heat-killed mycobacterium tuberculosis H37RA (Difco). Mice also received 200 ng of pertussis toxin (List Biological Laboratories) i.p. in 0.2 ml of PBS on the same day and 2 days later. Clinical signs of EAE were assessed daily with a 0–6 scoring system (0, no signs; 1, tail limpness; 2, impaired righting reflex; 3, hind limb weakness; 4, hind limb paralysis; 5, hind limb paralysis with fore limb weakness; 6, death).

Isolation of Mouse B Cells and Adoptive Transfer

For B cell isolation, splenic B cells were purified by negative selection of $CD43^+$ cells with anti-CD43 magnetic beads (Miltenyi Biotec). The enriched B cell population was >95% positive for B220 staining. The B cells (2×10^7 cells) from spleen of $Mb1^{Cre/+}$ and $Prdm1^{f/f}Mb1^{Cre/+}$ mice 28 days after MOG_{35-55} immunization or wild-type and $Sell^{-/-}$ mice were transferred intravenously into μMT mice 24 hr before EAE induction.

Isolation and Stimulation of Human B Cells from Healthy Blood Donors

Mononuclear cells were isolated from peripheral blood of healthy donors by centrifugation over Ficoll-Paque PLUS (GE Healthcare). B cells were enriched by positive selection of $CD19^+$ cells with anti-human CD19 magnetic beads (Miltenyi Biotec) and were routinely >95% positive for CD19 staining. The purified B cells (5×10^5 cells/ml) were cultured for 96 hr with IL-2 (10 ng/ml; R&D), IL-6 (10 ng/ml; R&D), and CpG ODN 2006 (CpG; 1 μg/ml; InvivoGen) in the presence of IFN-α (1,000 U/ml; PBL Biomedical Laboratories) or IFN-β (1,000 U/ml; PBL Biomedical Laboratories). This study was approved by the ethics committees of Research Institute for Microbial Diseases, Osaka University. Healthy volunteers were recruited after obtaining informed consent.

ELISA and ELISPOT

MOG-specific IgG in serum was measured by ELISA with a plate coated with 10 μg/ml MOG_{35-55} and then detected with goat anti-mouse IgG and HRP-conjugated anti-goat IgG Abs (SouthernBiotech). For measurement of cytokine release by autoantigen-reactive lymphocytes, single-cell suspensions of the dLNs prepared from mice 14 days after EAE induction were cultured with a range of MOG_{35-55} doses for 48 hr. For measurement of IL-10 production by mouse B cells, purified B cells (1×10^6 cells/ml) were cultured for 48 hr with 10 μg/ml of LPS (Sigma-Aldrich) and then stimulated with 10 μg/ml of anti-mouse IgM $F(ab)'_2$ (Jackson Immunoresearch). In some experiments, $CD19^+CD138^-$ and $CD138^+CD44^{hi}$ cells harvested from dLNs of wild-type mice 14 days after EAE induction were stimulated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) plus 1 μM ionomycin (Sigma-Aldrich) for 5 or 24 hr. For measurement of IL-10 and IgM production by human peripheral blood B cells, purified B cells (5×10^5 cells/ml) were cultured for 96 hr with IL-2, IL-6, IFN-α, and CpG. IFN-γ, IgM, IL-4, IL-6, IL-10, IL-13, IL-17a, IL-27, IL-35, and TGF-β1 in the culture medium were detected by ELISA and Bio-Plex suspension assay according to the manufacturer's protocol (Biolegend, BIO-RAD, Bethyl Laboratories, or R&D). IgM secretion by human B cells was detected by ELISPOT according to the manufacturer's protocol (R&D).

Statistical Analysis

We performed statistical evaluation with Prism software (GraphPad). A two-tailed, unpaired Student's t test was applied for statistical comparison of two groups. In case of unequal variance, t test with Welch's correction was used. Comparisons of two nonparametric data sets were done by the Mann-Whitney U test. A p value of less than 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.10.016.

AUTHOR CONTRIBUTIONS

M.M. planned and performed most of the experiments, analyzed data, and prepared the manuscript; A.B. assisted with experiments; T.Y. contributed to morphological characterization of cultured cells; Y.O. provided technical contributions to ChIP assays; H.K. and K.T. provided $Il10^{Venus/+}$ reporter mice; H.N. and S.S. collected human blood and provided reagents; A.K. and S.L.N. provided $Prdm1^{gfp/+}$ mice and edited the manuscript; Y.B. and T.K. supervised the project; T.K. contributed to manuscript writing; and Y.B. designed the study, performed some experiments, interpreted data, and wrote the manuscript.

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Current Opinion in Immunology

Regulatory T cells in cancer immunotherapy

Hiroyoshi Nishikawa and Shimon Sakaguchi

FOXP3$^+$CD25$^+$CD4$^+$ regulatory T (Treg) cells, crucial for the maintenance of immunological self-tolerance, are abundant in tumors. Most of them are chemo-attracted to tumor tissues, expanding locally and differentiating into a Treg-cell subpopulation that strongly suppresses the activation and expansion of tumor-antigen-specific effector T cells. Several cancer immunotherapies targeting FOXP3$^+$CD4$^+$ Treg cells, including depletion of Treg cells, are currently being tested in the clinic. In addition, clinical benefit of immune-checkpoint blockade, such as anti-CTLA-4 monoclonal antibody therapy, could be attributed at least in part to depletion of FOXP3$^+$CD4$^+$ Treg cells from tumor tissues. Thus, optimal strategies need to be established for reducing Treg cells or attenuating their suppressive activity in tumor tissues, together with activating and expanding tumor-specific effector T cells.

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Introduction

Since the molecular characterization of tumor antigens that are recognized by tumor-reactive antibodies (Ab) and cytotoxic T-lymphocytes (CTLs) in cancer patients, therapeutic vaccination with the tumor antigens has been explored in the clinic as an antigen-specific cancer immunotherapy [1–4]. However, only a minor fraction of patients have exhibited tumor regression after multiple vaccinations despite their development of measurable humoral and cellular immune responses against tumor antigens [5•,6,7]. To improve the efficacy of cancer vaccine, efforts have been made in these two decades to discover more immunogenic tumor-associated antigens and devise more effective ways of immunization, for example, by the use of various adjuvants, tumor antigen-expressing vectors and antigen-pulsed dendritic cells. In addition, it has become evident that the immunosuppressive elements present in cancer patients are critical impediments to the success of cancer immunotherapy [7–10]. One of the obstacles is CD25$^+$CD4$^+$ regulatory T (Treg) cells expressing the transcription factor FOXP3, which are physiologically present in the immune system and actively engaged in the maintenance of immunological self-tolerance by suppressing self-reactive T cells [11]. Considering that most tumor-associated antigens identified to date are antigenically normal self-constituents, it is likely that naturally occurring FOXP3$^+$ Treg cells also hamper effective anti-tumor immune responses in cancer patients and that they can be one of the cellular targets to evoke and augment anti-tumor immunity [2–4,9,12].

FOXP3$^+$ T cells in humans are heterogeneous in phenotype and function, including suppressive and non-suppressive subpopulations [13]. For example, naive CD4$^+$ T cells transiently express FOXP3 at a low level upon *in vitro* T-cell receptor (TCR) stimulation; yet they are hardly suppressive [13,14••]. The attempts to delineate suppressive or non-suppressive FOXP3$^+$CD4$^+$ T cells present in the peripheral blood have shown that FOXP3$^+$CD4$^+$ T cells can be dissected into three subpopulations by the expression levels of FOXP3 and the cell surface molecules CD45RA and CD25 (Figure 1): (i) FOXP3^{lo}CD45RA$^+$CD25^{lo} cells (Fraction [Fr.] I), designated naive or resting Treg cells, which differentiate into FOXP3^{hi}CD45RA$^-$CD25^{hi} cells (Fr. 2) upon antigenic stimulation; (ii) FOXP3^{hi}CD45RA$^-$CD25^{hi} cells (Fr.2), designated eTreg cells, which are terminally differentiated and highly suppressive; and (iii) FOXP3^{lo}CD45RA$^-$CD25^{lo} non-Treg cells (Fr. III), which do not possess suppressive activity but can secrete pro-inflammatory cytokines [14••]. This classification of FOXP3$^+$CD4$^+$ T cells is instrumental in defining suppressive or non-suppressive FOXP3$^+$ subpopulations, delineating developmental stages of Treg cells, and assessing their adaptive processes in physiological and pathological immune responses.

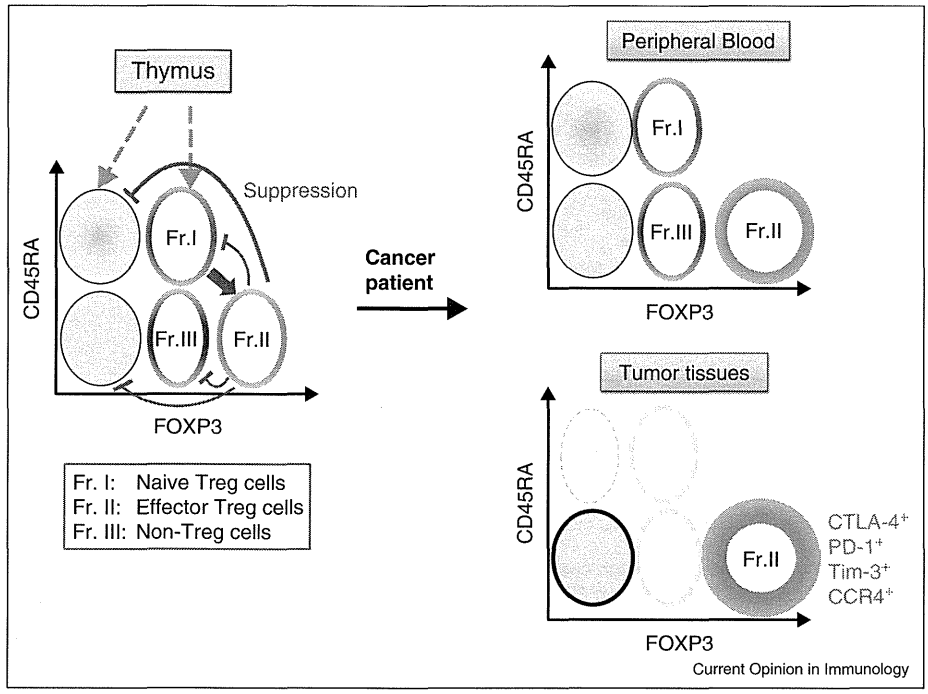
This review focuses on our current understanding of the roles of FOXP3$^+$ Treg cells in tumor immunity in humans and discusses a perspective for numerical or functional manipulation of Treg cells as a key strategy in cancer immunotherapy.

Roles of Treg cells in tumor immunity

Treg-cell infiltration is associated with tumor progression

Treg cells are found at high frequencies in tumor tissues of various types of cancers such as breast, lung, liver, pancreatic and gastrointestinal cancers and malignant melanoma (reviewed in [9]). The presence of large proportions of CD4$^+$ Treg cells among tumor-infiltrating

Figure 1



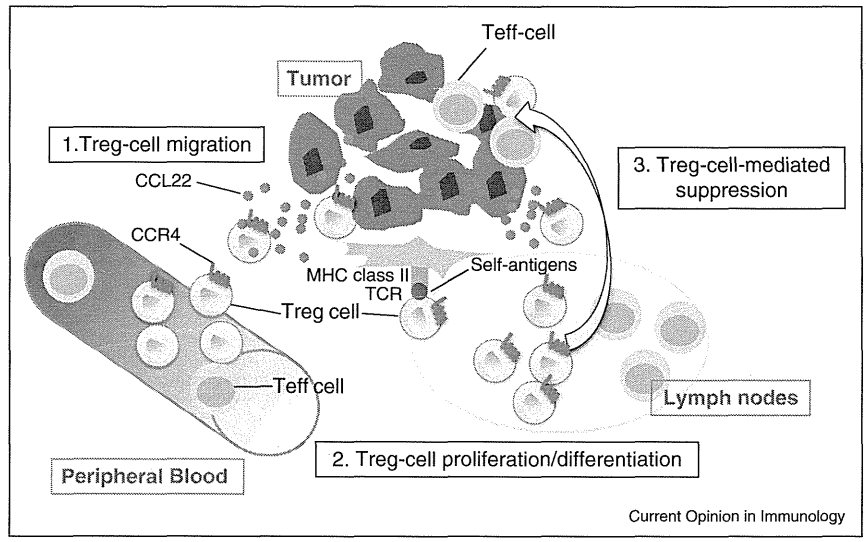
(Left) FOXP3$^+$CD4$^+$ T cells are dissected into three subpopulations by the expression levels of FOXP3 and the cell surface molecule CD45RA: FOXP3^{lo}CD45RA$^+$ cells (Fr. I), designated naive or resting Treg cells, which differentiate into FOXP3^{hi}CD45RA$^-$ cells (Fr. II), designated eTreg cells. FOXP3^{lo}CD45RA$^-$ non-Treg cells (Fr. III) are not suppressive. eTreg cells (Fr. II) are suppressive on other FOXP3$^+$ or FOXP3$^-$ T cells, in particular, on CD45RA^{hi} naive CD4$^+$ T cells. (Right) eTreg cells (Fr. II) are dominant in tumor tissues but not in the peripheral blood. These eTreg cells express CTLA-4, PD-1, CCR4 and Tim-3.

lymphocytes (TILs) [15•], in particular, decreased ratios of CD8$^+$ T cells to FOXP3$^+$CD25$^+$CD4$^+$ Treg cells among TILs [16•], is associated with poor prognosis in ovarian, breast, and gastric cancers (reviewed in [9]). These findings suggest that tumor-reactive CD8$^+$ CTLs are suppressed by FOXP3$^+$ Treg cells in tumor tissues. In contrast, there are some reports that high infiltration of FOXP3$^+$ Treg cells is associated with better prognosis in colon and head/neck cancers and Hodgkin lymphoma [17–19]. This apparent inconsistency can be, at least in part, attributed to different compositions of FOXP3$^+$ T-cell subpopulations in tumor tissues (Figure 1 and [14••]). Melanoma-infiltrating TILs predominantly contained eTreg cells (Fr. II), with very low frequencies of naive Treg cells (Fr. I) and FOXP3$^+$ non-Treg cells (Fr. III), compared with the composition of the corresponding FOXP3$^+$ subpopulations in the peripheral blood [20••]. In contrast, FOXP3$^+$ T cells infiltrating into colon cancers contained higher frequencies of non-Treg cells (Fr. III) as well as eTreg cells (Fr. II) (H.N. and S.S., unpublished data). The result indicates that the increased FOXP3$^+$ non-Treg cells, which are capable of secreting proinflammatory cytokines, could contribute to the better prognosis of some colon cancer patients even if they harbor high frequencies of total FOXP3$^+$ TILs. Further studies are required in various types of cancers to assess not only the number of FOXP3$^+$ TILs but also the composition of FOXP3$^+$ subpopulations among TILs, and to evaluate such parameters in terms of their relationship with the extent of anti-tumor immune responses and the prognosis of patients.

How do Treg cells infiltrate into tumor tissues?

As a likely mechanism by which Treg cells become abundant in tumor tissues, it has been proposed that tumor cells and/or tumor infiltrating macrophages produce the chemokine (C–C motif) ligand 22 (CCL22), which chemo-attracts and recruits to tumor tissues FOXP3$^+$CD4$^+$ Treg cells expressing C–C chemokine receptor type 4 (CCR4) (Figure 2 and [9,15•,20••,21]). Other combinations of chemokines and chemokine receptors, such as CCR10-CCL28 and CXC chemokine receptor (CXCR) 3-CXCR3 ligands (such as CXCL9, 10, and 11), also reportedly contribute to Treg-cell infiltration [22,23]. It remains obscure whether conventional T cells can differentiate into suppression-competent FOXP3$^+$ Treg cells in tumor microenvironments in humans. After the promoted migration to tumor tissues from the circulation, FOXP3$^+$CD4$^+$ Treg cells are activated and expand presumably via recognizing tumor-associated antigens or

Figure 2



FOXP3$^+$CD4$^+$ Treg cells infiltrate into tumor tissues through chemo-attraction mainly via CCR4–CCL22 and recognize self-antigens including tumor antigens present in tumor tissues. Proliferated/differentiated FOXP3$^+$CD4$^+$ Treg cells (mainly eTreg cells) efficiently suppress the activation of tumor antigen-specific effector T cells. To evoke and augment anti-tumor immune responses, Treg cells can be targeted at (1) Treg-cell migration, (2) Treg-cell proliferation/differentiation and (3) Treg-cell-mediated suppression.

self-antigens released from dying tumor cells (Figure 2). Indeed, Treg cells in cancer patients recognize a broad range of tumor antigens including NY-ESO-1 and Survivin, and suppress tumor-antigen-specific effector T cells [24–26,27••,28], as previously shown with mice [29]. Compared with tumor-reactive effector or memory CD4$^+$ T cells, natural FOXP3$^+$ Treg cells may be better at recognizing tumor-associated self-antigens because of their TCR repertoires being more self-reactive than those of conventional T cells and their higher level expression of T cell accessory molecules including adhesion molecules (such as LFA-1) indicative of their 'antigen-primed' states [30,31]. This Treg-cell dominant immune-suppressive tumor microenvironment implies that cancer vaccines composed of proteins or long peptides (>15 amino acids) of tumor antigens may preferentially activate tumor-antigen-specific Treg cells, rather than antigen-specific effector T cells [32], augmenting suppression by the former on the latter unless proper strategies to block Treg-cell activation or suppressive function are taken.

Treg cells suppress the activation of tumor-antigen-specific T cells

Are natural Treg cells indeed suppressing the activation and expansion of tumor-antigen-specific effector T cells in healthy individuals and cancer patients? Direct evidence for the case was provided by immune responses to NY-ESO-1, one of the most immunogenic cancer/testis antigens [6,33]. For example, *in vitro* NY-ESO-1 peptide stimulation of peripheral blood lymphocytes after depletion of CD25$^+$CD4$^+$ Treg cells was able to activate NY-ESO-1-specific naive CD4$^+$ T-cell precursors in healthy individuals and in melanoma patients who possessed NY-ESO-1-expressing tumors but failed to develop anti-NY-ESO-1 Ab [34•,35•]. In contrast, most NY-ESO-1-specific CD4$^+$ T cells in melanoma patients who had spontaneously developed anti-NY-ESO-1 Ab were derived from a memory population and could be activated even in the presence of CD25$^+$CD4$^+$ Treg cells [35•]. In addition, following vaccination of ovarian cancer patients with an HLA-DP-restricted NY-ESO-1 peptide, the development of NY-ESO-1-specific high-avidity effector T cells from naive T cells was hampered by the presence of CD25$^+$CD4$^+$ Treg cells, although the vaccination could expand low-avidity NY-ESO-1-specific CD4$^+$ T cells present in an effector/memory fraction before the vaccination [36]. These results collectively indicate that healthy individuals and cancer patients harbor potentially tumor-reactive T cells, whose activation and expansion are suppressed by natural Treg cells, and that Treg-cell depletion is able to activate and expand NY-ESO-1-specific high-avidity T cells from naive T-cell precursors, allowing their differentiation into high-avidity effector T cells capable of mediating potent anti-tumor immune responses.

Immunotherapy targeting Treg cells

Depletion of Treg cells or their functional alteration

As Treg cells constitutively express the high-affinity IL-2 receptor, CD25 (IL-2 receptor α-chain) can be suitable for Treg-cell depletion [37]. In animal models,

administration of cell-depleting anti-CD25 monoclonal Ab (mAb) before tumor inoculation resulted in tumor eradication [12,38]; in humans, removal of $CD25^+CD4^+$ Treg cells from PBLs induced tumor antigen-specific T cells *in vitro* as discussed earlier [34•,35•]. In clinical trials with anti-CD25 mAb or denileukin diftitox (DAB_{389}IL-2), which is an immunotoxin-conjugated IL-2, some studies have shown the potential of $CD25^+$ T cell depletion to augment anti-tumor immune responses; yet, other similar studies failed to obtain clinically evident augmentation [39–42]. Since activated effector T cells also express CD25 and their production of IL-2 is required for the expansion of $CD8^+$ CTLs [43], CD25-based cell depletion may reduce activated effector T cells as well, canceling the effect of Treg-cell depletion to augment anti-tumor immunity.

Chemokine receptors are another candidate for Treg-cell depletion. CCR4 was shown to be specifically expressed by $FOXP3^{hi}CD45RA^-CD25^{hi}$ eTreg cells, but not by $CD45RA^+FOXP3^{lo}CD4^+$ naive Treg cells or most effector T cells in peripheral blood (Figure 1 and [20••]). With depletion of $CCR4^+$ T cells and subsequent *in vitro* cancer/testis antigen NY-ESO-1 peptide stimulation, NY-ESO-1-specific $CD4^+$ T cells were efficiently activated in a similar manner as observed following $CD25^+$ T-cell depletion. $CCR4^+$ T-cell depletion also augmented *in vitro* induction of NY-ESO-1-specific $CD8^+$ T cells in melanoma patients. In addition, $CCR4^+$ eTreg cells were predominant among melanoma-infiltrating $FOXP3^+$ T cells and much higher in frequency compared with those in peripheral blood [20••]. Anti-CCR4 mAb is therefore instrumental for evoking and augmenting anti-tumor immunity in cancer patients by selectively depleting eTreg cells and is now being tested in the clinic.

Other molecules predominantly expressed by Treg cells can also be targeted for cell depletion as well as functional manipulation. GITR (glucocorticoid-induced TNF-receptor family related protein) is a co-stimulatory molecule expressed at low levels on resting $CD4^+$ and $CD8^+$ T cells and constitutively on $FOXP3^+CD4^+$ Treg cells at high levels [44]. Activation of GITR signaling with agonistic anti-GITR mAb or GITR ligands can inhibit the suppressive activity of $FOXP3^+CD4^+$ Treg cells and make effector T cells resistant to $FOXP3^+CD4^+$ Treg-cell-mediated suppression [44–46]. Another candidate molecule is OX40, a co-stimulatory molecule of the TNF receptor family. It is transiently expressed on activated T cells and constitutively expressed on $FOXP3^+CD4^+$ Treg cells. Previous studies using agonistic anti-OX40 mAb have shown that the mAb mediates anti-tumor effects by attenuating $FOXP3^+CD4^+$ Treg-mediated suppression and activating effector T-cell function [47,48]. Therapies targeting GITR and OX40 are currently in clinical trials.

Immune-checkpoint blockade with possible effects on Treg cells

Immune-checkpoint blockade by mAb such as anti-cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) mAb and anti-programmed cell death protein 1 (PD-1) mAb is currently being tested extensively with various types of solid tumors and has provided promising clinical results [10]. In fact, Ipilimumab, a humanized anti-CTLA-4 mAb, was approved by the FDA for the treatment of malignant melanoma based on the clinical data from phase III clinical trials [49••,50]. CTLA-4 is constitutively expressed by $FOXP3^+CD4^+$ Treg cells and is up regulated by $CD4^+$ and $CD8^+$ effector T cells after activation. It was originally thought that anti-CTLA mAb would block an inhibitory signal on activated $CD4^+$ and $CD8^+$ effector T cells and recover their anti-tumor activity [51,52]. Recent animal studies using anti-CTLA-4 mAb lacking the ADCC (antibody-dependent cellular cytotoxicity) activity by modulating Fc portion or Fc receptor knockout mice have shown that anti-tumor activity of anti-CTLA-4 mAb was attributed to depletion of $FOXP3^+CD4^+$ Treg cells from tumor tissues, rather than direct activation of effector T cells [53•,54•,55•]. Indeed, decreased numbers of $FOXP3^+$ Treg cells in tumor tissues following anti-CTLA-4 mAb (Ipilimumab, IgG1 subclass) treatment were strongly correlated with clinical benefit [56,57]. In addition, the crucial roles of CTLA-4 for $FOXP3^+CD4^+$ Treg-cell function was revealed in animal studies, which showed that specific deficiency of CTLA-4 in $FOXP3^+CD4^+$ Treg cells impaired their suppressive function and thereby augmented anti-tumor immunity [58,59].

While most anti-PD-1 mAbs and anti-PD-L1 mAbs investigated in the clinic are of the IgG4 subclass [60–62], which is not cell-depleting, it remains to evaluate Treg-cell function in tumor tissues because PD-1 is also highly expressed by tumor-infiltrating Treg cells ([63] and H.N. and S.S., unpublished data). As the PD-1 mAb CT-011 is of the IgG1 subclass, it is of interest to compare clinical benefits among the anti-PD-1 mAbs with different isotypes [64]. Immune-checkpoint blockade targeting other molecules such as LAG3 is also under clinical studies (http://clinicaltrials.gov/). Considering the expression of these molecules on $FOXP3^+CD4^+$ Treg cells as well, it is worth exploring whether mAbs specific for the molecules may have some effects on $FOXP3^+CD4^+$ Treg cells, in particular, those in tumor tissues.

Chemicals or drugs preferentially affecting Treg cells

In addition to biologicals for Treg-cell depletion or functional alteration, anti-cancer drugs such as cyclophosphamide and fludarabine can selectively affect Treg cells [65,66], presumably because natural Treg cells are physiologically in a more proliferative state than other

T cells via recognizing self-antigens or commensal microbes. Supporting this effect, multiple peptide vaccine combined with a single dose of cyclophosphamide reduced the number of Treg cells and induced strong immune responses against multiple tumor antigen peptides, with longer survival of renal cell carcinoma patients [66]. In addition to such radiomimetic drugs affecting proliferative T cells, there may be other chemicals that differentially affect Treg cells and effector T cells via exploiting, for example, different cytokine or metabolic sensitivities, thereby tipping their balance toward the dominance of effector T cells to augment anti-tumor immunity.

Conclusions and perspectives

The vital roles of Treg cells in tumor immunity are now widely accepted and Treg-cell targeting therapy is under active investigation. For clinical application of these therapies, there are some issues to be considered. One is how deleterious autoimmunity possibly accompanying Treg-cell depletion can be circumvented. In addition to optimizing the degree and duration of the depletion, it is critical to target a Treg-cell subpopulation, rather than whole FOXP3$^+$ cells, to evoke effective anti-tumor immunity while avoiding autoimmunity. One possible way is to specifically control eTreg cells, which are predominant in tumor tissues and most suppressive. For example, cell-depleting anti-CCR4 mAb and anti-CTLA-4 mAb deplete eTerg cells but not naive Treg cells because the latter expresses CCR4 and CTLA-4 at much lower levels than the former. The preserved naive Treg cells are sufficient to suppress serious autoimmunity [14••,20••]. It also needs to consider that Treg cells and activated effector T cells share common phenotypes; for example, both express CD25, CTLA-4, PD-1 and GITR, although at different levels. This implies that cell-depleting mAbs specific for these molecules could deplete tumor-reactive activated effector T cells as well, reducing the anti-tumor effects by Treg-cell depletion [45]. The timing and dose of mAb administration can be essential factors for differential control of Treg cells and effector T cells involved in tumor immunity. It is also critically important to monitor tumor-infiltrating Treg cells, in particular Treg-cell subpopulations, rather than circulating Treg cells, before and after immunological intervention to predict the efficacy of Treg-cell depletion or functional modulation for evoking or augmenting anti-tumor immunity [7]. It is envisaged that combinations of Treg-cell targeting (e.g., by reducing Treg cells or attenuating their suppressive activity in tumor tissues) with the activation of tumor-specific effector T cells (e.g., by cancer vaccine) will make the current cancer immunotherapy more effective in the clinic.

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RNAi-induced loss of *pat-10* disrupts endocytosis through impairment of the actin cytoskeleton (*12*, *13*, *15*). To assay the role of *pat-10* in endocytosis, we used a secretion and endocytosis reporter designed to actively secrete GFP (ssGFP) from muscle cells into the pseudocoelomic fluid, where it is endocytosed by the coelomocyte cells and degraded (fig. S9A) (*16*). Therefore, the ssGFP reports upon effective muscular secretion and endocytosis by coelomocytes. Fitting the hypothesis that *pat-10* overexpression improves transport and cellular processing through improved subcellular scaffolding, the *pat-10 OE* strain had a decrease in overall ssGFP fluorescence (Fig. 3, E and F). The decrease in ssGFP resulted from improved secretion and uptake, as shown by the absence of fluorescence in the muscle and pseudocoelomic fluid (Fig. 3E). This decrease was not due to an overall decrease in expression of GFP (fig. S9B). Conversely, RNAi of *pat-10* increased overall fluorescence through decreased muscle secretion and coelomocytic endocytosis (Fig. 3, E and G). To fully block coelomocytic uptake and degradation of ssGFP, RNAi of *cup-4*, a ligand-gated ion channel required in endocytosis (*17*), showed an even higher increase in fluorescence (Fig. 3G) and also reduced thermotolerance in the wild type (Fig. 3H). Collectively, these data indicate *pat-10* has an active role in cytoskeletal maintenance, which is critical to cellular transport.

To test for conservation, we disrupted the actin cytoskeleton in human embryonic kidney (HEK) 293T cells using cytochalasin D, which blocks the addition of actin monomers to filaments (*18*), or latrunculin A, which binds actin monomers and prevents polymerization (Fig. 4A) (*19*). Inhibiting filamentous actin formation with either cytochalasin D or latrunculin A significantly reduced thermotolerance in human cells without causing death at permissive temperatures (Fig. 4B and fig. S10). Similar to our *C. elegans* data, these findings reiterate the importance of the actin cytoskeleton during times of cellular stress.

Elevated levels of *hsf-1* have been shown to benefit multiple organisms, yet its oncogenic properties are a major therapeutic drawback (*20*, *21*). Because the inducible chaperone network promotes survival and proliferation of metastasizing cells (*22*), the ability to harness protective, non-chaperone components within the HSF-1 signal transduction cascade appears essential for future drug development. Identification of *pat-10* as a modifier of thermotolerance and longevity may apply to mammalian systems without the typical oncogenic dangers associated with increased chaperone levels.

The *hsf-1(CT)* strain was still able to mount a transcriptional response to heat shock, albeit reduced in complexity of *hsf-1(FL)*. The molecular mechanism remains unclear by which *hsf-1(CT)* regulates transcription without the C-terminal activation domain, but possible explanations include HSF-1 containing multiple activation domains. Alternatively, the *hsf-1(CT)* modification may alter affinities to DNA-binding sites or different cofactors, which would modify the transcriptional profile.

Our findings underscore the importance of maintaining filamentous actin, as opposed to total levels of actin. We propose a model in which HSF-1 regulates chaperones and actin cytoskeletal genes in parallel to promote thermotolerance and longevity (Fig. 4C). In the absence of chaperone induction, stabilization of the actin cytoskeleton is sufficient to promote survival under conditions of cellular stress and aging.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/346/6207/360/suppl/DC1
Materials and Methods
Figs. S1 to S10
Tables S1
References (*23–40*)

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AUTOIMMUNITY

Detection of T cell responses to a ubiquitous cellular protein in autoimmune disease

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T cells that mediate autoimmune diseases such as rheumatoid arthritis (RA) are difficult to characterize because they are likely to be deleted or inactivated in the thymus if the self antigens they recognize are ubiquitously expressed. One way to obtain and analyze these autoimmune T cells is to alter T cell receptor (TCR) signaling in developing T cells to change their sensitivity to thymic negative selection, thereby allowing their thymic production. From mice thus engineered to generate T cells mediating autoimmune arthritis, we isolated arthritogenic TCRs and characterized the self antigens they recognized. One of them was the ubiquitously expressed 60S ribosomal protein L23a (RPL23A), with which T cells and autoantibodies from RA patients reacted. This strategy may improve our understanding of the underlying drivers of autoimmunity.

T cells mediate a variety of autoimmune diseases (*1*, *2*), likely through the recognition of self antigens. However, identification of the self antigens targeted by T cells in systemic autoimmune diseases such as rheumatoid arthritis (RA) has been technically difficult (*3–5*). This is because pathogenic T cells expressing high-affinity T cell receptors (TCRs) for ubiquitous self antigens may be largely deleted (i.e., negatively selected) in the thymus and

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scarcely detectable in the periphery or, if detected, in an inactivated state (*6*). This can be circumvented by altering TCR signaling, which changes the sensitivity of developing T cells to thymic selection and results in new dominant self-reactive TCR specificities that are causative of systemic autoimmune diseases (*7*–*11*). For example, a hypomorphic point mutation of ζ-associated protein 70 (ZAP-70), a TCR-proximal signaling molecule, causes T cell–mediated spontaneous autoimmune arthritis in mice, which resembles RA (*8*).

To identify ubiquitously expressed self antigens commonly targeted in mouse and human systemic autoimmune disease, we first examined whether the arthritogenic $CD4^+$ T helper (T_H) cells in BALB/c SKG mice, which develop autoimmune arthritis due to the ZAP-70 mutation, made use of a specific dominant TCR. We compared the arthritogenic capacity of SKG $CD4^+$ T cells expressing different TCR Vβ subfamilies (fig. S1). Transfer of SKG $CD4^+$ T cells expressing Vβ6, Vβ8.1/8.2, or Vβ10 into BALB/c *Rag2*$^{-/-}$ mice induced arthritis with similar severities. In addition, CDR3 gene segments of $Vβ6^+$ $CD4^+$ T cells in arthritic joints were diverse, with few common sequences among individual arthritic SKG mice (fig. S2 and tables S1 and S2). Thus, under the assumption that arthritogenic SKG $CD4^+$ T cells are highly polyclonal and make use of various Vα and Vβ TCR chains, we attempted to isolate a single arthritogenic $CD4^+$ T cell from a particular $CD4^+$ T cell subpopulation—for example, those expressing Vα2 and Vβ6, which constituted ~1% of joint-infiltrating $CD4^+$ T cells. To differentiate arthritogenic $CD4^+$ T cells from forkhead box P3 (Foxp3)–expressing $CD4^+$ regulatory T (T_{reg}) cells (*1*), we used SKG mice with knock-in of enhanced green fluorescent protein (EGFP)–Foxp3 fusion protein, designated eFOX SKG mice, which also spontaneously developed arthritis (fig. S3). We cloned a single TCR pair

A

Clone	Vα	Jα	CDR3	Vβ	Jβ	CDR3	Arthritogenic	Positive cells / Analyzed cells
1-23	14D-3/DV8*03	26*01	CAAS KG YAQGLTF	19*01	2-4*01	CASSI GGTGP SQNTLYF	No	1 / 12 (8.3%)
7-39	14D-3/DV8*03	40*01	CA AIY TGNYKYVF	19*01	2-1*01	CASS WRWGGER AEQFF	Yes	27 / 30 (90%)
6-39	14D-3/DV8*03	52*01	CAAS AA GANTGKLTF	19*01	2-7*01	CAS TPTF SYEQYF	Yes	2 / 26 (8%)

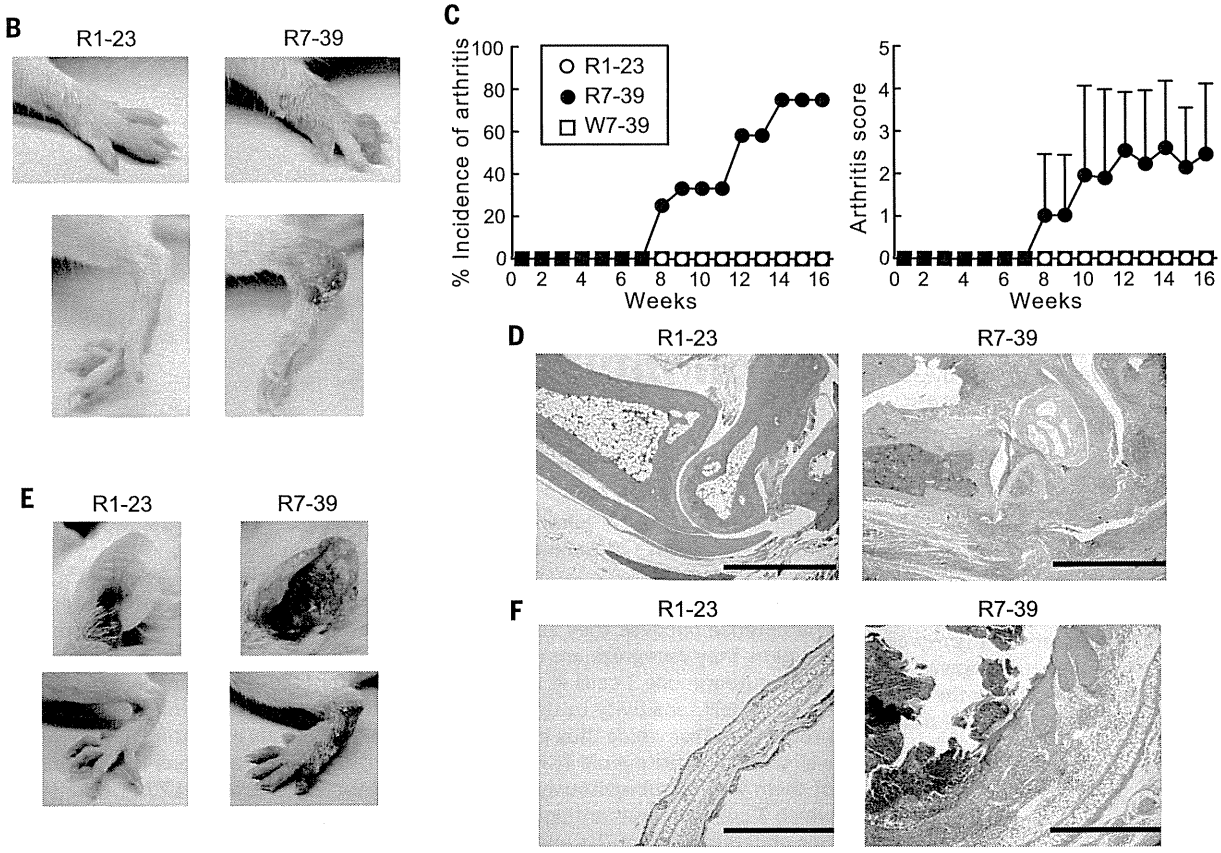

Fig. 1. Arthritis-inducing activity of two TCRs individually expressed in retrogenic mice. (**A**) Amino acid sequences and frequencies of two arthritogenic TCRs (7-39 and 6-39) and the nonarthritogenic 1-23 TCR. These three TCRs were obtained from three different mice. CDR, complementarity-determining region. Amino acid abbreviations: A, Ala; C, Cys; E, Glu; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. (**B**) Joint swelling in R7-39 retrogenic mice. (**C**) Incidence and scores of spontaneous arthritis in R7-39 (n = 11), R1-23 (n = 14), and W7-39 mice (n = 8). Error bars indicate means ± SD. (**D**) Hematoxylin and eosin (HE) staining of arthritic joints; scale bar, 1 mm. (**E**) Ears and hind paws of R7-39 and R1-23 mice. (**F**) HE staining of ears from R7-39 and R1-23 mice; scale bar, 500 μm. Results in (B) and in (D) to (F) represent three independent experiments.

from individual GFP$^-$ Vα2$^+$ Vβ6$^+$ CD4$^+$ T cells present in arthritic joints of eFOX SKG mice, transfected *Rag2*$^{-/-}$ SKG bone marrow (BM) cells with the TCR gene, and transferred the BM cells into *Rag2*$^{-/-}$ mice to construct retrogenic mice expressing the TCR pair in developing T cells (*12–15*). Among nine retrogenic strains each expressing a distinct TCR, those expressing 7-39 or 6-39 TCRs spontaneously developed arthritis at incidences of 80.0% and 27.3%, respectively (Fig. 1, A to C, and fig. S4, A to C). The two arthritogenic TCRs and a control nonarthritogenic 1-23 TCR used the same Vα and Vβ gene segments but different Jα and Jβ genes and CDR3 sequences (Fig. 1A). Arthritic joints in retrogenic 7-39 (R7-39) mice showed mononuclear cell infiltration, pannus formation, and cartilage destruction (Fig. 1D). Some (66.7%) of the R7-39, but not the R6-39, mice also developed chronic dermatitis, which exhibited hyperkeratosis and parakeratosis, histopathological features of human psoriasis (*16*) (Fig. 1, E and F, and fig. S5). Other organs were histologically intact (fig. S6).

In R7-39 mice, 7-39 TCR–transduced cells preferentially differentiated into monoclonal CD4$^+$ T cells with an activated and memory phenotype (fig. S7), and were able to transfer both arthritis and dermatitis into other *Rag2*$^{-/-}$ mice. Both arthritic R7-39 and nonarthritic R1-23 mice failed to develop Foxp3$^+$ T_{reg} cells (fig. S8). In contrast to 7-39 TCR gene–transfected *Rag2*$^{-/-}$ BM cells with the SKG ZAP-70 mutation, 7-39 TCR gene–transfected ZAP-70–intact *Rag2*$^{-/-}$ BALB/c BM cells did not cause arthritis in retrogenic mice (designated W7-39 mice). In W7-39 mice, the majority of 7-39 TCR–expressing CD4$^+$ T cells were negatively selected in the thymus, and those that had escaped thymic negative selection exhibited a naïve nonactivated phenotype, indicating their dormant or anergic state (Fig. 1C and fig. S9).

Taken together, these results demonstrate that CD4$^+$ T cells with a specific TCR mediate autoimmune arthritis and also dermatitis, and that more than one TCR specificity is individually able to confer T cell arthritogenicity.

We next constructed T cell hybridomas expressing 7-39 or 6-39 TCRs and attempted to determine the self antigens recognized by these TCRs. The 7-39 hybridoma cells produced interleukin-2 (IL-2) when stimulated by cell extracts not only from SKG fibroblast-like synoviocytes (FLSs) but also from P3U1 cells, a BALB/c plasma cell–derived cell line (fig. S10). In contrast, syngeneic antigen-presenting cells (APCs) were sufficient to induce IL-2 production by 6-39 hybridoma cells, indicating that the 6-39 TCRs recognized a self antigen constitutively displayed by APCs (fig. S4D). To further characterize the self antigen recognized by 7-39 TCRs, we reconstituted *Rag2*$^{-/-}$ mice with a mixture of 7-39 TCR–transfected *Rag2*$^{-/-}$ SKG BM cells and *TCR*β$^{-/-}$ BALB/c BM cells on the assumption that the autoantibodies produced by B cells might specifically react with the self antigen recognized by 7-39 TCRs because T cell help came solely from 7-39 T_H cells. The sera from these "B cell–reconstituted" mice specifically reacted with an 18-kD protein from the cell extract of P3U1 cells (Fig. 2A). Mass spectrometric analysis identified this protein as RPL23A, a component of the 60*S* subunit of ribosomes (*17, 18*) (fig. S11). Various organs were found to express *RPL23A* mRNA at high levels (Fig. 2B). The amino acid sequence of RPL23A is 100% conserved between mice and humans (*18*). The sera from the B cell–reconstituted R7-39 mice indeed recognized recombinant RPL23A, but not histone H1.2 protein, another candidate protein indicated by the mass spectrometric analysis (Fig. 2C). In addition, recombinant RPL23A protein specifically stimulated the 7-39 hybridoma cells in a dose-dependent, class II major histocompatibility complex (MHC) I-A^d–dependent manner (Fig. 2D and fig. S12). Among 20-amino acid RPL23A peptides with consecutive overlapping of 5 amino acid residues, $RPL23A_{71\text{-}90}$ peptide stimulated 7-39 TCRs most potently (table S3 and fig. S13A).

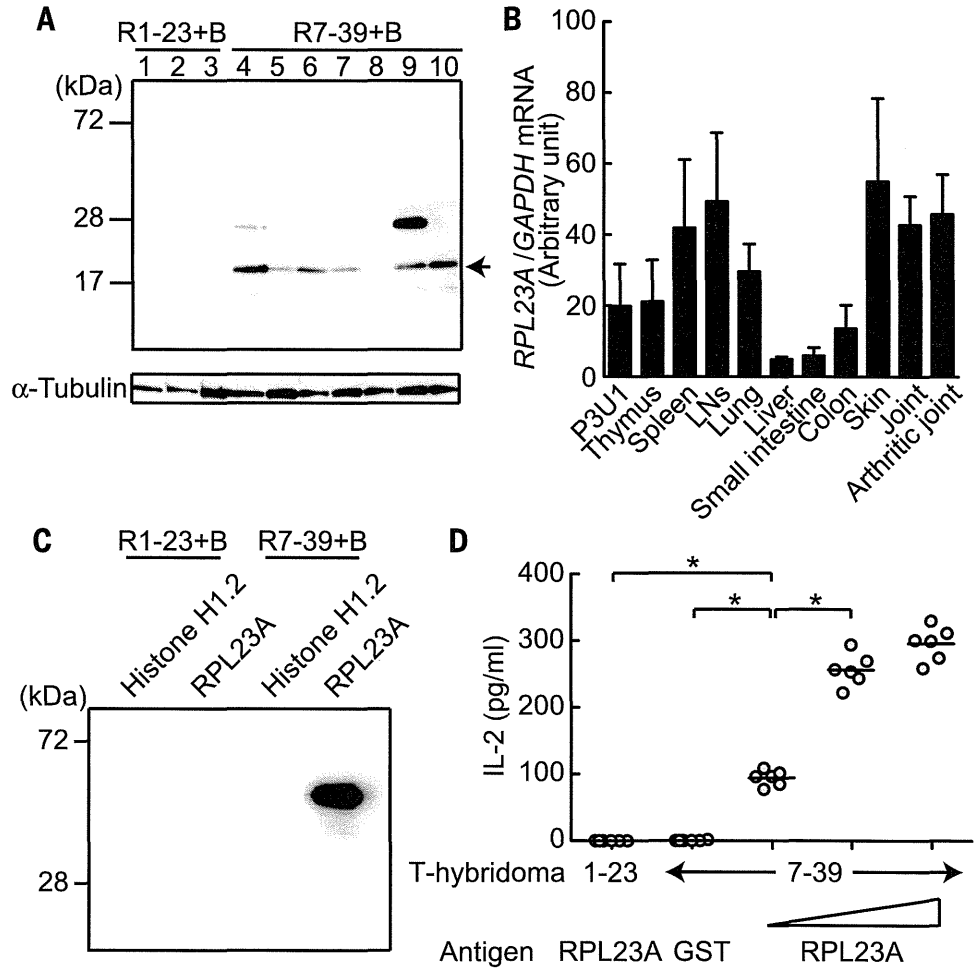

Fig. 2. Identification of the self antigen recognized by arthritogenic 7-39 TCRs. (**A**) Immunoblot analysis by sera from B cell–reconstituted R7-39 mice (n = 7) and B cell–reconstituted R1-23 mice (n = 3). Arrow indicates the commonly recognized protein. (**B**) Quantitative real-time polymerase chain reaction (qPCR) analysis for RPL23A gene expression in various tissues from SKG mice (n = 3). Error bars indicate means ± SD. (**C**) Recombinant RPL23A protein revealed by immunoblotting with sera from the indicated mice. (**D**) IL-2 production by 7-39 or 1-23 T cell hybridomas stimulated with the indicated recombinant proteins (n = 6). Horizontal bars indicate the means. *P < 0.05 (Kruskal-Wallis test followed by Steel-Dwass test). Results represent two [(A) to (C)] or three (D) independent experiments.

B cell–reconstituted R7-39 mice and arthritic SKG mice developed antibodies reacting with cyclic citrullinated peptides (CCP), as also observed in RA patients (*19*) (fig. S14A), yet there was no significant difference in titer of antibodies to RPL23A whether this was assessed with citrullinated or noncitrullinated RPL23A protein (fig. S14, B and C). In addition, the $RPL23A_{71\text{-}90}$ peptide recognized by 7-39 TCRs contained no arginine residue to be converted to citrulline (table S3).

Taken together, these results indicate that the ubiquitously expressed protein RPL23A can be a target antigen of both arthritis and dermatitis. Furthermore, more than one systemic antigen can be targeted for arthritis induction, because the 6-39 TCRs did not react to peptides derived from RPL23A (fig. S13B).

Upon transfer, $CD4^+$ T cells, but not sera, from B cell–reconstituted R7-39 mice induced arthritis in $Rag2^{-/-}$ mice (fig. S15). Indeed, $CD4^+$ T cells from arthritic joints or the regional lymph nodes of R7-39 mice produced inflammatory cytokines [including IL-17A, interferon-γ (IFN-γ), and granulocyte macrophage-colony stimulating factor (GM-CSF)] upon activation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, RPL23A protein, or $RPL23A_{71\text{-}90}$ peptide (Fig. 3, A to D, fig. S16, A to D, and fig. S17). In addition, RPL23A stimulated nonarthritic SKG, but not BALB/c, $CD4^+$ T cells to produce IL-17A in vitro (Fig. 3E). It also augmented the production of IL-17A by $CD4^+$ T cells from SKG mice treated with mannan, which can trigger autoimmune arthritis in SKG mice by promoting T_H17 differentiation of arthritogenic $CD4^+$ T cells (*20*, *21*). An arthritic joint of SKG mice indeed harbored $CD4^+$ T cells possessing the Vβ CDR3 of 7-39 TCRs (table S2).

We next evaluated the contribution of T_{reg} cells to controlling arthritogenic $CD4^+$ T cells. T_{reg} cells

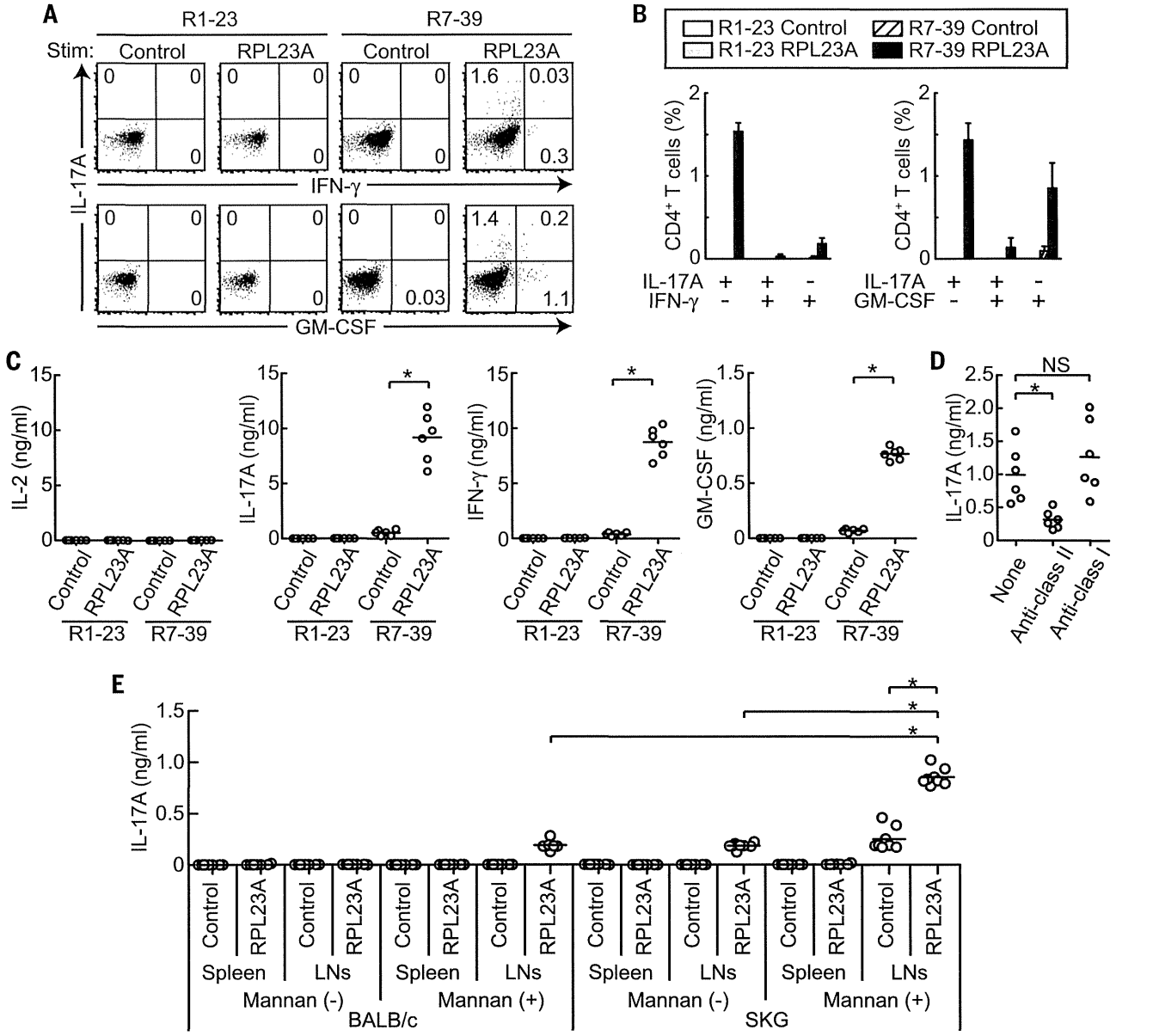

Fig. 3. RPL23A-reactive T_H cells in R7-39 mice. (**A**) Cytokine production by $CD4^+$ T cells from regional lymph nodes of R7-39 or R1-23 mice after in vitro stimulation with recombinant RPL23A or control glutathione *S*-transferase (GST) protein. Stim, stimulation. Data are representative of three independent experiments. (**B**) Percentages of cytokine-producing $CD4^+$ T cells in (A) (n = 3). (**C**) Cytokine amounts in culture supernatants in (A) (n = 6). (**D**) IL-17A production by RPL23A-stimulated lymphocytes from R7-39 mice in the presence or absence of blocking antibodies to MHC class I or class II (n = 6). (**E**) IL-17A production by lymphocytes stimulated with recombinant RPL23A or control GST proteins (n = 8). Lymphocytes were taken from SKG or BALB/c mice with or without mannan treatment. In (B), results are shown as means ± SD. In (C) to (E), horizontal bars indicate the means; *P < 0.05 (Kruskal-Wallis test followed by Steel-Dwass test); NS, not significant. Results represent two independent experiments in (B) and (C).

from either ZAP-70–intact BALB/c or ZAP-70–mutant SKG mice failed to suppress arthritis development in *Rag2*$^{-/-}$ mice when cotransferred with phenotypically activated or memory 7-39 TCR$^+$ CD4$^+$ T cells (figs. S7 and S18), although T_{reg} cells were capable of suppressing naïve arthritogenic T cells effectively (*9*).

These results collectively indicate that RPL23A is able to stimulate CD4$^+$ T cells in R7-39 mice via RPL23A-derived peptide–MHC class II complexes, driving them to differentiate into arthritogenic effector T_H cells (*20*), which are capable of mediating arthritis even in the presence of T_{reg} cells.

Lastly, we examined possible immune responses to RPL23A in RA patients. *RPL23A* mRNA was found to be ubiquitously expressed in healthy human tissues (Fig. 4A). In synovial tissues of RA patients and also in the apparently normal synovial tissues of osteoarthritis (OA) patients, RPL23A was detected in the cytoplasm of synovial cells, including CD55$^+$ FLSs (Fig. 4B). Relative to healthy controls (1.3%, n = 74), a significantly higher proportion of RA patients (16.8%, n = 374) were positive for serum immunoglobulin G–type autoantibodies to RPL23A (Fig. 4C). Two out of 23 psoriatic arthritis (PsA) patients (8.7%) were positive for the autoantibody, whereas all of the OA patients (n = 11), systemic lupus erythematosus (SLE) patients (n = 30), or

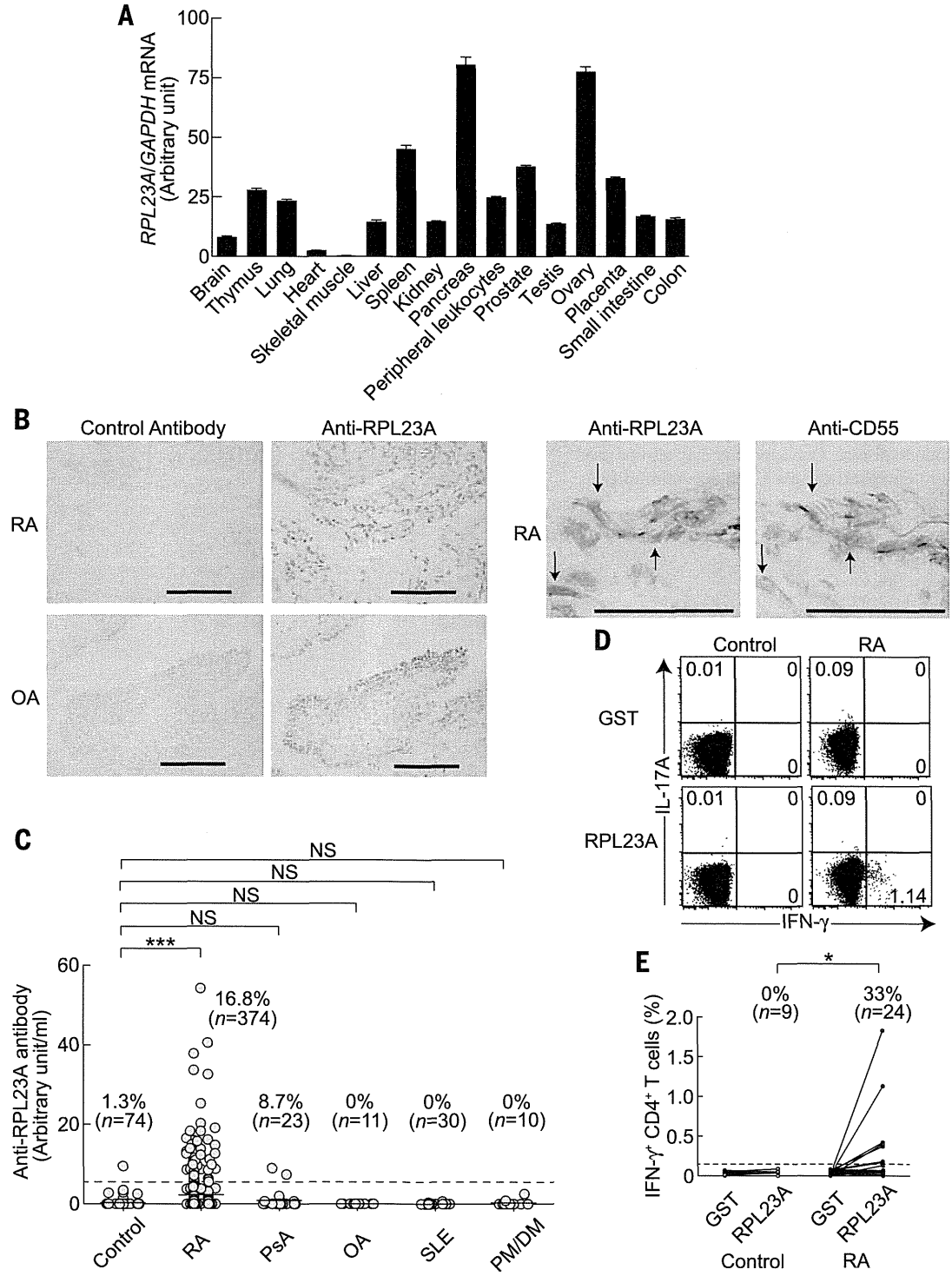

Fig. 4. Anti-RPL23A humoral and cellular immune responses in RA patients. (**A**) qPCR analysis of RPL23A gene expression in various tissues from healthy human subjects (n = 3). Results are shown as means ± SD and represent two independent experiments. (**B**) Immunohistochemical staining of synovial tissues from RA or OA patients for RPL23A or CD55 expression (scale bars, four images at left, 200 μm; two images at right, 50 μm). Serial sections were stained by anti-RPL23A, anti-CD55, or control antibody. Arrows indicate cells that are both RPL23A- and CD55-positive. Representative results from three patients are shown. (**C**) Serum levels of autoantibodies to RPL23A assessed by enzyme-linked immunosorbent assay (ELISA) in RA, PsA, OA, SLE, and PM/DM patients or healthy individuals. Horizontal bars indicate the medians. ***P < 0.001 (Kruskal-Wallis test followed by Dunn's multiple comparison test). (**D**) Cytokine production from CD4$^+$ T cells stimulated with recombinant RPL23A or GST protein. (**E**) Percentages of IFN-γ$^+$ cells in RPL23A- or GST-stimulated CD4$^+$ T cells in RA patients (n = 24) or healthy individuals (n = 9). *P < 0.05 (χ^2 test). Dashed lines indicate the threshold in (C) and (E).

polymyositis/dermatomyositis (PM/DM) patients (n = 10) were negative. In addition, in the synovial fluid of a subset of RA patients, we detected $CD4^+$ T cells producing IFN-γ upon stimulation with RPL23A (Fig. 4, D and E). These findings in humans, together with the key role of anti-RPL23A T cell responses for autoimmune arthritis and psoriasis-like dermatitis in mice, suggest that the responses may play a pathogenic role at least in a subset of patients with RA or PsA.

Our results show that by attenuating TCR signal intensity in developing T cells (hence reducing their sensitivity to thymic negative selection by natural self ligands), T cells reactive with ubiquitously expressed self antigens can be generated as dominant pathogenic clones causing systemic autoimmune disease. Because similar attenuation of TCR signaling at various degrees in conjunction with T_{reg} cell depletion is able to produce a variety of other autoimmune diseases in mice (*9*, *22*), this strategy of generating pathogenic T cells and characterizing the self antigens they recognize would facilitate our understanding of the mechanisms of other autoimmune diseases of currently unknown etiology. In addition, given that genetic polymorphism in a signaling molecule in T cells is a major determinant of genetic susceptibility to various human autoimmune diseases including RA (*23*), such a genetic variation might, at least in part, alter thymic selection, hence forming a TCR repertoire for causing autoimmune disease. Our approach may also be useful in deciphering how T cell autoimmunity to a ubiquitous self antigen triggers localized tissue damage in RA and other human autoimmune diseases, and in devising effective means of systemic or local intervention in the disease process.

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SUPPLEMENTARY MATERIALS

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High expression of MAGE-A4 and MHC class I antigens in tumor cells and induction of MAGE-A4 immune responses are prognostic markers of CHP-MAGE-A4 cancer vaccine☆

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ABSTRACT

Purpose: We conducted a cancer vaccine clinical trial with MAGE-A4 protein. Safety, clinical response, and antigen-specific immune responses were analyzed and the prognostic factors by vaccination were investigated.
Experimental design: Twenty patients with advanced esophageal, stomach or lung cancer were administered MAGE-A4 vaccine containing 300 μg protein subcutaneously once every 2 weeks in six doses. Primary endpoints of this study were safety and MAGE-A4 immune responses.
Results: The vaccine was well tolerated. Fifteen of 20 patients completed one cycle of vaccination and two patients showed SD. A MAGE-A4-specific humoral immune response was observed in four patients who had high expression of MAGE-A4 and MHC class I on tumor cells. These four patients showed significantly longer overall survival than patients without an antibody response after vaccination ($p = 0.009$). Patients with tumor cells expressing high MAGE-A4 or MHC class I antigen showed significantly longer overall survival than those with low expression. Induction of CD4 and CD8T cell responses was observed in three and six patients, respectively, and patients with induction of MAGE-A4-specific IFNγ-producing CD8T cells, but not CD4T cells, lived longer than those without induction.
Conclusions: The CHP-MAGE-A4 vaccine was safe. Expression of MAGE-A4 and MHC class I in tumor tissue and the induction of a MAGE-A4-specific immune response after vaccination would be feasible prognostic markers for patients vaccinated with MAGE-A4.

1. Introduction

The expression of cancer/testis (CT) antigens is normally limited to human germ line cells in the testis and to various types of human cancers [1,2]. Among CT antigens, the melanoma-associated antigen gene (MAGE) family is also known to show such unique expression and to induce spontaneous humoral and cellular immune responses in MAGE-expressing cancer patients [3,4], with the result that they are feasible targets for tumor immunotherapy.

Numerous cancer vaccine strategies are under development and some patients have experienced clinical benefits after vaccination. Among the MAGE family, a phase II cancer vaccine trial with MAGE-A3 protein in non-small-cell lung cancer patients showed 8% reduction of the recurrence rate [5]. Based on the outcome of this phase II study, a randomized double-blind phase III study (MAGRIT trial) with MAGE-A3 protein vaccination was performed [6].

MAGE-A4 is also reported to be expressed in a wide variety of tumors, e.g., 60% esophageal cancer, 50% head and neck cancer, 24% non-small-cell lung cancer, 33% gastric tumor, and 21% Hodgkin's

☆ The study was registered in the University hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) Clinical Trial (Unique trial number: UMIN000003188) on February 15, 2010 (UMIN-CTRURL: http://www.umin.ac.jp/ctr/index.htm).

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disease but not in normal tissues besides the testis. MAGE-A4 elicits spontaneous humoral or cellular immune responses in patients with MAGE-A4-expressing non-small-cell lung cancer, head and neck cancer and adult T cell leukemia/lymphoma [3,4,7,8]. High expression of MAGE-A4, as well as other CT antigens, in tumors was correlated with the poor prognosis of patients with bladder cancer, ovarian cancer, non-small-cell lung cancer and head and neck cancer [9–14]. Many MAGE-A4 epitope peptides recognized by CD4 and CD8 T cells in the context of human leukocyte antigen (HLA) class I and class II have been identified, e.g., HLA-A0201 [15,16], HLA-A2401 [17], HLA-B3701 [18], HLA-DP0501, and HLA-DR1403 [19].

Because tumor-specific T cells are considered to be a direct effector of tumor immunity, the expression level of MHC class I on cancer cells is crucial for the prognosis of cancer patients, especially in the case of an immune therapy such as a cancer vaccine. It is reported that deficient MHC class I surface expression is associated with reduced patient survival in colon cancer, gastric cancer and non-small-cell lung cancer [20–23], and is considered to be one of the causes of the immune escape of tumor cells [24,25]. In patients vaccinated with tumor antigens, some papers reported the effect of the expression level of MHC class I on cancer cells on the clinical effect of vaccinated patients, but there are few reports on their prognosis after vaccination [26,27].

In this study, we conducted a cancer vaccine clinical trial with a complex of MAGE-A4 protein and cholesteryl pullulan (CHP) nanoparticles in advanced cancer patients. We monitored and analyzed the safety, clinical effect, humoral and cellular immune responses and expression of antigens in these patients.

2. Materials and methods

2.1. CHP-MAGE-A4 vaccine

The complex of cholesterol-bearing hydrophobized pullulan (CHP) and MAGE-A4 protein (CHP-MAGE-A4) was provided by ImmunoFrontier, Inc. (Tokyo, Japan) [28]. The synthesis, production, formulation and packaging of the investigational agent were performed in accordance with current Good Manufacturing Practices (cGMP) and met the applicable criteria for use in humans. The toxicity of the drug products was assessed using animal models, and stability was monitored during the clinical trial using representative samples of the investigational drug product.

2.2. Study design

A phase I, open-label, single-institutional clinical trial of the CHP-MAGE-A4 vaccine was designed to evaluate the safety, immune response and clinical response. Patients eligible for entry were those who had advanced cancers that were refractory to standard therapy and expressed MAGE-A4 antigen as assessed by immunohistochemistry (IHC). The CHP-MAGE-A4 vaccine containing 300 μg MAGE-A4 protein was administered subcutaneously once every 2 weeks in six doses. Two weeks after the last administration, the safety, immune response and clinical response were evaluated. Thereafter, the vaccine was administered additionally. Clinical response was assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST ver1.1) [29]. Safety was evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events ver.3.0 (NCI-CTCAE ver.3.0) [30]. The protocol was approved by the Ethics Committee of Osaka Universities according to the Declaration of Helsinki. Written informed consent was obtained from each patient before enrolling in the study. The study was conducted in compliance with Good Clinical Practice and was registered in the University hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) Clinical Trial (Unique trial number: UMIN000003188) on February 15, 2010 (UMIN-CTRURL: http://www.umin.ac.jp/ctr/index.htm).

2.3. MAGE-A4 protein and peptides

For ELISA, recombinant N-His-tagged MAGE-A4 protein was given by Mie University. For Western blot analysis, the MAGE-A4 open reading frame was given by Hokkaido University and was cloned into pGEX-HT plasmid given by Dr. J. Takagi (Osaka University, Osaka, Japan). N-GST-His-tagged MAGE-A4 protein was expressed in M15 *Escherichia coli* cells and purified by Glutathione Sepharose 4B. Finally, recombinant MAGE-A4 protein without a His-tag was purified by TEV protease [31]. For in vitro stimulation of T cells, the following series of 31 MAGE-A4 overlapping peptides spanning the protein was synthesized: 1–20, 11–30, 21–40, 31–50, 41–60, 51–70, 61–80, 71–90, 81–100, 91–110, 101–120, 111–130, 121–140, 131–150, 141–160, 151–170, 161–180, 171–190, 181–200, 191–210, 201–220, 211–230, 221–240, 231–250, 241–260, 251–270, 261–280, 271–290, 281–300, 291–310, and 300–317.

2.4. ELISA

Recombinant protein (0.4 μg/ml) in coating buffer was adsorbed onto 96-well plates and incubated overnight at 4 °C. Plates were washed with phosphate-buffered saline (PBS) and blocked with 1% bovine serum albumin (BSA). 100 μl of serially diluted serum was added to each well and incubated for 2 h at room temperature. Horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Medical & Biological Laboratories, Nagoya, Japan) was added to the wells. Ovalbumin (OVA, albumin from chicken egg white; Sigma, St. Louis, MO) was used as the control protein in each assay. The cut-off value of the antibody reaction was 0.47 O.D., calculated from the results of 47 healthy donors with the average + 2 SD.

2.5. Immunohistochemistry (IHC)

IHC was performed using formalin-fixed paraffin-embedded cancer specimens obtained from all patients enrolled in this trial and 57 esophageal cancer patients who had received surgical treatment. Monoclonal antibodies were anti-MAGE-A4 protein (57B), anti-HLA class I (EMR 8–5) and anti-CD8 (clone C8/144B). The reaction was evaluated as +++ (>50% stained cells), ++ (25–50%), + (5–25%), ± (1–5%) and − (<1%) for MAGE-A4 and HLA class I expression.

2.6. In vitro stimulation of CD4 and CD8 T cells

CD8 and CD4 T cells were purified from peripheral blood mononuclear cells (PBMCs) using CD8 Microbeads and a CD4+ T Cell Isolation Kit (Miltenyi Biotec). The remaining cells were used as antigen-presenting cells (APCs) after pulsing with a mixture of 31 MAGE-A4 overlapping peptides. Then, 5×10^5 CD4 or CD8 T cells were cultured with 10×10^5 APCs after irradiation with IL-2 (10 U/mL; Roche Diagnostics) and IL-7 (20 ng/mL; R&D Systems) for 21 days or 8 days, respectively. CD4 or CD8 T cells harvested were re-stimulated with T-APCs pulsed with a mixture of 31 MAGE-A4 overlapping peptides or HIV (p17, 39–51) peptide as the control for 6 h [32].

2.7. IFNγ intracellular staining (ICS)

ICS was performed with an ICS kit (BD Biosciences) according to the manufacturer's instructions followed by treatment with GolgiStop reagent containing monensin (BD Biosciences) for 1 h. Cells

Table 1
Immune responses and clinical responses following CHP-MAGE-A4 vaccination.

Patient ID	Immunization	MAGE-A4-specific immune response							
		Antibody[a]		CD4[b]		CD8[b]		Clinical response	OS (days)
		Pre	Post	Pre	Post	Pre	Post		
P-1	16	–	+	–	+	–	+	PD	218
P-2	13	–	–	–	–	–	–	PD	254
P-3	5	+	nd	nd		nd		NE	(74)
P-4	6	–	–	–	–	–	–	PD	82
P-5	7	+	+	–	–	–	+	PD	206
P-6	15	–	–	–	–	–	–	SD	228
P-7	31	–	++	–	+	–	+	PD	436
P-8	2	–	nd	nd		nd		NE	(42)
P-9	16	–	–	–	–	–	–	PD	340
P-10	7	–	–	–	–	–	–	PD	90
P-11	5	+	nd	nd		nd		NE	(81)
P-12	35	–	+	–	+	–	+	SD	767
P-13	7	–	–	–	–	–	–	PD	129
P-14	9	–	–	–	–	–	–	PD	179
P-15	7	–	–	–	–	–	+	PD	96
P-16	40	–	++	–	–	–	+	PD	1029
P-17	4	–	nd	nd		nd		NE	(63)
P-18	4	–	nd	nd		nd		NE	(66)
P-19	6	–	–	–	–	–	–	PD	92
P-20	7	+	+	–	–	–	–	PD	116

OS: overall survival; PD: progressive disease; SD: stable disease; NE: not evaluated; nd: not done.

[a] Antibody response was determined by ELISA. Antibody response shown here represents O.D. for MAGE-A4 protein: ++ ≥1.0; + >1.0 to ≥0.47; – >0.47.

[b] CD4 and CD8 T cell responses were determined by IFN-γ intracellular cytokine staining with those cells stimulated in vitro once. IFN-γ-positive cells: +++ >10%; ++ >5% to ≤10%; + >1% to ≤5%; – ≤1%.

were stained with CD8-V450 (clone RPA-T8; BD Biosciences), CD4-V450 (clone RPA-T4; BD Biosciences), CD3-Alexafluor 700 (clone UCHT1; BD Biosciences), eFluor 780-fixable viability dye (eBioscience, San Diego, CA) and IFNγ-FITC (clone 4S.B3; BD Biosciences).

2.8. Western blot

Recombinant protein (20 ng) in sample buffer was boiled for 5 min and subjected to SDS-PAGE with 10–20% polyacrylamide Bio-Rad Ready-Gels (Bio-Rad). After electrophoresis, the membrane was blocked with 5% FCS/PBS and then incubated with patients' sera diluted 1:100 for 1 h at room temperature. Horseradish peroxidase (HRP)-conjugated goat anti-human IgG (MBL) was added to the membrane. Signals were developed with a 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium chromogenic substrate kit (Bio-Rad). Anti-MAGE-A4 monoclonal antibody (57B) used as the positive control at 1:200 dilution was given by Dr G.C. Spagnoli (University Hospital Basel, Basel, Switzerland).

2.9. Activated regulatory T cells in PBMC

Activated regulatory T cells (Treg) were analyzed by a flow cytometer using CD3-PerCPCy5.5 (clone OKT3; eBioscience), CD4-Alexafluor 700 (clone RPA-T4; eBioscience), CD8-V500 (clone RPA-T8; BD Biosciences), CD45RA-FITC (clone HI100; BD Biosciences), eFluor 780-fixable viability dye (eBioscience) and FoxP3-PE (clone 236A/E7; eBioscience). The details of the assay and the definition of activated Tregs were described previously [33].

2.10. Statistics analysis

Rates of the immune responses were compared by Fisher's exact test, and the survival curve was estimated using the Kaplan–Meier method and compared by the log-rank test. All analyses were performed using the SPSS statistical package, version 15.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. Patient characteristics

Twenty advanced cancer patients were enrolled: 18 patients with esophageal cancer, a patient with lung cancer and a patient with gastric cancer expressing MAGE-A4 antigen (Supplementary Table). They received 2–40 immunizations and 15 patients completed a cycle of vaccination (Table 1).

3.2. Safety

Grade 1 fever and Grade 1 injection site reactions, e.g., skin redness or pruritus, were observed in 4 and 13 patients, respectively, after vaccination, and improved without any treatment (Supplementary Table). No severe adverse event was observed.

3.3. Clinical response

All patients underwent image analysis and routine physical checks during and after vaccination. An SD response was observed in two esophageal cancer patients, P-6 and P-12, out of 15 patients who completed vaccination (Table 1). In patient P-6, relapsed lymph node metastasis in the right neck after radical esophagectomy showed a 9% increase in its diameter after 6 immunizations with CHP-MAGE-A4. In patient P-12, although the main tumor disappeared after chemotherapy, metastasis in the left lung was observed with a 15% increase in its diameter after a cycle of vaccination. Both patients received additional cycles of CHP-MAGE-A4 vaccination; however, these target lesions showed rapid enlargement after the second cycle.

3.4. Monitoring of humoral immune response

MAGE-A4 antibody in sera obtained from all patients at baseline and 15 vaccine-completed patients two weeks after the final immunization were analyzed by ELISA. Four patients, P-3, P-5, P-11

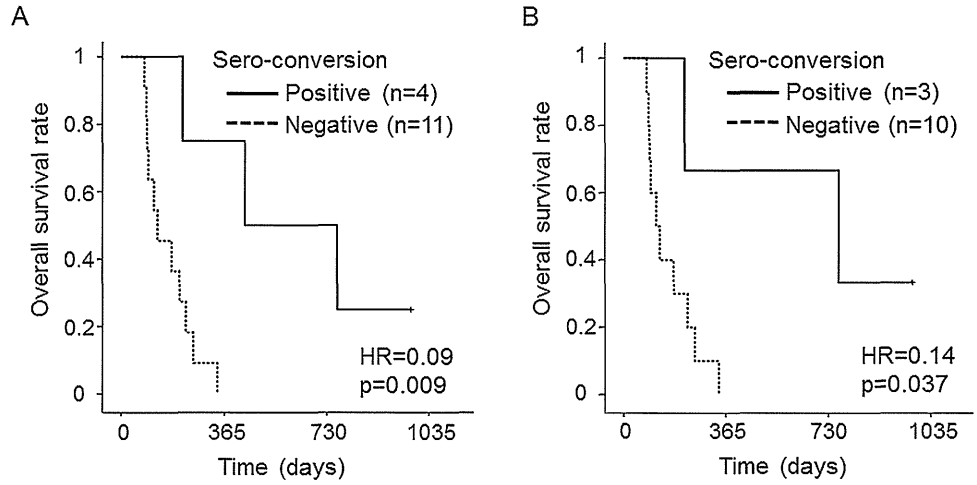

Fig. 1. Antibody production and prognosis. Overall survival of 15 patients and 13 esophageal cancer patients who completed 1 cycle of vaccination and the antibody response determined by ELISA were analyzed. Kaplan–Meier curves illustrate the duration of overall survival of sero-converted patients (solid line) and patients without an antibody response (dotted line) in 15 patients (A) and 13 esophageal cancer patients (B). The hazard ratio (HR) and log-rank *P* value for overall survival comparing patients with positive against negative antibody responses were calculated.

and P-20, showed the production of MAGE-A4 antibody at baseline (sero-positive) while others did not (sero-negative) (Table 1). After vaccination, 4 of 13 sero-negatives among 15 vaccine-completed patients showed increased O.D. values by ELISA and were considered positive serological responses (Supplementary Fig. 1A). No increased response was observed with sera from two sero-positives. These sero-conversions were observed just after a cycle of vaccination in all four patients. Anti-MAGE-A4, but not anti-His-tag, antibody responses in sera from patients P-1, P-12 and P-16 were analyzed by Western blot analysis using recombinant MAGE-A4 protein without any tags (Supplementary Fig. 1B).

Then, the overall survival after the first immunization in sero-conversion positives and negatives was analyzed in 15 vaccine-completed patients. The four sero-converted patients showed prolonged overall survival, significantly longer than that of patients without an antibody response after vaccination (Fig. 1A). When the analysis was limited to esophageal cancer patients, the overall survival of the three sero-converted patients was also significantly longer than that of patients without a MAGE-A4 antibody response (Fig. 1B).

3.5. Immunohistochemical analysis of MAGE-A4, MHC class I and CD8

Expression of MAGE-A4 and MHC Class I antigens on tumor cells was analyzed by IHC using formalin-fixed paraffin-embedded tumor tissues obtained from all enrolled patients (Supplementary Table). Among 15 vaccine-completed patients, high expression of MAGE-A4 (>25% tumor cells) and MHC class I (>5% tumor cells) was observed in tumor tissues from 12 and 12 patients, respectively. Then, we analyzed whether there is any relation between the expression of MAGE-A4 and MHC class I antigens on tumor cells and the induction of immune responses by CHP-MAGE-A4 vaccination. Four of eight patients with high expression of MAGE-A4 or MHC class I antigen on tumor cells showed an antibody response while no patients with low expression of either antigen on tumors showed an antibody response. High expression of both MAGE-A4 and MHC class I antigens was observed on tumor cells from sero-converted patients (Fig. 2A and B). Next, we analyzed whether there is any relation between the expression of those antigens and overall survival by CHP-MAGE-A4 vaccination. Patients with tumor cells expressing high MAGE-A4 or MHC class I antigen showed significantly longer overall survival than those with lower expressions (Fig. 2C and D).

3.6. Induction of MAGE-A4-specific CD4 and CD8 T cell responses

MAGE-A4-specific CD4 and CD8 T cell responses were analyzed by ICS assay using PBMCs obtained from 15 vaccine-completed patients at baseline and 2 weeks after the 6th immunization (Supplementary Fig. 2). MAGE-A4-specific IFNγ-producing CD4 and CD8 T cells were observed in no patient at baseline. After vaccination, induction of a CD4 T cell response was observed in three patients, P-1, P-7, P-12, who showed sero-conversion, and induction of a CD8 T cell response was observed in six patients, P-1, P-5, P-7, P-12, P-15, P-16, who showed antibody production (Table 1). Patients with induction of MAGE-A4-specific IFNγ-producing CD8 T cells, but not CD4 T cells, lived longer than those without induction (Supplementary Fig. 3).

3.7. Impact of CD4+ FoxP3 high+ regulatory T cells on overall survival

The ratio of CD4+ Foxp3 high+ cells in CD3+ T cells was analyzed using PBMCs obtained at baseline from 15 vaccine-completed patients. When the patients were divided by the mean of the ratio, the two SD patients, P-6 and P-12, belonged in the low ratio group (Supplementary Fig. 4A and B). Patients with a low ratio of CD4+ Foxp3 high+ cells in CD3+ T cells showed longer overall survival than patients with a high ratio after vaccination, although it was not significant (Supplementary Fig. 4C).

4. Discussion

We showed that the induction of MAGE-A4-specific immune responses correlated well with the prognosis of patients vaccinated with CHP-MAGE-A4. In our previous study of cancer vaccines with NY-ESO-1 protein [34–39], NY-ESO-1f peptide [40] and NY-ESO-1 overlapping peptide [41], feasible clinical responses were observed in several patients; however, we could not confirm the effects of NY-ESO-1 vaccines on the good prognosis of enrolled patients. There are several reports of successful cancer vaccines which prolonged the overall survival of vaccinated patients [42,43], and some studies revealed that patients with the induction of an antigen-specific CD8 T cell response, but not an antibody response,