (PBMCs) following cancer vaccine. However, measurable tumor regression is observed in a limited number of patients receiving cancer vaccines. There is a need to re-evaluate systematically the immune responses induced by cancer vaccines. Here, we established animal models targeting two human cancer/testis antigens, NY-ESO-1 and MAGE-A4. Cytotoxic T lymphocyte (CTL) epitopes of these antigens were investigated by immunizing BALB/c mice with plasmids encoding the entire sequences of NY-ESO-1 or MAGE-A4. CD8<sup>+</sup> T cells specific for NY-ESO-1 or MAGE-A4 were able to be detected by ELISPOT assays using antigen presenting cells pulsed with overlapping peptides covering the whole protein, indicating the high immunogenicity of these antigens in mice. Truncation of these peptides revealed that NY-ESO-1-specific CD8<sup>+</sup> T cells recognized D<sup>d</sup>-restricted 8mer peptides, NY-ESO-1<sub>81-88</sub>. MAGE-A4-specific CD8<sup>+</sup> T cells recognized D<sup>d</sup>-restricted 9mer peptides, MAGE-A4<sub>265-273</sub>. MHC/peptide tetramers allowed us to analyze the kinetics and distribution of the antigen-specific immune responses, and we found that stronger antigen-specific CD8<sup>+</sup> T cell responses were required for more effective anti-tumor activity. Taken together, these animal models are valuable for evaluation of immune responses and optimization of the efficacy of cancer vaccines.

© 2013 Elsevier Ltd. All rights reserved.

### 1. Introduction

A number of cancer vaccine strategies targeting tumor antigens recognized by the human immune system have been tested [1–3]. While many of these cancer vaccines elicited measurable

immune responses detectable in peripheral blood mononuclear cells (PBMCs), only a subset of treated patients experienced clinical benefits, such as tumor regression [4,5]. Because of the weak clinical effectiveness of currently available cancer vaccines, not only new immunogenic antigens, effective adjuvant formulations, vectors or vaccination methods but also new methodologies to evaluate efficacy of cancer vaccines are required.

NY-ESO-1, a germ line cell protein detected by SEREX (serological identification of antigens by recombinant expression cloning) using the serum of an esophageal cancer patient, is often expressed by cancer cells, but not by normal somatic cells [3,6]. This ideal expression pattern facilitated the study of this antigen; including immuno-monitoring of cancer patients with NY-ESO-1-expressing tumors and clinical trials that focused on NY-ESO-1 [3]. While these studies have revealed that a number of different cancer vaccines, including short and overlapping peptides, protein, viral vectors and DNA, resulted in development of measurable immune responses,

*Abbreviations:* APC, antigen presenting cells; CTL, cytotoxic T lymphocyte; dLN, draining lymph node; ELISPOT assay, enzyme-linked immunospot assay; IFN, interferon; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells.

\* Corresponding author at: Experimental Immunology, Immunology Frontier Research Center, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 4963; fax: +81 6 6879 4464.

*E-mail addresses:* nisihiro@ifrec.osaka-u.ac.jp (H. Nishikawa), shiku@clin.medic.mie-u.ac.jp (H. Shiku).

<sup>1</sup> Departments of Cancer Vaccine and Immuno-Gene Therapy, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan. Tel.: +81 59 231 5062; fax: +81 59 231 5276.

correlations between immunological and clinical responses were often weak or difficult to observe [3].

MAGE-A4, another cancer/testis (CT) antigen, elicits MAGE-A4-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses in some patients with MAGE-A4-expressing cancers, indicating that MAGE-A4 is also an immunogenic protein [7–9]. We have recently reported a novel MAGE-A4 epitope presented by human leukocyte antigen (HLA)-A\*2402 using a CD8<sup>+</sup> T cell clone 2-28 [9]. As this clone effectively killed tumor cell lines that expressed both MAGE-A4 and HLA-A\*2402, this antigen could be a candidate for a cancer vaccine.

Given the poor correlation between the immune responses detected in PBMCs and clinical responses [2,3,5], it is necessary to re-evaluate existing cancer immunotherapy strategies in detail using animal models, namely reverse translational research. To this end, we developed animal models involving human tumor antigens, such as NY-ESO-1 or MAGE-A4 in this study.

## 2. Materials and methods

### 2.1. Mice

Female BALB/c mice were purchased from SLC Japan (Shizuoka, Japan) and used at 7–10 weeks of age. They were maintained at the Animal Center of Mie University Graduate School of Medicine (Mie, Japan). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Mie University Graduate School of Medicine.

### 2.2. Antibodies and reagents

Anti-H2-K<sup>d</sup> (KD40, mouse IgG2a), anti-H2-D<sup>d</sup> (DD98, mouse IgG2a), and anti-H2-L<sup>d</sup> (30-5-7, mouse IgG2a) were produced and purified from each hybridoma. FITC-conjugated anti-CD8 mAb (53-6.7, rat IgG2a) and APC-conjugated anti-CD4 mAb (GK1.5, rat IgG2b) were purchased from BD Biosciences (Franklin Lakes, NJ). PE-conjugated anti-Foxp3 mAb (Fjk16s, rat IgG2a) was purchased from eBiosciences (San Diego, CA). Synthetic NY-ESO-1 and MAGE-A4 peptides (summarized in Supplementary Table 1) were obtained from Sigma Genosys (Hokkaido, Japan).

### 2.3. Immunization using a gene gun

Naive BALB/c mice were immunized twice at two-week intervals. Gold particles coated with 1 µg of each plasmid DNA were prepared and delivered into the shaved skin of the abdominal wall of BALB/c mice using a Helios Gene Gun System (BioRad, Hercules, CA) at a helium discharge pressure of 350–400 psi, as described previously [10,11].

### 2.4. Cell isolation

Spleen cell suspensions were mixed with CD8 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and separated into CD8<sup>+</sup> T cells by positive selection on a MACS column. The isolated CD8<sup>+</sup> T cell populations were confirmed to contain >95% CD8<sup>+</sup> T cells.

### 2.5. Enzyme-linked immunospot (ELISPOT) assay

The number of IFN-γ secreting antigen-specific CD8<sup>+</sup> T cells was assessed by ELISPOT assay as described previously [10,11]. Briefly, purified CD8<sup>+</sup> T cells were cultured for 24 hours with 5 × 10<sup>5</sup> irradiated CD90-depleted splenocytes pulsed with the indicated peptides in 96-well nitrocellulose-coated microtiter plates (Millipore, Bedford, MA) coated with rat anti-mouse IFN-γ mAb (R4-6A2,

BD Biosciences). Spots were developed using biotinylated anti-mouse IFN-γ mAb (XMG1.2, BD Biosciences), alkaline phosphatase conjugated streptavidin (MABTECH, Sweden) and alkaline phosphatase substrate kit (BioRad), and subsequently counted.

### 2.6. ELISA

96-well flat-bottomed microliter plates (Immuno-NUNC) were coated with 20 ng/50 µml of NY-ESO-1 or MAGE-A4 protein, respectively, at 4 °C overnight. Wells were blocked with 1% BSA/PBS for 1 hour at room temperature and washed three times. Serum (1:100 dilution) was added and incubated at 4 °C overnight. After washing, goat anti-mouse IgG antibody conjugated with horseradish peroxidase (Promega, Madison, WI) was added (1:5000 dilution). Two hours later, color was developed with TMB substrate solution (Thermo scientific, IL) and stopped with H<sub>2</sub>SO<sub>4</sub>. The absorbance was measured at 450 nm and calculated after subtraction of the absorbance value of control wells without sera.

### 2.7. Flow cytometry and tetramer staining

Tetramer staining was performed as described previously [11]. Briefly, cells were stained with PE-labeled NY-ESO-1<sub>81-88</sub>/D<sup>d</sup> or MAGE-A4<sub>265-273</sub>/D<sup>d</sup> tetramers (prepared at the Ludwig Institute Core Facility, Lausanne, Switzerland) for 10 minutes at 37 °C before additional staining of surface markers for 15 minutes at 4 °C. After washing, the results were analyzed on FACSCanto (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR).

### 2.8. Tumors

CT26 is a colon epithelial tumor derived by intrarectal injections of N-nitroso-N-methylurethane in BALB/c mice [12]. CT26 expressing NY-ESO-1 or MAGE-A4, a human cancer/testis antigen were established as described previously [11,13].

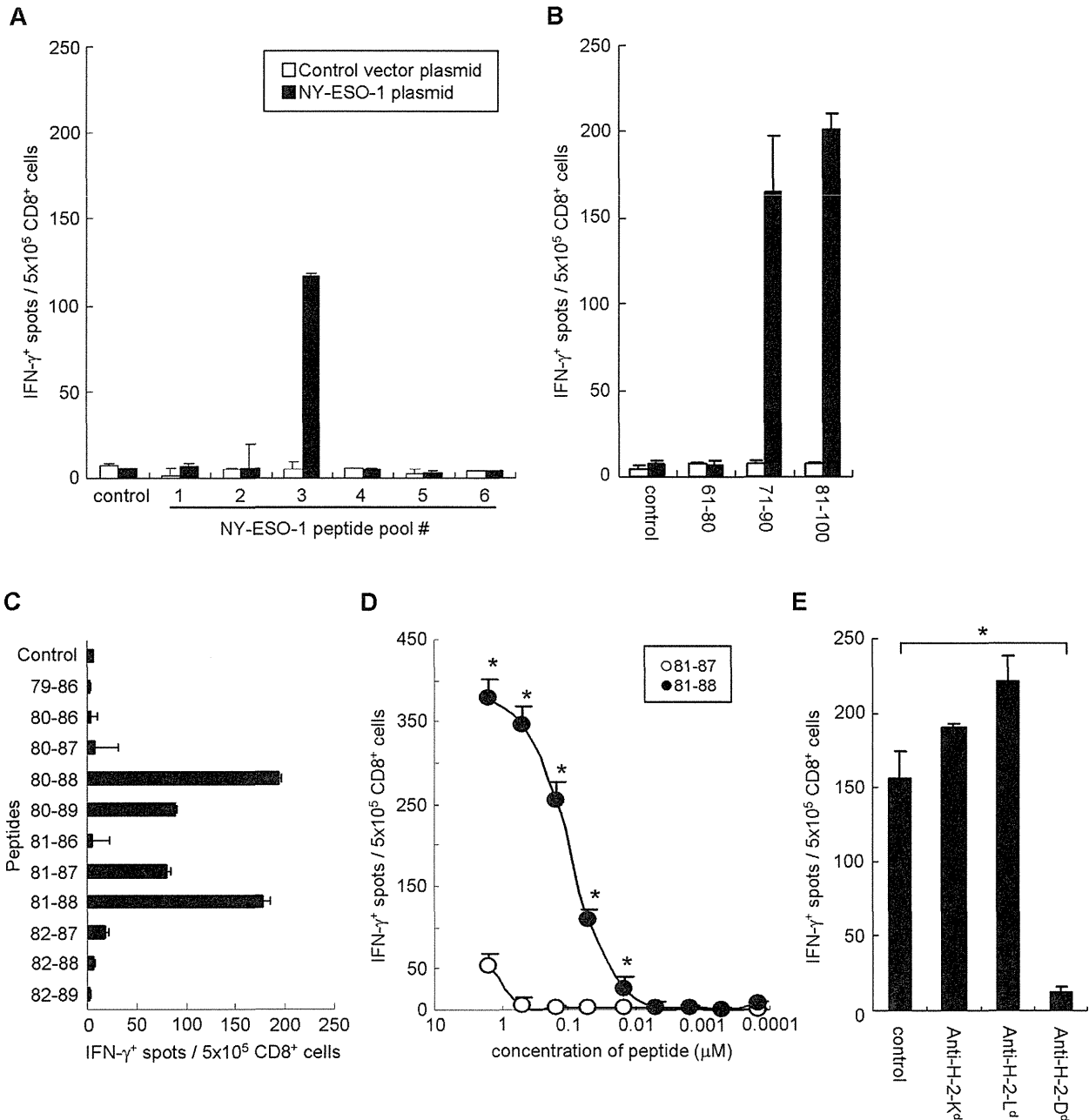
### 2.9. Statistical analysis

Values were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Student's *t*-test. *p* values <0.05 were considered statistically significant.

## 3. Results

### 3.1. NY-ESO-1-specific CD8<sup>+</sup> T cells recognize D<sup>d</sup>-restricted NY-ESO-1<sub>81-88</sub> peptide

We analyzed the minimal epitope recognized by NY-ESO-1-specific CD8<sup>+</sup> T cells after immunization with NY-ESO-1. To this end, we employed a Helios Gene Gun System as we have previously detected NY-ESO-1-specific CD8<sup>+</sup> T cell responses [10,11]. To identify minimal epitopes, naive BALB/c mice were immunized twice at two-week intervals with plasmids encoding the entire sequence of NY-ESO-1. CD8<sup>+</sup> T cells were obtained from spleens and specific T cell responses were analyzed by ELISPOT assay using peptide pools shown in Supplementary Table 1. A significant number of NY-ESO-1-specific CD8<sup>+</sup> T cells was detected against peptide pool #3 (Fig. 1A). To identify the NY-ESO-1 epitope, NY-ESO-1-specific CD8<sup>+</sup> T cells were stimulated with each of these peptides. IFN-γ secretion was observed when NY-ESO-1-specific CD8<sup>+</sup> T cells were stimulated with 71–90 and 81–100 NY-ESO-1 peptides, suggesting the presence of a minimal epitope within 81–90 residues (Fig. 1B). To determine the minimal epitope, the 81–90 peptide of NY-ESO-1 was further truncated. NY-ESO-1-specific CD8<sup>+</sup> T cells recognized the 80–88 and 81–88, but not 80–87 or 82–88 peptides, thus



**Fig. 1.** NY-ESO-1-specific CD8<sup>+</sup> T cells recognize D<sup>d</sup>-restricted NY-ESO-1<sub>81-88</sub> peptide. (A–C) BALB/c mice were immunized by gene gun twice at two-week intervals with plasmids encoding the entire sequence of NY-ESO-1. CD8<sup>+</sup> T cells were obtained from spleens and specific T cells were analyzed with ELISPOT assay using APCs pulsed with the indicated peptides. (D) Avidity of induced NY-ESO-1-specific CD8<sup>+</sup> T cells was analyzed with ELISPOT assay using APCs pulsed with graded doses of peptides ranging from 3 to 0.0001  $\mu$ M. (E) MHC restriction of induced NY-ESO-1-specific CD8<sup>+</sup> T cells was analyzed with ELISPOT assay by the addition of anti-H-2-K<sup>d</sup>, anti-H-2-D<sup>d</sup> or anti-H-2-L<sup>d</sup> mAbs. These experiments were repeated two to four times with similar results. Data are mean  $\pm$  SD.

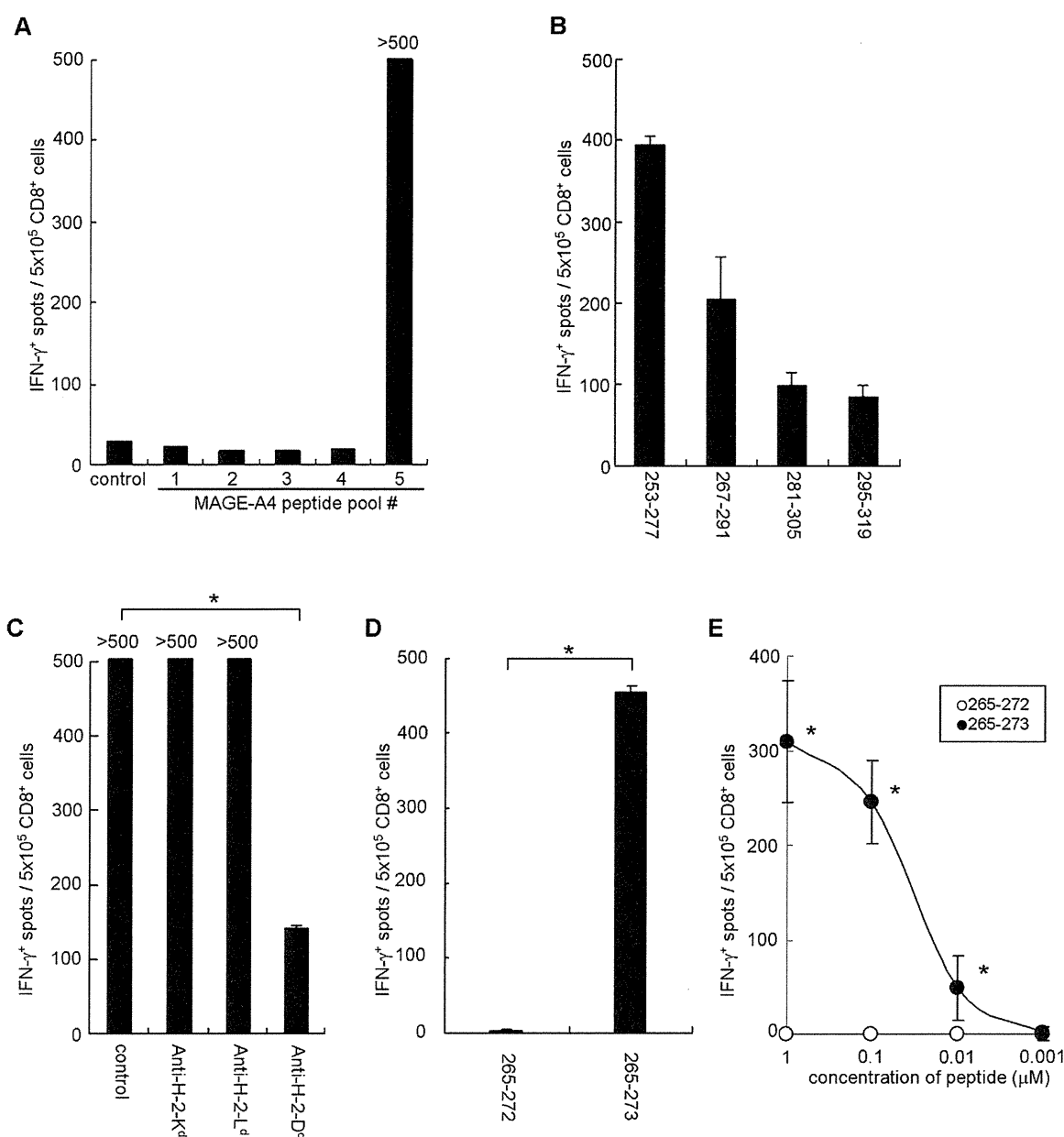
the minimal epitope was identified to be NY-ESO-1<sub>81-88</sub> peptide (Fig. 1C).

To confirm this, graded doses of the peptides were pulsed on antigen presenting cells (APCs) and specific IFN- $\gamma$  secretion was analyzed by ELISPOT assay. NY-ESO-1-specific CD8<sup>+</sup> T cells were high-avidity, and capable to recognize as little as 30 nM of peptide (Fig. 1D), confirming that NY-ESO-1<sub>81-88</sub> peptide is the minimal epitope. Next, we assessed the restriction of this response using blocking antibodies. NY-ESO-1-specific CD8<sup>+</sup> T cell responses were completely blocked by addition of anti-H-2-D<sup>d</sup> mAb (Fig. 1E). Taken together, NY-ESO-1-specific CD8<sup>+</sup> T cells recognize D<sup>d</sup>-restricted NY-ESO-1<sub>81-88</sub> peptide.

### 3.2. MAGE-A4-specific CD8<sup>+</sup> T cells recognize D<sup>d</sup>-restricted MAGE-A4<sub>265-273</sub> peptide

To establish a MAGE-A4 animal model, we determined the minimal epitope of MAGE-A4-specific CD8<sup>+</sup> T cells after immunization with MAGE-A4. Naive BALB/c mice were immunized twice at two-week intervals with plasmids encoding the entire sequence of MAGE-A4. Splenic CD8<sup>+</sup> T cells were prepared and specific T cell responses were analyzed by ELISPOT assay using peptide pools shown in Supplementary Table 1. MAGE-A4-specific CD8<sup>+</sup> T cells were induced in mice immunized with plasmids encoding MAGE-A4 within peptide pool #5 (Fig. 2A). To elucidate the dominant





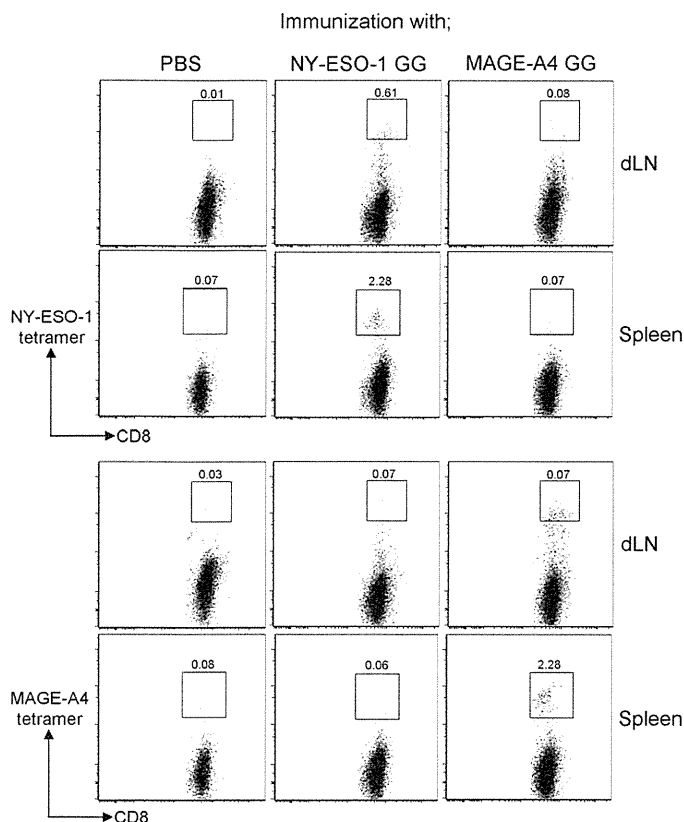
**Fig. 2.** MAGE-A4-specific CD8<sup>+</sup> T cells recognize D<sup>d</sup>-restricted MAGE-A4<sub>265-273</sub> peptide. (A, B) BALB/c mice were immunized by gene gun twice at two-week intervals with plasmids encoding the entire sequence of MAGE-A4. CD8<sup>+</sup> T cells were obtained from spleens, and specific T cells were analyzed with ELISPOT assay using APCs pulsed with the indicated peptides. (C) MHC restriction of induced MAGE-A4-specific CD8<sup>+</sup> T cells was analyzed with ELISPOT assay by the addition of the indicated mAb. (D) MAGE-A4<sub>253-277</sub> was subjected to BIMAS program and the highest score within MAGE-A4<sub>253-277</sub> for a D<sup>d</sup> binding motif was predicted in 265–272 and 265–273 of MAGE-A4. These predicted peptides were analyzed with ELISPOT assay for identification of MAGE-A4 epitope peptide. (E) Avidity of MAGE-A4-specific CD8<sup>+</sup> T cells was analyzed with ELISPOT assay using APCs pulsed with graded doses of peptides. These experiments were repeated two to four times with similar results. Data are mean ± SD.

MAGE-A4 epitope, MAGE-A4-specific CD8<sup>+</sup> T cells were stimulated with each of the peptides from pool #5. The 253–277 peptide was most effective for stimulating MAGE-A4-specific CD8<sup>+</sup> T cells (Fig. 2B). We next assessed the restriction of this response using blocking antibodies. MAGE-A4-specific CD8<sup>+</sup> T cell responses were completely blocked by anti-H-2 D<sup>d</sup> mAb (Fig. 2C). Given the H-2 D<sup>d</sup> restriction of this CD8<sup>+</sup> T cell response, we employed computer-based BIMAS program to predict optimized MHC class I epitope within the MAGE-A4<sub>253-277</sub> peptide. This program ranks all the possible MHC class I epitopes within a given polypeptide sequence. MAGE-A4<sub>253-277</sub> was subjected to this program and the highest score within MAGE-A4<sub>253-277</sub> for a D<sup>d</sup> binding motif was predicted in 265–272 and 265–273 of MAGE-A4 (Supplementary Table 2). MAGE-A4-specific CD8<sup>+</sup> T cells recognized the 265–273, but not

265–272 peptides, thus the minimal epitope was considered to be the MAGE-A4<sub>265-273</sub> peptide (Fig. 2D). To confirm this minimal epitope, graded doses of peptides were pulsed on APC and specific IFN-γ secretion was analyzed by ELISPOT assay. MAGE-A4-specific CD8<sup>+</sup> T cells were high avidity, and could recognize as little as 10 nM of the peptide (Fig. 2E). We conclude that MAGE-A4-specific CD8<sup>+</sup> T cells recognize D<sup>d</sup>-restricted MAGE-A4<sub>265-273</sub> peptide.

### 3.3. Kinetics and distribution of NY-ESO-1/MAGE-A4-specific CD8<sup>+</sup> T cells after gene gun immunization

Next, we generated MHC/peptide tetramers based on the data of minimal epitope and MHC restriction for NY-ESO-1/MAGE-A4-specific CD8<sup>+</sup> T cells. BALB/c mice were immunized with plasmids



**Fig. 3.** NY-ESO-1/MAGE-A4-specific CD8<sup>+</sup> T cells are detected after immunization with a gene gun. BALB/c mice were immunized twice at two-week intervals with plasmids encoding the entire sequences of NY-ESO-1 or MAGE-A4 using a gene gun. Seven days after the second vaccination, CD8<sup>+</sup> T cells were obtained from the draining lymph nodes (dLNs) and spleens, and specific T cells were analyzed with MHC/peptide tetramer assay. These experiments were repeated two to four times with similar results. GG: gene gun.

encoding the whole sequences of NY-ESO-1 or MAGE-A4 by gene gun and the kinetics and distribution of NY-ESO-1/MAGE-A4-specific CD8<sup>+</sup> T cells were analyzed with MHC/peptide tetramers. NY-ESO-1-specific CD8<sup>+</sup> T cells were detected 7–10 days after the primary immunization both in draining lymph nodes and spleens in mice immunized with plasmids encoding NY-ESO-1, but not in mice immunized with MAGE-A4 or control mice (Fig. 3, 4A and 4B). NY-ESO-1-specific T cell responses were further enhanced by the secondary vaccination in both the draining lymph nodes and spleens (Fig. 4A and B). Similarly, MAGE-A4-specific CD8<sup>+</sup> T cells were detected 7–10 days after the primary immunization by gene gun in both the draining lymph nodes and spleens and were enhanced after the booster vaccination (Figs. 3, 4C and 4D), suggesting that these assays are useful tools for analyzing the kinetics and distribution of these antigen-specific CD8<sup>+</sup> T cells.

#### 3.4. NY-ESO-1-specific CD8<sup>+</sup> T cell responses are primed spontaneously after tumor inoculation and these cells partially inhibit tumor growth

It is important to establish tumor models to re-evaluate cancer immunotherapy strategies in detail. To this end, we employed CT26 (a murine colon carcinoma) tumor transplantation model with stable expression of NY-ESO-1 and examined NY-ESO-1-specific CD8<sup>+</sup> T cell and humoral responses spontaneously primed in tumor bearing animals. BALB/c mice were inoculated with CT26-NY-ESO-1 and specific CD8<sup>+</sup> T cell and Ab responses were analyzed with MHC/peptide tetramers and ELISA, respectively. NY-ESO-1-specific

CD8<sup>+</sup> T cells were spontaneously primed 7 days after tumor inoculation in the draining lymph nodes, spleens and peripheral blood in mice inoculated with CT26-NY-ESO-1 and augmented thereafter (Fig. 5A). We then addressed whether these spontaneously-primed NY-ESO-1-specific CD8<sup>+</sup> T cells were involved in tumor growth inhibition. To deplete these CD8<sup>+</sup> T cells, tumor bearing mice were injected with anti-CD8 mAb and tumor growth was analyzed. Anti-CD8 mAb administration augmented tumor growth compared with the control group without any treatment (Fig. 5C), suggesting an anti-tumor role of spontaneously-primed NY-ESO-1-specific CD8<sup>+</sup> T cells. On the other hand, NY-ESO-1-specific Ab responses were not observed 7 days after tumor inoculation, but detected 21 days after tumor inoculation (Fig. 5B). This is compatible with immunological monitoring in humans showing that higher stage of melanoma patients frequently develop humoral immune responses against NY-ESO-1 [3,14].

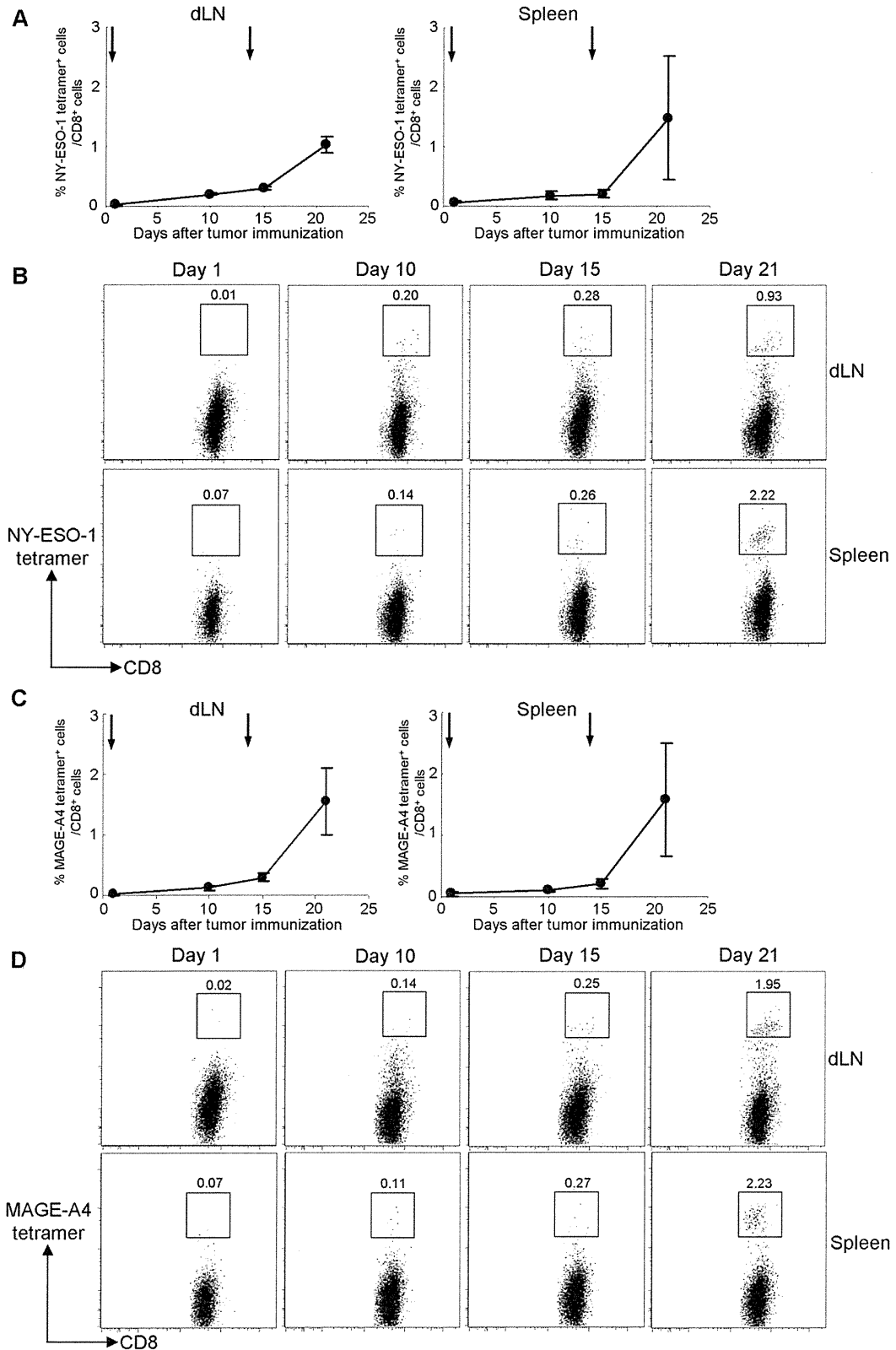
We next immunized these mice with plasmids encoding the entire sequence of NY-ESO-1 and anti-tumor activity was examined. Tumor growth was significantly reduced by immunization with NY-ESO-1 as compared to the control group without treatment (Fig. 5C). Furthermore, CD8<sup>+</sup> T cell depletion totally abolished the anti-tumor effects induced by DNA vaccine (Fig. 5C). As CD4<sup>+</sup> regulatory T cells (Tregs) are reportedly associated with spontaneously-primed and treatment-induced anti-tumor immune responses [15], we also investigated tumor-infiltrating Tregs. While Tregs were present in tumors, their frequency was not associated with anti-tumor activity induced by immunization with plasmids encoding NY-ESO-1 (Fig. 5D). Together, CD8<sup>+</sup> T cell and Ab responses to NY-ESO-1 in this tumor model closely parallel NY-ESO-1 immune responses in humans. Spontaneous tumor antigen-specific immune responses restrained, albeit incomplete, tumor growth, but tumor growth were vigorous and overwhelmed the tumor growth inhibition, thus additional augmentation of these immune responses are required for effective control of tumor growth.

#### 3.5. MAGE-A4-specific CD8<sup>+</sup> T cell responses is primed spontaneously after tumor inoculation

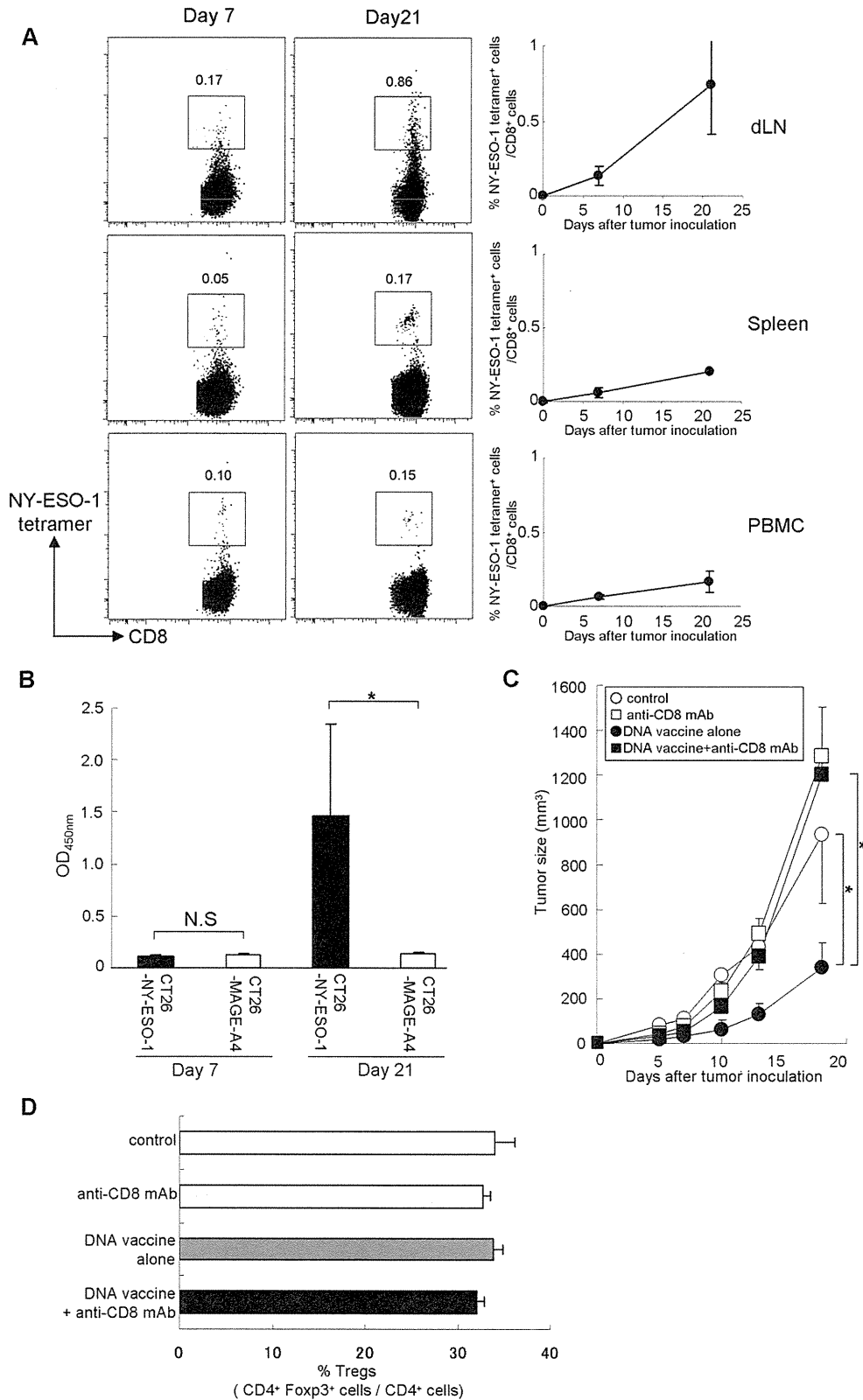
We established another tumor transplantation model with stable expression of MAGE-A4 and examined MAGE-A4-specific CD8<sup>+</sup> T cell and humoral responses spontaneously primed in tumor bearing mice. BALB/c mice were inoculated with CT26-MAGE-A4 and specific CD8<sup>+</sup> T cell and humoral responses were analyzed with MHC/peptide tetramers and ELISA. In these mice, MAGE-A4-specific CD8<sup>+</sup> T cells were spontaneously primed 7 days after tumor inoculation in the draining lymph nodes, spleen and PBMC, and augmented thereafter (Fig. 6A). MAGE-A4-specific Ab responses were not observed 7 days after tumor inoculation, but detected 21 days after tumor inoculation (Fig. 6B). Like as the result of NY-ESO-1 models, cellular and humoral immune responses to MAGE-A4 in this model closely parallel MAGE-A4 immune responses in humans.

## 4. Discussion

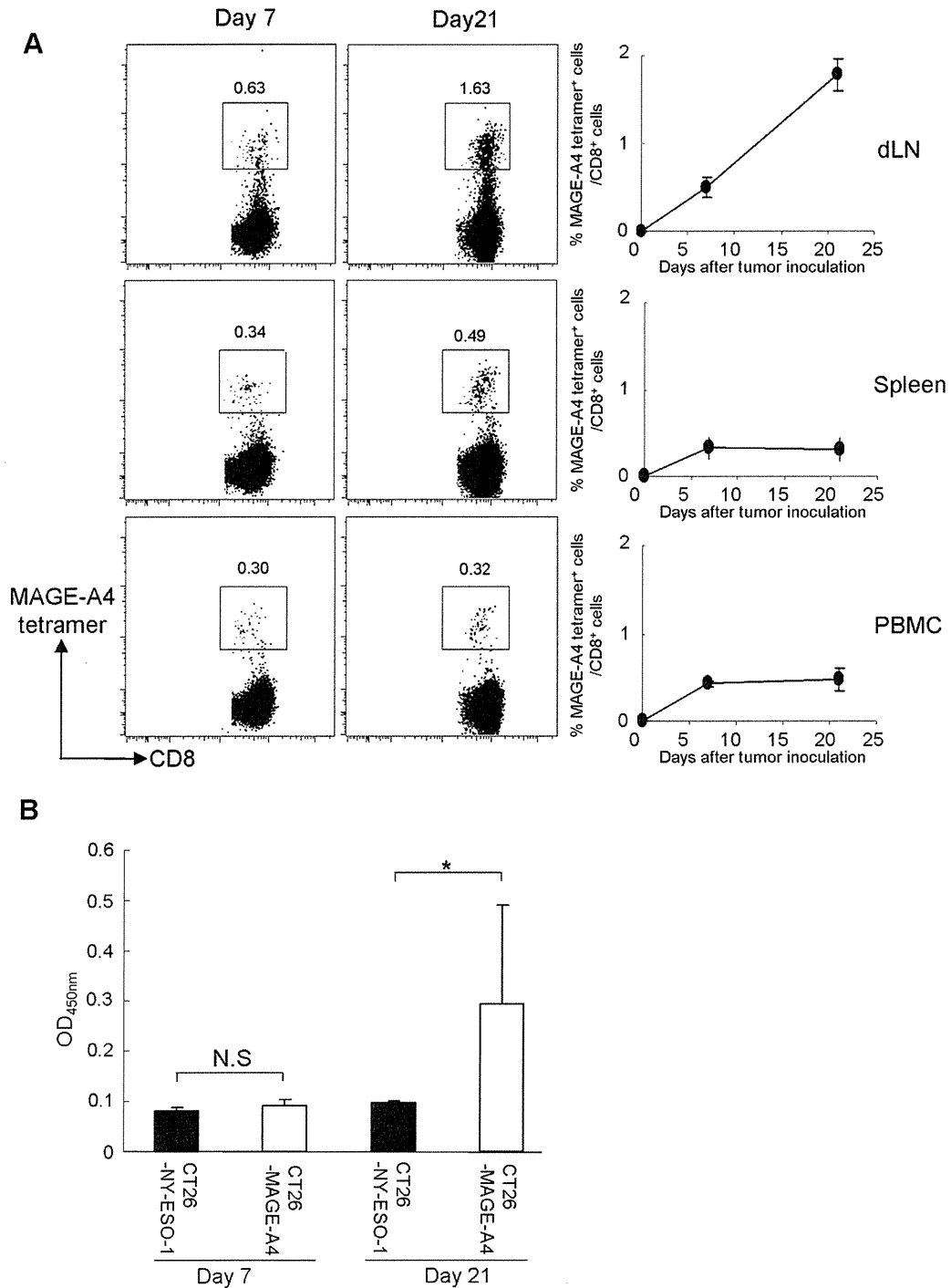
We have established mouse models that allowed studies on NY-ESO-1 and MAGE-A4 immunity. Using these models, we evaluated the kinetics and distribution of antigen-specific CD8<sup>+</sup> T cells after tumor growth and immunization. While it has been recently shown that CD4<sup>+</sup>CD25<sup>+</sup> Tregs and the ratio of CD8<sup>+</sup> effector T cells to Tregs in tumors critically influenced the prognosis of cancer patients [16,17], limitation of samples usually makes it difficult to investigate the function of effector T cells and Tregs at tumor sites in humans. Given the importance of tumors as active sites of anti-tumor responses, it is important to examine not only the draining



**Fig. 4.** Kinetics and distribution of NY-ESO-1/MAGE-A4-specific CD8<sup>+</sup> T cells after immunization with a gene gun. Kinetics and distribution of NY-ESO-1 (A and B)/MAGE-A4 (C and D)-specific CD8<sup>+</sup> T cells were analyzed by MHC/peptide tetramer assay. BALB/c mice were immunized twice at two-week intervals with plasmids encoding the entire sequence of either NY-ESO-1 or MAGE-A4 using a gene gun. CD8<sup>+</sup> T cells were obtained from the draining lymph nodes (dLNs) and spleens on the indicated days, and specific T cells was analyzed with MHC/peptide tetramer assay. These experiments were repeated two to four times with similar results. Data in (A) and (C) are mean  $\pm$  SD. Arrows in (A) and (C) represent the first and second immunization.



**Fig. 5.** NY-ESO-1-specific CD8<sup>+</sup> T cells are primed spontaneously after tumor inoculation, and these cells partially inhibit tumor growth. BALB/c mice were inoculated with CT26-NY-ESO-1. (A) CD8<sup>+</sup> T cells were isolated from the draining lymph nodes (dLNs), spleens and PBMCs on the indicated days, and NY-ESO-1-specific T cells were analyzed with MHC/peptide tetramer assay. (B) Sera were obtained on the indicated days and Ab responses against NY-ESO-1 were analyzed with ELISA. These experiments were repeated twice with similar results. Data are mean ± SD. \**p* < 0.05 as compared to control. (C) BALB/c mice were immunized with plasmids encoding the entire sequence of NY-ESO-1, and CT26-NY-ESO-1 was inoculated on day 21 (one week after final immunization). Indicated groups of mice were administered with anti-CD8 mAb (clone 19/178, 100 μg/mouse, every 12 days). More than 95% of CD8<sup>+</sup> T cells were depleted by this method (data not shown). Each group consisted of four mice. Mice were monitored thrice a week. These experiments were repeated two times with similar results. (D) Tumor infiltrating lymphocytes were collected 21 days after tumor inoculation and the frequency of Foxp3<sup>+</sup> cells in CD4<sup>+</sup> T cells was analyzed.



**Fig. 6.** Spontaneous MAGE-A4-specific CD8<sup>+</sup> T cells are primed after tumor inoculation. BALB/c mice were inoculated with CT26-MAGE-A4. (A) CD8<sup>+</sup> T cells were isolated from the draining lymph nodes (dLNs), spleens and PBMCs on the indicated days, and MAGE-A4-specific T cells was analyzed with MHC/peptide tetramer assay. (B) Sera were obtained on the indicated days, and Ab responses against MAGE-A4 were analyzed with ELISA. These experiments were repeated twice with similar results. Data are mean ± SD. \**p* < 0.05 as compared to control.

lymph nodes, spleens and PBMCs, but also tumor local sites. Our models could be valuable tools for analyzing antigen-specific T cells in both novel cancer immunotherapy and cancer immunotherapy that have been already tested in humans.

In our models, we found that spontaneous CD8<sup>+</sup> T cell and Ab responses were primed and increased along with tumor progression in both NY-ESO-1 and MAGE-A4 models [3,9]. Accumulating data show that induction/augmentation of anti-tumor immune responses are often detected in patients with larger tumors [3,14],

suggesting that immune responses found in our NY-ESO-1 and MAGE-A4 tumor models closely parallel NY-ESO-1 and MAGE-A4 immune responses in humans. It has been a long debate whether spontaneous anti-tumor responses detected in cancer patients impact on tumor growth, as tumors continuously grow in patients harboring spontaneous anti-tumor immune responses. Our tumor model provides a clear answer for this conundrum. Although the immune responses spontaneously primed in tumor-bearing hosts partly inhibit a tumor growth, this immune response

is not strong enough to completely reject the tumor in the host. This means that further activation of immune responses by appropriate immunotherapy is essential for tumor rejection. Indeed, when these spontaneous immune responses were augmented by DNA vaccine, tumor growth was significantly inhibited. In contrast, we have reported that peptide vaccine using this NY-ESO-1 peptide enhanced tumor growth rather than inhibiting tumor growth unless it is vaccinated with proper adjuvants [13].

In fact, antigen-specific antibody may not play an important role for tumor rejection in our models, because 1) most tumor antigens including NY-ESO-1 and MAGE-A4 focused on this study, was exclusively expressed intracellularly by the tumor cells, thus not accessible for antibody [3] and 2) Ab responses were detected on day 21, namely later than CD8<sup>+</sup> T cell responses. Nevertheless, we have reported that injection of NY-ESO-1 mAb with chemotherapy, that can accentuate the release of intracellular tumor antigens to facilitate mAb access to intracellular target molecules, augmented anti-tumor effect in the same model system, though Ab administration alone did not inhibit tumor growth [18]. In our mouse model, spontaneously-primed anti-NY-ESO-1 Ab was detected when tumors reached a larger size. The level of spontaneously-primed antibody was, however, about 10-times lower than that achieved by mAb injection [18], suggesting that spontaneously-primed Ab responses may potentially have some anti-tumor effects, but the amount of Abs is too low to exhibit effective anti-tumor activity.

Since no NY-ESO-1 homologue is present in mice, the detected immune responses against NY-ESO-1 are considered to reflect a foreign antigen, rather than a self-antigen [3]. Whereas cancer-testis antigens like NY-ESO-1 are only expressed by cancer cells and testis, but not by normal somatic cells, mimicking foreign antigens, some cancer-testis antigens are reportedly expressed in medullary thymic epithelial cells under control of AIRE (Autoimmune regulator) [3,19]. It is plausible that cancer-testis antigens like NY-ESO-1 could be considered self-antigens during thymic selection, resulting in a repertoire of NY-ESO-1-specific T cells that are either subject to central or peripheral tolerance [3,20–22]. Thus, studies using mice in which NY-ESO-1 is a self-antigen should allow resolving this issue.

A unique finding of our study is that NY-ESO-1-specific CD8<sup>+</sup> T cell epitopes were present in an immunogenic part defined in humans [3]. This finding implies that immunogenicity may be characterized with similar components between humans and mice, further supporting the usefulness of our models. Our animal models provide important tools for the development of effective cancer vaccines.

In conclusion, we established animal models involving human tumor antigens, such as NY-ESO-1 or MAGE-A4 protein. These models allowed us to study the kinetics and distribution of antigen-specific immune responses in detail, and hence providing tools to optimize the efficacy of current cancer immunotherapy.

## Acknowledgments

This article is dedicated to the memory of Lloyd J. Old, M.D. We thank Dr. J. Wing for critical reading and Ms. K. Mori and K. Sasada for excellent technical assistance.

This study was supported by Grants-in-Aid for Scientific Research (B) (HN) and Grants-in-Aid for Scientific Research on Priority Areas (HN and HS) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Cancer Research Institute Investigator Award (HN), Cancer Research Grant from Foundation of Cancer Research Promotion (HN), the Sagawa Foundation for Promotion of Cancer Research (HN) and Senri Life Science Foundation (HN).

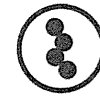
**Contributions:** Designed the experiments: DM, HN, TK, HS. Performed the experiments: DM, HN, TN, LW, ES. Analyzed the data: DM, HN, NH, TK, HS. Contributed reagents/materials/analysis tools: IL, EN. Wrote the paper: DM, HN, TK. **Conflicts of interest:** D.M. and N.H. are employees of ImmunoFrontier, Inc. The other authors have no potential conflicts of interest.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2013.02.056>.

## References

- Scanlan MJ, Gure AO, Jungbluth AA, Old LJ, Chen Y-T. Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. *Immunol Rev* 2002;188:22–32.
- Boon T, Coulie PG, Van den Eynde BJ, Human van der Bruggen P. T cell responses against melanoma. *Annu Rev Immunol* 2006;24:175–208.
- Gnjatic S, Nishikawa H, Jungbluth AA, Gure AO, Ritter G, Jager E, et al. NY-ESO-1: review of an immunogenic tumor antigen. *Adv Cancer Res* 2006;95:1–30.
- Belardelli F, Ferrantini M, Parmiani G, Schlom J, Garaci E. International meeting on cancer vaccines: how can we enhance efficacy of therapeutic vaccines? *Cancer Res* 2004;64(18):6827–30.
- Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. *Nat Med* 2004;10(9):909–15.
- Chen Y-T, Scanlan MJ, Sahin U, Tureci O, Gure AO, Tsang S, et al. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci USA* 1997;94(5):1914–8.
- De Plaen E, De Backer O, Arnaud D, Bonjean B, Chomez P, Martelange V, et al. A new family of mouse genes homologous to the human MAGE genes. *Genomics* 1999;55(2):176–84.
- Duffour MT, Chaux P, Lurquin C, Cornelis G, Boon T, van der Bruggen P. A MAGE-A4 peptide presented by HLA-A2 is recognized by cytolytic T lymphocytes. *Eur J Immunol* 1999;29(10):3329–37.
- Miyahara Y, Naota H, Wang L, Hiasa A, Goto M, Watanabe M, et al. Determination of cellularly processed HLA-A2402-restricted novel CTL epitopes derived from two cancer germ line genes, MAGE-A4 and SAGE. *Clin Cancer Res* 2005;11(15):5581–9.
- Nishikawa H, Tanida K, Ikeda H, Sakakura M, Miyahara Y, Aota T, et al. Role of SEREX-defined immunogenic wild-type cellular molecules in the development of tumor-specific immunity. *Proc Natl Acad Sci USA* 2001;98(25):14571–6.
- Nishikawa H, Sato E, Briones G, Chen LM, Matsuo M, Nagata Y, et al. In vivo antigen delivery by a *Salmonella typhimurium* type III secretion system for therapeutic cancer vaccines. *J Clin Invest* 2006;116(7):1946–54.
- Griswold DP, Corbett TH. A colon tumor model for anticancer agent evaluation. *Cancer* 1975;36(6 Suppl):2441–4.
- Muraoka D, Kato T, Wang LA, Maeda Y, Noguchi T, Harada N, et al. Peptide vaccine induces enhanced tumor growth associated with apoptosis induction in CD8<sup>+</sup> T cells. *J Immunol* 2010;185(6):3768–76.
- Jager E, Stockert E, Zidianakis Z, Chen Y-T, Karbach J, Jager D, et al. Humoral immune responses of cancer patients against Cancer-Testis antigen NY-ESO-1: Correlation with clinical events. *Int J Cancer* 1999;84(5):506–10.
- Nishikawa H, Sakaguchi S. Regulatory T cells in tumor immunity. *Int J Cancer* 2010;127(4):759–67.
- Curjel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004;10(9):942–9.
- Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, et al. Intraepithelial CD8<sup>+</sup> tumor-infiltrating lymphocytes and a high CD8<sup>+</sup>/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci USA* 2005;102(51):18538–43.
- Noguchi T, Kato T, Wang LN, Maeda Y, Ikeda H, Sato E, et al. Intracellular tumor-associated antigens represent effective targets for passive immunotherapy. *Cancer Res* 2012;72(7):1672–82.
- Gotter J, Brors B, Hergenbahn M, Kyewski B. Medullary epithelial cells of the human thymus express a highly diverse selection of tissue-specific genes colocalized in chromosomal clusters. *J Exp Med* 2004;199(2):155–66.
- Danke NA, Koelle DM, Yee C, Beheray S, Kwok WW. Autoreactive T cells in healthy individuals. *J Immunol* 2004;172(10):5967–72.
- Nishikawa H, Jager E, Ritter G, Old LJ, Gnjatic S. CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells control the induction of antigen-specific CD4<sup>+</sup> helper T cell responses in cancer patients. *Blood* 2005;106:1008–11.
- Nishikawa H, Qian F, Tsuji T, Ritter G, Old LJ, Gnjatic S, et al. Influence of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells on low/high-avidity CD4<sup>+</sup> T cells following peptide vaccination. *J Immunol* 2006;176(10):6340–6.



# A novel human-derived antibody against NY-ESO-1 improves the efficacy of chemotherapy

Anurag Gupta<sup>1</sup>, Natko Nuber<sup>1,6</sup>, Christoph Esslinger<sup>2</sup>, Mareike Wittenbrink<sup>2</sup>, Martin Treder<sup>2</sup>, Alexandro Landshammer<sup>1</sup>, Takuro Noguchi<sup>3</sup>, Marcus Kelly<sup>3</sup>, Sacha Gnjatic<sup>3</sup>, Erika Ritter<sup>3</sup>, Lotta von Boehmer<sup>1</sup>, Hiroyoshi Nishikawa<sup>4</sup>, Hiroshi Shiku<sup>5</sup>, Lloyd Old<sup>3</sup>, Gerd Ritter<sup>3</sup>, Alexander Knuth<sup>1</sup> and Maries van den Broek<sup>1</sup>

<sup>1</sup>Department of Oncology, University Hospital Zürich, Zürich, Switzerland

<sup>2</sup>CT-Atlantic, Schlieren, Switzerland

<sup>3</sup>Ludwig Institute for Cancer Research, New York Branch, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

<sup>4</sup>Experimental Immunology, Immunology Frontier Research Center, Osaka University, Osaka, Japan

<sup>5</sup>Departments of Cancer Vaccine and Immuno-Gene Therapy, Mie University Graduate School of Medicine, Tsu, Mie, Japan

<sup>6</sup>Department of Biomedicine, University of Basel, Basel, Switzerland

We investigated whether antibodies against intracellular tumor-associated antigens support tumor-specific immunity when administered together with a treatment that destroys the tumor. We propose that released antigens form immune complexes with the antibodies, which are then efficiently taken up by dendritic cells. We cloned the first human monoclonal antibodies against the Cancer/Testis (CT) antigen, NY-ESO-1. We tested whether the monoclonal anti-NY-ESO-1 antibody (12D7) facilitates cross-presentation of a NY-ESO-1-derived epitope by dendritic cells to human CD8<sup>+</sup> T cells, and whether this results in the maturation of dendritic cells *in vitro*. We investigated the efficacy of 12D7 in combination with chemotherapy using BALB/c mice bearing syngeneic CT26 tumors that express intracellular NY-ESO-1. Human dendritic cells that were incubated with NY-ESO-1:12D7 immune complexes efficiently stimulated NY-ESO-1<sub>157-165</sub>/HLA-A2-specific human CD8<sup>+</sup> T cells to produce interferon- $\gamma$ , whereas NY-ESO-1 alone did not. Furthermore, the incubation of dendritic cells with NY-ESO-1:12D7 immune complexes resulted in the maturation of dendritic cells. Treatment of BALB/c mice that bear CT26/NY-ESO-1 tumors with 5-fluorouracil (5-FU) plus 12D7 was significantly more effective than chemotherapy alone. We propose systemic injection of monoclonal antibodies (mAbs) against tumor-associated antigens plus a treatment that promotes the local release of those antigens resulting in immune complex formation as a novel therapeutic modality for cancer.

**Keywords:** NY-ESO-1, antibody, chemotherapy

## Introduction

Cancer/Testis (CT) antigens form an extended family of proteins that are frequently expressed in a large variety of malignancies but are absent from healthy tissue, except for the testis and placenta. Cancer patients often develop spontaneous immune responses toward CT antigens, which illustrate their immunogenicity (1-3). Their apparent immunogenicity and unique expression pattern make CT antigens attractive targets for immunotherapy, and a number of clinical trials in which cancer patients were immunized with CT antigens in different forms have been completed, some of which show objective

clinical responses (4-12).

Dendritic cell (DC) maturation is a key prerequisite for the activation of T cells, and moreover, antigen presentation by steady-state DCs results in peripheral tolerance induction, a process that is considered crucial for the protection against autoimmunity (13, 14). DC maturation usually is induced by infection or inflammation—or by adjuvants for that matter—and can be a local event. Insufficient maturation of tumor-associated DCs may be one of multiple reasons for the compromised response of tumor-infiltrating T cells compared to peripheral T cells (15, 16). Cross-presentation of sufficient amounts of tumor-derived antigens may be another limiting factor, especially because the number of tumor-associated DCs often is low and cross-presentation is inefficient (17, 18). Therefore, we developed a novel immunotherapeutic approach that combines enhanced cross-presentation of epitopes derived from intracellular proteins with concomitant DC maturation. We hypothesized that administration of monoclonal antibodies (mAbs) against CT antigens together with a therapy that releases these usually intracellular antigens may support the local formation of immune complexes, which are efficiently taken up by DCs (19, 20) resulting in increased presentation of CT antigen-derived epitopes to CD8<sup>+</sup> T cells. Because there is evidence that the uptake of immune complexes by DCs through the activating receptor for IgG (Fc $\gamma$ RIIA) results in DC maturation (21), the use of mAbs against CT antigens may serve both purposes: DC activation and enhanced cross-presentation.

The fact that NY-ESO-1 is one of the best-characterized and most immunogenic CT antigens known to date (22, 23) and is frequently expressed by tumors of different origin (6, 24) prompted us to clone human-derived mAbs against NY-ESO-1 from patients who had high serum levels of NY-ESO-1-specific IgG and, thus, presumably a high frequency of NY-ESO-1-specific B cells. The obvious advantage of cloning a therapeutic antibody from humans is that adverse side effects of such an antibody are very unlikely and that it therefore can relatively be quickly tested in clinical trials. We report here the generation of the first human-derived IgG1 mAbs against NY-ESO-1 and the selection of a lead development candidate (12D7). We show that 12D7 facilitates cross-presentation of a NY-ESO-1-derived epitope to CD8<sup>+</sup> T cells, that 12D7:NY-ESO-1 immune

complexes induce the maturation of human monocyte-derived DCs *in vitro*, and that 12D7 significantly enhances the therapeutic efficacy of chemotherapy using a preclinical syngeneic mouse model.

**Table 1**  
**Binding of human monoclonal anti-NY-ESO-1 antibodies to NY-ESO-1. Comparison of EC<sub>50</sub> and equilibrium affinity constants for the binding between NY-ESO-1 and different anti-NY-ESO-1 antibodies.**

Antibody	EC <sub>50</sub> [pM] (prok. NY-ESO-1)	K <sub>D</sub> [M] (prok. NY-ESO-1)	K <sub>D</sub> [M] (euk. NY-ESO-1)
12D7	1.14	2.08x10 <sup>-10</sup>	1.56x10 <sup>-10</sup>
1D4	2.23	1.62x10 <sup>-9</sup>	2.24x10 <sup>-10</sup>
30D6	1.09	4.35x10 <sup>-9</sup>	2.65x10 <sup>-9</sup>
31E4	9.52	1.9x10 <sup>-9</sup>	2.23x10 <sup>-9</sup>
15B12	72.6	---	---
E978 control	6.66	2.56x10 <sup>-8</sup>	1.56x10 <sup>-11</sup>

**Results**

**Cloning of human-derived monoclonal antibodies from cancer patients**

We cloned eight different NY-ESO-1-specific human-derived monoclonal antibodies (HD mAbs) from a melanoma patient, of which the following five were selected for further analysis based on their affinity to the target: 1D4, 12D7, 15B12, 30D6, and 31E4. All HD mAbs were of IgG1 isotype.

**In vitro characterization of HD mAbs**

To compare the binding properties of five different anti-NY-ESO-1 HD mAbs to recombinant NY-ESO-1 protein, we determined the half-maximal effective concentration (EC<sub>50</sub>) using a protein ELISA. All antibodies bound recombinant NY-

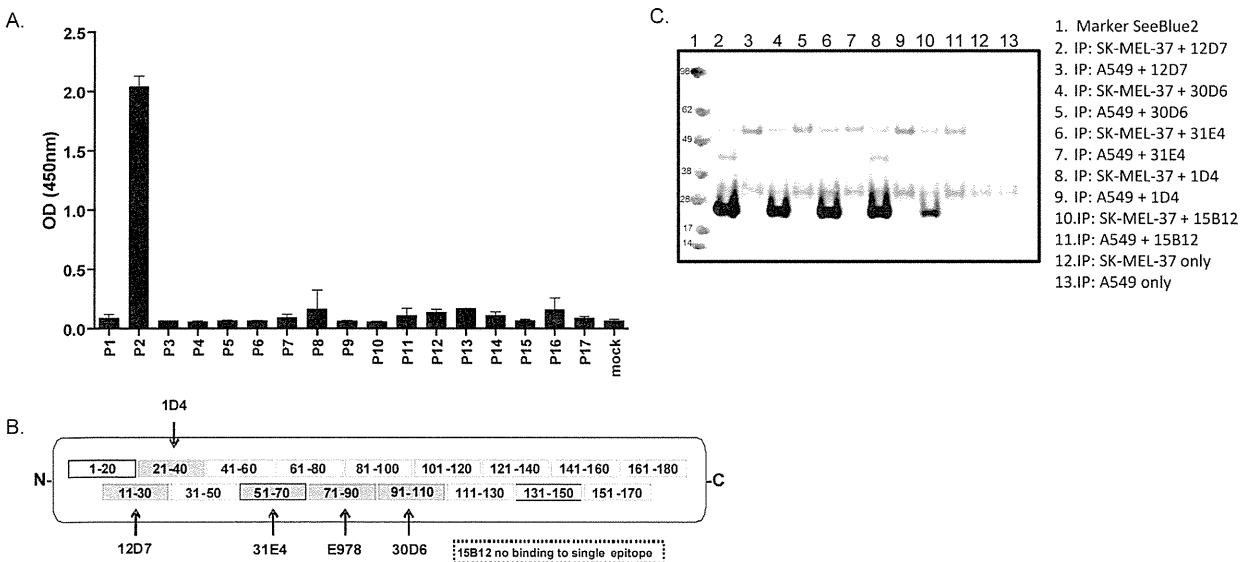
ESO-1 produced in bacteria in the low pM range. Actual binding constants to recombinant NY-ESO-1 produced in bacteria and in eukaryotic cells were determined by surface plasmon resonance (Biacore Systems) (Table 1).

To determine the epitopes recognized by the different mAbs, we used a set of overlapping peptides spanning the complete NY-ESO-1 protein as coating antigen in ELISA. As shown in Figure 1A, 12D7 binds to a peptide representing the amino acids 11 to 30 from the NY-ESO-1 protein, but not to the two adjacent peptides that span amino acids 1-20 or 21-40. This suggests that the epitope recognized by 12D7 lies at the junction of these two peptides around amino acid 20 of NY-ESO-1. Figure 1B summarizes the epitope-specificity of all five anti-NY-ESO-1 antibodies. In addition, all antibodies were tested for binding to endogenous NY-ESO-1 from the human melanoma cell line SK-MEL-37 by immunoprecipitation. All antibodies precipitate NY-ESO-1 from a cell lysate of an NY-ESO-1+ cell line (SK-MEL-37) (Figure 1C). Because 12D7 had the highest affinity for eukaryotic NY-ESO-1, we performed further experiments with this mAb.

**12D7 facilitates cross-presentation of NY-ESO-1 by DCs and induces concomitant DC maturation**

To test whether 12D7 facilitates the cross-presentation of NY-ESO-1-derived epitopes *in vitro*, we generated monocyte-derived, HLA-A\*0201+ DCs and fed them with 12D7:NY-ESO-1 immune complexes, NY-ESO-1, or 12D7. DCs were subsequently incubated with cloned NY-ESO-1<sub>157-165</sub>/HLA-A\*0201-specific CD8+ T cells, and the percentage of T cells that produced IFN-γ was used as readout for antigen recognition. Mature DCs fed with NY-ESO-1 protein induced IFN-γ production in a low but discernible percentage of T cells (Figure 2A, black bars), which did not occur when DCs were not matured (Figure 2A, white bars). DCs fed with 12D7:NY-ESO-1 immune complexes induced the production of IFN-γ in a much

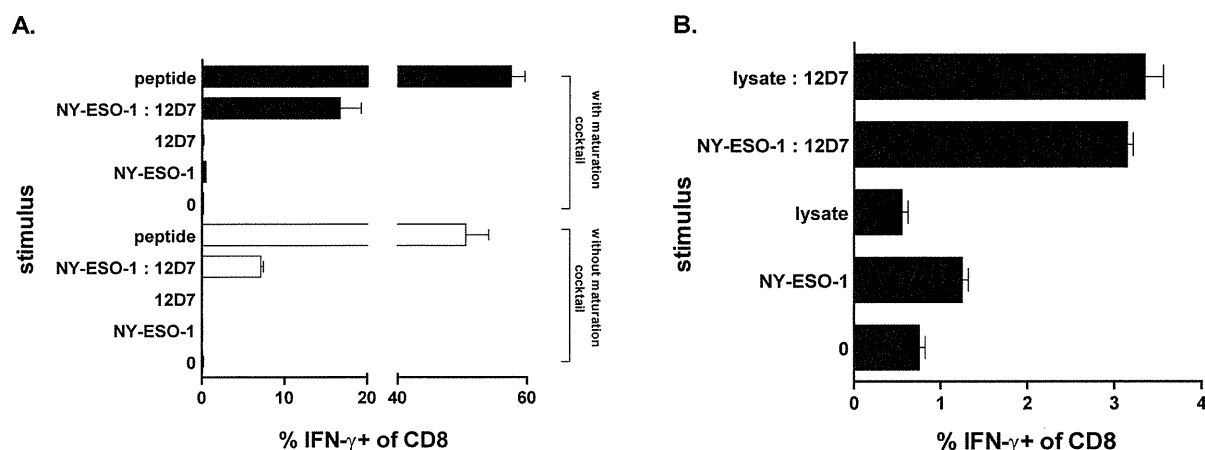
**Figure 1**



**Epitope mapping of anti-NY-ESO-1 human monoclonal antibodies.** (A) Representative peptide ELISA for antibody 12D7, where P1-P17 represent overlapping NY-ESO-1 peptides. (B) Overview of the specificities of different NY-ESO-1 specific human-derived mAbs. (C) Immunoprecipitation of NY-ESO-1 from a cell lysate of a NY-ESO-1+ cell line SK-MEL-37 or a NY-ESO-1- cell line A549 by human anti-NY-ESO-1 mAbs.



Figure 2



**Human monoclonal anti-NY-ESO-1 antibody (12D7) facilitates cross-presentation of a NY-ESO-1-derived, HLA-A2-restricted epitope (NY-ESO-1<sub>157-165</sub>).** (A) HLA-A2+, monocyte-derived DCs were incubated with 20  $\mu$ g NY-ESO-1 protein, 200  $\mu$ g human monoclonal anti-NY-ESO-1 antibody (12D7), with immune complexes (12D7:NY-ESO-1) or with media for 3 h, were washed and cultured for 36 h with (black bars) or without (white bars) 25 ng/mL TNF- $\alpha$  + 1  $\mu$ g/mL sCD40L (maturation cocktail).  $6 \times 10^4$  cloned, NY-ESO-1<sub>157-165</sub>/HLA-A2-specific CD8+ T cells were added to  $10^5$  DCs in the presence of 10  $\mu$ g/mL Brefeldin A, followed by a 5 h incubation and subsequent surface staining for CD8 and intracellular staining for IFN- $\gamma$ .  $10^{-6}$  M peptide was added to DCs as positive control. All cultures were performed in triplicate. (B) HLA-A2+, monocyte-derived DCs were incubated with 20  $\mu$ g NY-ESO-1 protein, 200  $\mu$ g human monoclonal anti-NY-ESO-1 antibody (12D7), with lysate of  $10^7$  NY-ESO-1+ SK-MEL-37 cells (lysate), with immune complexes (NY-ESO-1:12D7 or lysate:12D7), or with media (0) for 3 h, were washed and cultured for 36 h with 25 ng/mL TNF- $\alpha$  + 1  $\mu$ g/mL sCD40L (maturation cocktail).  $6 \times 10^4$  cloned, NY-ESO-1<sub>157-165</sub>/HLA-A2-specific CD8+ T cells were added to  $10^5$  DCs in the presence of 10  $\mu$ g/mL Brefeldin A, followed by a 5 h incubation and subsequent surface staining for CD8 and intracellular staining for IFN- $\gamma$ . All cultures were performed at least in duplicate.

higher percentage of T cells and, importantly, also did so when DCs that were not deliberately matured were used (Figure 2A, compare black and white bars). None of the negative controls—DCs fed with 12D7, mock immune complexes, or medium—induced IFN- $\gamma$  production (Figure 2A and data not shown). To exclude that our observations are a peculiarity of recombinant NY-ESO-1, we incubated 12D7 with a cell lysate of SK-MEL-37 cells, which naturally express NY-ESO-1, and subsequently fed this mixture to DCs. DCs fed with the 12D7:lysate or with 12D7:NY-ESO-1 presented NY-ESO-1-derived epitopes approximately equally well (Figure 2B).

Because presentation of 12D7:NY-ESO-1 immune complexes seemed not to require deliberate DC maturation, we addressed whether the uptake of immune complexes, but not the uptake of uncomplexed protein, induced DC maturation *in vitro*. We therefore compared the expression of three surface molecules that are upregulated on mature DCs (CD83, CD86, and MHC class II) after incubation with media, 12D7, NY-ESO-1, or with 12D7:NY-ESO-1 immune complexes in the absence of maturation cocktail. We found that only immune complexes induced an upregulation of CD86, CD83, and MHC II (Figure 3A; left, middle, and right panels, respectively). We then compared the expression of CD83, CD86, and MHC II on DCs that were incubated with the maturation cocktail, with immune complexes, or with both, in order to determine the relative potency of immune complexes with respect to DC maturation. We found that immune complexes were almost as potent in inducing DC maturation as the classical maturation cocktail (sCD40L plus TNF- $\alpha$ ) (Figure 3B). A combination of immune complexes plus maturation cocktail resulted in the most pronounced upregulation of CD86 and CD83 (Figure 3B; left and middle panels, respectively), whereas MHC II was not further upregulated compared to any of the two treatments

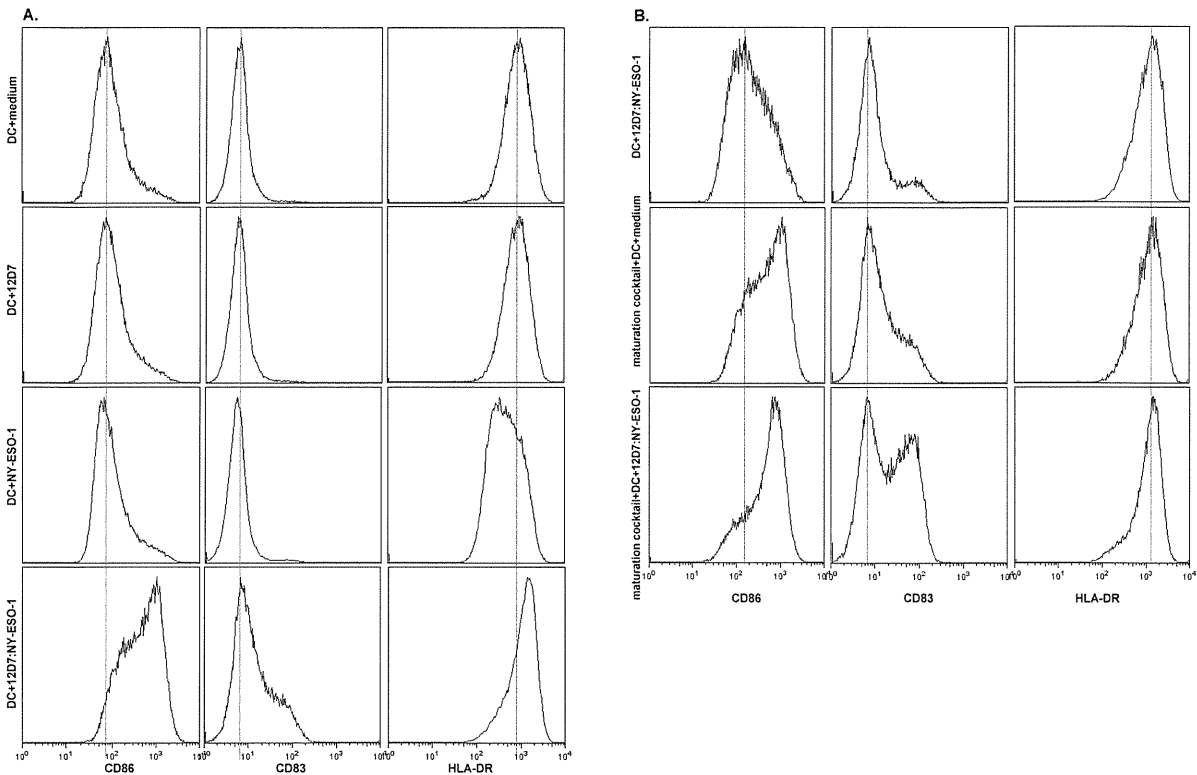
alone (Figure 3B, right panels).

#### 12D7 increases the therapeutic efficacy of chemotherapy in mice with NY-ESO-1+ tumors

To test the therapeutic efficacy of 12D7 *in vivo*, we injected  $10^6$  syngeneic, NY-ESO-1-transfected CT26 tumor cells s.c. in BALB/c mice. To induce release of intracellular NY-ESO-1, mice were treated with 75 mg/kg 5-FU when tumors reached a size of approximately 25 mm<sup>2</sup>, which was typically around 2 weeks after injection of tumor cells. The treatment with 5-FU was repeated one week later and, in some groups, was combined with 100  $\mu$ g 12D7 given systemically 2 d after each 5-FU injection. As can be seen from the growth curves, 5-FU has the expected therapeutic effect. Importantly, this was enhanced by 12D7 (Figure 4A). Treatment with 12D7 alone had no effect, presumably because the amount of spontaneously released antigen is not sufficient in this particular model. A compilation of end-point tumor sizes from 4 independent experiments shows a highly significant difference between mice treated with 5-FU plus 12D7, and mice treated with 5-FU alone (Figure 4B).

To investigate whether treatment with 5-FU plus 12D7 supported tumor-specific immunity, we injected mice with Brefeldin A 4 h before euthanasia, followed by staining for CD45.2, CD8, and intracellular IFN- $\gamma$ . This way of analysis shows which cells actually are making IFN- $\gamma$  *in vivo* and not which cells potentially can do this upon *in vitro* restimulation with peptide. This method obviously does not allow discrimination between single peptide specificities, but it is of higher biological relevance (25) particularly because we envisaged that DC activation, which we have shown to occur upon cross-presentation (Figure 3), may also support the presentation of other epitopes besides those derived from NY-ESO-1. Treatment with 5-FU plus 12D7 supported CD8+ and

**Figure 3**



**Immune complexes induce maturation of monocyte-derived DCs *in vitro*.** (A) CD14-derived DCs were incubated with media, 200 µg NY-ESO-1, 20 µg 12D7 or preformed immune complexes of 20 µg 12D7 + 200 µg NY-ESO-1, and were analyzed 36 h later for surface expression of CD86, CD83, or MHC II. (B) CD14-derived DCs were incubated with preformed immune complexes of 20 µg 12D7 + 200 µg NY-ESO-1, maturation cocktail (sCD40L + TNF- $\alpha$ ) or with preformed immune complexes plus maturation cocktail, and were analyzed 36 h later for surface expression of CD86, CD83, or MHC II.

effector function in the tumor (Figure 4C). Treatment with 5-FU (Figure 4C) or 12D7 (data not shown) did not have this effect.

**Discussion**

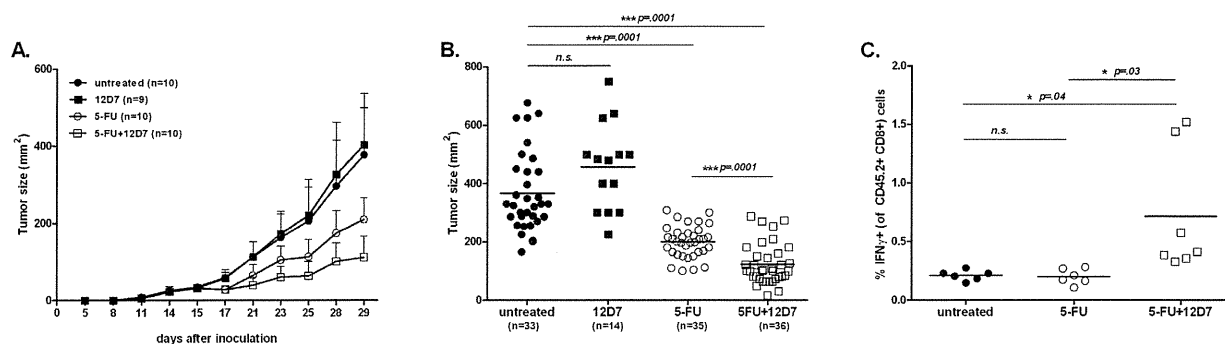
We hypothesized that antibodies against intracellular, tumor-associated antigens support tumor-specific immunity when used in combination with a therapy that induces cell death such as chemo- or radiotherapy. We envisaged that such antibodies form immune complexes with the released tumor antigens. These immune complexes are subsequently taken up with higher efficiency compared to protein (fragments) by DCs (26), which then cross-present relevant epitopes to local CD8+, tumor-specific T cells. This presumed sequence of events may be of particular interest as evidence is accumulating that both chemo- and radiotherapy support tumor-specific immunity (27), and we therefore reasoned that additional stimulation of tumor-specific immunity could further improve the efficacy of these standard therapies.

For this purpose, we have cloned the first fully human mAbs to NY-ESO-1 using Epstein-Barr virus (EBV)-transformed B cells from a melanoma patient and subjected those to preclinical experiments to obtain proof of principle. We found that 12D7, a fully human IgG1 mAb specific for the immunogenic CT antigen NY-ESO-1, supported cross-presentation of NY-ESO-1 *in vitro* resulting in an approximate 15-fold increase of the number of responding CD8+ T cells. Of the other four NY-ESO-

1-specific mAbs we generated here, 1D4 and 30D6 improved cross-presentation of NY-ESO-1 (data not shown), whereas 15B12 and 31E4 seemed not effective (data not shown). This difference may be explained by the difference in affinity, as 15B12 did not show binding to NY-ESO-1 by Biacore—although it did bind weakly to NY-ESO-1 in ELISA—and 31E4 had at least a 1-log lower affinity than 12D7, 1D4, and 30D6. At present, we have no reason to think that the epitope recognized by the mAb impacts on its ability to support cross-presentation. Our observation that 12D7:NY-ESO-1 immune complexes are considerably less efficient than peptide-loaded DCs in stimulating IFN- $\gamma$  production illustrates that cross-presentation is a rather inefficient process, but underscores the therapeutic potential of antibodies against tumor-associated antigens.

It is well accepted now that activation of T cells *in vivo* crucially depends on antigen presentation by mature or activated DCs (14, 28). Many cues, including inflammation and infection but also endogenous signals, can induce DC maturation (29), and the lack of such signals in the tumor environment may be one reason why tumor-infiltrating T cells often have compromised functions (16, 30). Because the uptake of immune complexes was shown to result in DC maturation (19), we specifically addressed this issue here. We found that the *in vitro* uptake of immune complexes resulted in DC maturation that was comparable to sCD40L plus TNF- $\alpha$ , which is a classical maturation cocktail. Therefore, the use of mAbs against CT antigens may serve both purposes: DC activation and enhanced

Figure 4



**A human, monoclonal anti-NY-ESO-1 antibody (12D7) increases the therapeutic efficacy of 5-FU chemotherapy in mice bearing NY-ESO-1+ syngeneic tumors.** Female BALB/c mice were injected s.c. with  $10^6$  CT26/NY-ESO-1+ cells and treatment was started when tumors reached a surface of approximately  $25 \text{ mm}^2$  (~ d13-15). (A) Mice received 75 mg/kg 5-FU i.p. at days 15 and 22 and/or 100  $\mu\text{g}$  12D7 i.p. on days 17 and 24. The results are shown as mean $\pm$ SD. A representative experiment of 4 experiments is shown. (B) Compilation of 4 independent experiments, each symbol represents the tumor surface of an individual mouse at the end of the experiment (d 29). (C) Mice were injected i.p. 1 week after the last injection with 12D7 (d 29) with 250  $\mu\text{g}$  Brefeldin A and were euthanized 4 h later. Processing of tumors and staining with antibodies for CD45.2, CD8 (surface), and IFN- $\gamma$  (intracellular) was performed in the presence of 10  $\mu\text{g}/\text{mL}$  Brefeldin A. Each symbol represents values from individual mice at the end of the experiment.

cross-presentation at the relevant anatomic location. This is not trivial, as systemic activation of DCs may not be without risk as systemic side effects such as the release of cytokines or autoimmunity may ensue (31, 32).

We found that 12D7 improved the efficacy of chemotherapy in a preclinical mouse model of transplanted, syngeneic NY-ESO-1-expressing tumors, thus supporting our concept. Further support comes from the fact that more CD8+ T cells infiltrate the tumor and that those cells have increased effector function. By itself, however, 12D7 had no therapeutic effect, suggesting that the amount of released tumor antigen is limiting without deliberate destruction of the tumor. Our *in vivo* experiments require the binding of human IgG to mouse Fc $\gamma$  receptors (Fc $\gamma$ R), which was previously described (33, 34). Improved efficacy of chemotherapy by the use of tumor-associated antigen-specific antibodies will presumably work for chemotherapies especially, which are not immunosuppressive or—even more important—promote immunogenic cell death (35).

We propose the concept of antibody-facilitated T cell induction in cancer (AFTIC) as a novel type of immunotherapy. AFTIC is based on the application of mAbs against tumor-associated antigens, including CT antigens, plus a treatment that promotes the local release of those antigens, such as chemo- or radiotherapy. The locally released antigens and the mAb form immune complexes, which facilitate the uptake and subsequent presentation of antigen-derived peptides by tumor-associated DCs. As the uptake of immune complexes induces concomitant maturation of DCs, AFTIC supports boosting as well as *de novo* activation of tumor-specific CD8+ T cells. Furthermore, administration of antibodies against a particular tumor-associated antigen may promote the presentation of the same antigen when administered as a cancer vaccine, thereby improving the efficacy of immunotherapy. Alternatively, better antigen presentation of immune complexes and concomitant DC maturation may support the activity of adoptively transferred T cells provided they have the same antigen specificity as the therapeutic antibodies.

## Abbreviations

CT, Cancer/Testis; DC, dendritic cell; mAb, monoclonal antibody

## Acknowledgements

We thank Alexandre Ruffieux and Victor Escalante (Institute of Laboratory Animal Science, University of Zürich) for expert animal care. This work was supported by Atlantic Philanthropies (New York, USA), the Cancer Research Institute, the Science Foundation for Oncology (Zürich), the Ellinger Foundation Zürich, the Hartmann Müller Foundation Zürich, and the Hanne Liebermann Foundation (Zürich).

## References

- Jäger D, Jäger E, Knuth A. Immune responses to tumour antigens: implications for antigen specific immunotherapy of cancer. *J Clin Pathol* 2001; **54**: 669-674. (PMID: 11533070)
- Scanlan MJ, Güre AO, Jungbluth AA, Old LJ, Chen YT. Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. *Immunol Rev* 2002; **188**: 22-32. (PMID: 12445278)
- Scanlan MJ, Simpson AJ, Old LJ. The cancer/testis genes: review, standardization, and commentary. *Cancer Immun* 2004; **4**: 1. (PMID: 14738373)
- Jäger E, Gnjatich S, Nagata Y, Stockert E, Jäger D, Karbach J, Neumann A, Rieckenberg J, Chen YT, Ritter G, Hoffman E, Arand E, Old LJ, Knuth A. Induction of primary NY-ESO-1 immunity: CD8+ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1+ cancers. *Proc Natl Acad Sci U S A* 2000; **97**: 12198-12203. (PMID: 11027314)
- Davis ID, Chen W, Jackson H, Parente P, Shackleton M, Hopkins W, Chen Q, Dimopoulos N, Luke T, Murphy R, Scott AM, Maraskovsky E, McArthur G, MacGregor D, Sturrock S, Tai TY, Green S, Cuthbertson A, Maher D, Miloradovic L, Mitchell SV, Ritter G, Jungbluth AA, Chen YT, Gnjatich S, Hoffman EW, Old LJ, Cebon JS.

- Recombinant NY-ESO-1 protein with ISCOMATRIX adjuvant induces broad integrated antibody and CD4(+) and CD8(+) T cell responses in humans. *Proc Natl Acad Sci U S A* 2004; **101**: 10697-10702. (PMID: 15252201)
6. Gnjjatic S, Nishikawa H, Jungbluth AA, Güre AO, Ritter G, Jäger E, Knuth A, Chen YT, Old LJ. NY-ESO-1: review of an immunogenic tumor antigen. *Adv Cancer Res* 2006; **95**: 1-30. (PMID: 16860654)
  7. Jäger E, Karbach J, Gnjjatic S, Neumann A, Bender A, Valmori D, Ayyoub M, Ritter E, Ritter G, Jäger D, Panicali D, Hoffman E, Pan L, Oettgen H, Old LJ, Knuth A. Recombinant vaccinia/fowlpox NY-ESO-1 vaccines induce both humoral and cellular NY-ESO-1-specific immune responses in cancer patients. *Proc Natl Acad Sci U S A* 2006; **103**: 14453-14458. (PMID: 16984998)
  8. Bender A, Karbach J, Neumann A, Jäger D, Al-Batran SE, Atmaca A, Weidmann E, Biskamp M, Gnjjatic S, Pan L, Hoffman E, Old LJ, Knuth A, Jäger E. LUD 00-009: phase 1 study of intensive course immunization with NY-ESO-1 peptides in HLA-A2 positive patients with NY-ESO-1-expressing cancer. *Cancer Immunol* 2007; **7**: 16. (PMID: 17944437)
  9. Nicholaou T, Ebert LM, Davis ID, McArthur GA, Jackson H, Dimopoulos N, Tan B, Maraskovsky E, Miloradovic L, Hopkins W, Pan L, Venhaus R, Hoffman EW, Chen W, Cebon J. Regulatory T-cell-mediated attenuation of T-cell responses to the NY-ESO-1 ISCOMATRIX vaccine in patients with advanced malignant melanoma. *Clin Cancer Res* 2009; **15**: 2166-2173. (PMID: 19276262)
  10. Karbach J, Gnjjatic S, Bender A, Neumann A, Weidmann E, Yuan J, Ferrara CA, Hoffmann E, Old LJ, Altorki NK, Jäger E. Tumor-reactive CD8+ T-cell responses after vaccination with NY-ESO-1 peptide, CpG 7909 and Montanide ISA-51: association with survival. *Int J Cancer* 2010; **126**: 909-918. (PMID: 19728336)
  11. Karbach J, Neumann A, Atmaca A, Wahle C, Brand K, von Boehmer L, Knuth A, Bender A, Ritter G, Old LJ, Jäger E. Efficient *in vivo* priming by vaccination with recombinant NY-ESO-1 protein and CpG in antigen naive prostate cancer patients. *Clin Cancer Res* 2011; **17**: 861-870. (PMID: 21163871)
  12. Valmori D, Souleimanian NE, Tosello V, Bhardwaj N, Adams S, O'Neill D, Pavlick A, Escalon JB, Cruz CM, Angiulli A, Angiulli F, Mears G, Vogel SM, Pan L, Jungbluth AA, Hoffmann EW, Venhaus R, Ritter G, Old LJ, Ayyoub M. Vaccination with NY-ESO-1 protein and CpG in Montanide induces integrated antibody/Th1 responses and CD8 T cells through cross-priming. *Proc Natl Acad Sci U S A* 2007; **104**: 8947-8952. (PMID: 17517626)
  13. Hawiger D, Inaba K, Dorsett Y, Guo M, Mahnke K, Rivera M, Ravetch JV, Steinman RM, Nussenzweig MC. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions *in vivo*. *J Exp Med* 2001; **194**: 769-779. (PMID: 11560993)
  14. Probst HC, McCoy K, Okazaki T, Honjo T, van den Broek M. Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. *Nat Immunol* 2005; **6**: 280-286. (PMID: 15685176)
  15. Vicari AP, Chiodoni C, Vaure C, Ait-Yahia S, Dercamp C, Matsos F, Reynard O, Taverne C, Merle P, Colombo MP, O'Garra A, Trinchieri G, Caux C. Reversal of tumor-induced dendritic cell paralysis by CpG immunostimulatory oligonucleotide and anti-interleukin 10 receptor antibody. *J Exp Med* 2002; **196**: 541-549. (PMID: 12186845)
  16. Middel P, Brauneck S, Meyer W, Radzun HJ. Chemokine-mediated distribution of dendritic cell subsets in renal cell carcinoma. *BMC Cancer* 2010; **10**: 578. (PMID: 20969772)
  17. Otahal P, Hutchinson SC, Mylin LM, Tevethia MJ, Tevethia SS, Schell TD. Inefficient cross-presentation limits the CD8+ T cell response to a subdominant tumor antigen epitope. *J Immunol* 2005; **175**: 700-712. (PMID: 16002665)
  18. Petersen TR, Dickgreber N, Hermans IF. Tumor antigen presentation by dendritic cells. *Crit Rev Immunol* 2010; **30**: 345-386. (PMID: 20666707)
  19. Regnault A, Lankar D, Lacabanne V, Rodriguez A, Théry C, Rescigno M, Saito T, Verbeek S, Bonnerot C, Ricciardi-Castagnoli P, Amigorena S. Fcγ receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. *J Exp Med* 1999; **189**: 371-380. (PMID: 9892619)
  20. Cioca DP, Deak E, Cioca F, Paunescu V. Monoclonal antibodies targeted against melanoma and ovarian tumors enhance dendritic cell-mediated cross-presentation of tumor-associated antigens and efficiently cross-prime CD8+ T cells. *J Immunother* 2006; **29**: 41-52. (PMID: 16365599)
  21. Nimmerjahn F, Ravetch JV. Fc-receptors as regulators of immunity. *Adv Immunol* 2007; **96**: 179-204. (PMID: 17981207)
  22. Jäger E, Chen YT, Drijfhout JW, Karbach J, Ringhoffer M, Jäger D, Arand M, Wada H, Noguchi Y, Stockert E, Old LJ, Knuth A. Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. *J Exp Med* 1998; **187**: 265-270. (PMID: 9432985)
  23. Maio M, Coral S, Sigalotti L, Elisei R, Romei C, Rossi G, Cortini E, Colizzi F, Fenzi G, Altomonte M, Pinchera A, Vitale M. Analysis of cancer/testis antigens in sporadic medullary thyroid carcinoma: expression and humoral response to NY-ESO-1. *J Clin Endocrinol Metab* 2003; **88**: 748-754. (PMID: 12574209)
  24. Fratta E, Coral S, Covre A, Parisi G, Colizzi F, Danielli R, Marie Nicolay HJ, Sigalotti L, Maio M. The biology of cancer testis antigens: putative function, regulation and therapeutic potential. *Mol Oncol* 2011; **5**: 164-182. (PMID: 21376678)
  25. Liu F, Whitton JL. Cutting edge: re-evaluating the *in vivo* cytokine responses of CD8+ T cells during primary and secondary viral infections. *J Immunol* 2005; **174**: 5936-5940. (PMID: 15879085)
  26. Melief CJ. Cancer immunotherapy by dendritic cells. *Immunity* 2008; **29**: 372-383. (PMID: 18799145)
  27. Lugade AA, Moran JP, Gerber SA, Rose RC, Frelinger JG, Lord EM. Local radiation therapy of B16 melanoma tumors increases the generation of tumor antigen-specific effector cells that traffic to the tumor. *J Immunol* 2005; **174**: 7516-7523. (PMID: 15944250)
  28. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998; **392**: 245-252. (PMID: 9521319)