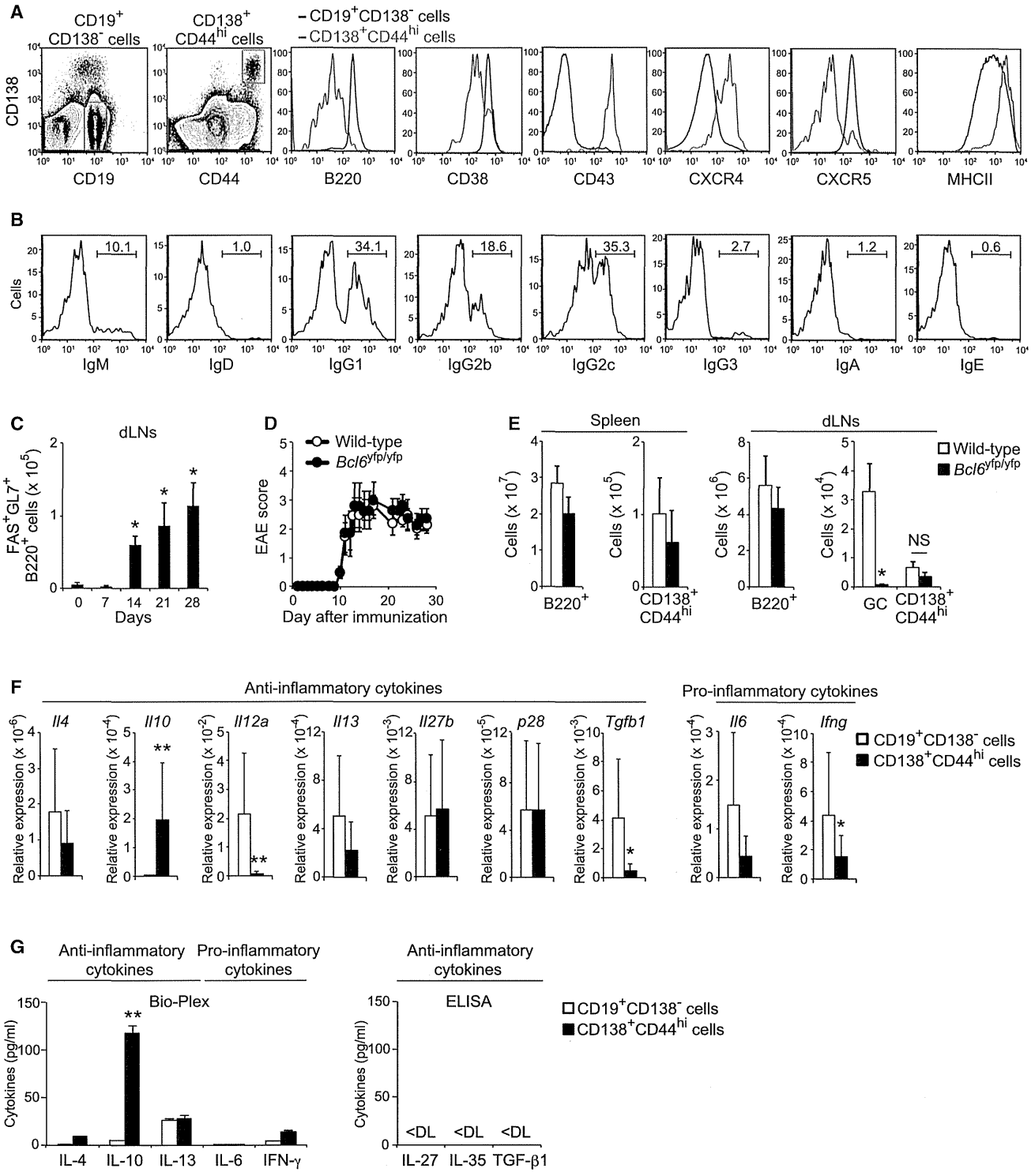


Figure 3. EAE Induces Generation of GC-Independent Plasmablasts that Produce IL-10 Preferentially

(A) Flow cytometry of $CD19^+CD138^-$ (black histogram, gated as in the left-most panel) and $CD138^+CD44^{hi}$ cells (red histogram, gated as in second panel from the left) harvested from dLNs of wild-type mice 14 days after MOG_{35-55} immunization.

(B) Flow cytometry of $CD138^+CD44^{hi}$ cells harvested from dLNs of wild-type mice 14 days after MOG_{35-55} immunization. Percentages of Ig^+ cells are shown.

(C) Absolute number of $FAS^+GL7^+B220^+$ (GC) B cells from dLNs harvested from wild-type mice before and 7, 14, 21, and 28 days after MOG_{35-55} immunization. Data are presented as mean ± SEM for five to six mice. *$p < 0.05$ versus day 0 (Mann-Whitney U test).

(D) Clinical EAE scores for wild-type and $Bcl6^{yfp/yfp}$ mice immunized with MOG_{35-55}. The EAE score is shown as mean ± SEM for seven mice.

(legend continued on next page)

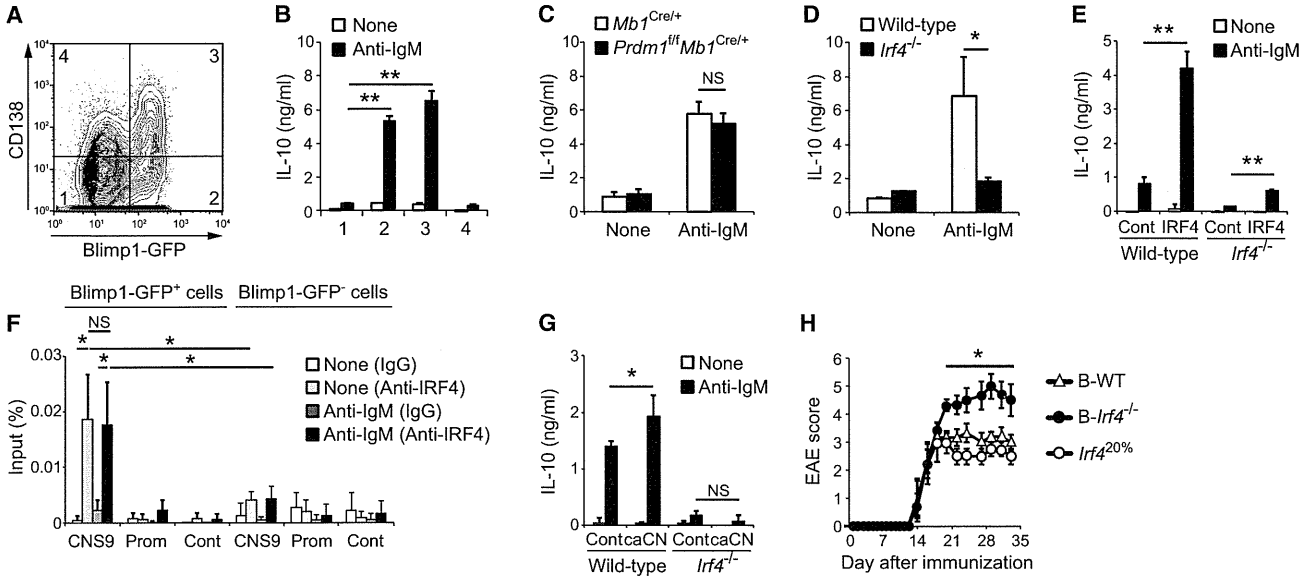


Figure 4. IRF4 Is Essential for Plasmablast IL-10 Production

(A) Flow cytometry of B cells isolated from spleen of *Prdm1*$^{gfp/+}$ mice and cultured with LPS for 48 hr. Four populations—GFP$^-$CD138$^-$ (fraction 1), GFP$^+$CD138$^-$ (fraction 2), GFP$^+$CD138$^+$ (fraction 3), and GFP$^-$CD138$^+$ (fraction 4) cells—were sorted and assayed in (B).

(B) ELISA of IL-10 secreted by the sorted B cells after stimulation with anti-IgM for 24 hr.

(C) ELISA of IL-10 secreted by B cells isolated from peripheral LNs of *Mb1*$^{Cre/+}$ and *Prdm1*$^{f/f}$*Mb1*$^{Cre/+}$ mice and cultured with LPS for 48 hr followed by stimulation with anti-IgM for 24 hr.

(D) ELISA of IL-10 secreted by B cells isolated from peripheral LNs of wild-type and *Irf4*$^{-/-}$ mice and cultured with LPS for 48 hr followed by stimulation with anti-IgM for 24 hr.

(E) ELISA of IL-10 secreted by GFP$^+$ cells sorted from LPS-activated wild-type and *Irf4*$^{-/-}$ B cells retrovirally transduced with GFP alone (Cont) or IRF4 followed by stimulation with anti-IgM for 24 hr.

(F) ChIP analysis of GFP$^+$ plasmablasts and GFP$^-$ B cells sorted from LPS-activated *Prdm1*$^{gfp/+}$ B cells, stimulated with anti-IgM for 30 min, and then precipitated with anti-IRF4 Ab or goat IgG. Input DNA and precipitated DNA were quantified by RT-PCR with PCR primers specific for CNS9 and promoter (Prom) regions of *Il10* or 3′ region of *Cd19* (Cont). Data shown are pooled from two independent experiments.

(G) ELISA of IL-10 secreted by GFP$^+$ cells sorted from LPS-activated wild-type and *Irf4*$^{-/-}$ B cells retrovirally transduced with GFP alone (Cont) or constitutively active calcineurin (caCN) followed by stimulation with anti-IgM for 24 hr. NS, not significant.

(H) Clinical EAE scores for chimeric mice in which only B cells lacked IRF4 (B-*Irf4*$^{-/-}$; wild-type mice lethally irradiated and reconstituted with 80% μMT plus 20% *Irf4*$^{-/-}$ bone marrow) and two control chimera groups: wild-type mice lethally irradiated and reconstituted with 80% μMT plus 20% wild-type bone marrow (B-WT) or reconstituted with 80% wild-type plus 20% *Irf4*$^{-/-}$ bone marrow (*Irf4*$^{20\%}$). The EAE score is shown as mean ± SEM for five to six mice. *p < 0.05 versus B-WT mice (Mann-Whitney U test).

(B–G) Data are presented as mean ± SD. *p < 0.05, **p < 0.001 (Student's t test).

Data are representative of three (A–E and G) or two (H) independent experiments. See also Figures S3 and S4.

cell generation (Figures 4C, S3A, and S3B), suggesting that Blimp1 in developing plasmablasts is dispensable for IL-10 production. Importantly, B cells lacking Blimp1 fail to fully differentiate into plasma cells, but rather initiate this differentiation pathway (Kallies et al., 2007; Kallies and Nutt, 2007; Shapiro-Shelef et al., 2003). Therefore, we next focused on the functional importance of IRF4 because it is a critical factor in the early phase of plasma cell differentiation and one of the downstream targets of TLR and BCR signaling (Mittrücker et al., 1997; Oracki et al., 2010). LPS-activated *Irf4*$^{-/-}$ B cells had impaired IL-10 secretion after BCR ligation (Figure 4D). Reciprocally, retroviral expression of IRF4 in wild-type B cells substantially increased IL-10 production and partially rescued it in *Irf4*$^{-/-}$ B cells (Figure 4E). Furthermore, chromatin immunoprecipitation (ChIP) analysis revealed that IRF4 in plasmablasts bound to the *Il10* CNS9 region, which controls *Il10* expression and is located approximately 9.1 kbp upstream of the transcription start site (Lee et al., 2009), though this binding frequency was unaffected by BCR stimulation (Figure 4F). These results imply that IRF4 not only induces plasmablast generation but also directly regulates *Il10* expression. Nuclear factor

(E) Absolute number of each B cell subset from spleen and dLNs harvested from wild-type and *Bcl6*$^{yfp/yfp}$ mice 28 days after MOG_{35-55} immunization. Data are presented as mean ± SEM for six mice. *p < 0.05 versus wild-type mice (Mann-Whitney U test). NS, not significant.

(F and G) Quantitative RT-PCR (F) and ELISA and Bio-Plex cytokine (G) analysis of CD19$^+$CD138$^-$ and CD138$^+$CD44^{hi} cells harvested from dLNs of wild-type mice 14 days after MOG_{35-55} immunization. For ELISA and Bio-Plex suspension assay, the isolated CD19$^+$CD138$^-$ and CD138$^+$CD44^{hi} cells were stimulated with PMA and ionomycin for 5 hr (G). Data are presented as mean ± SD. Abbreviations: <DL, below detection limit. *p < 0.05, **p < 0.001 versus CD19$^+$ cells (Student's t test).

Data are representative from three (A, B, and F) or two (C–E and G) independent experiments.

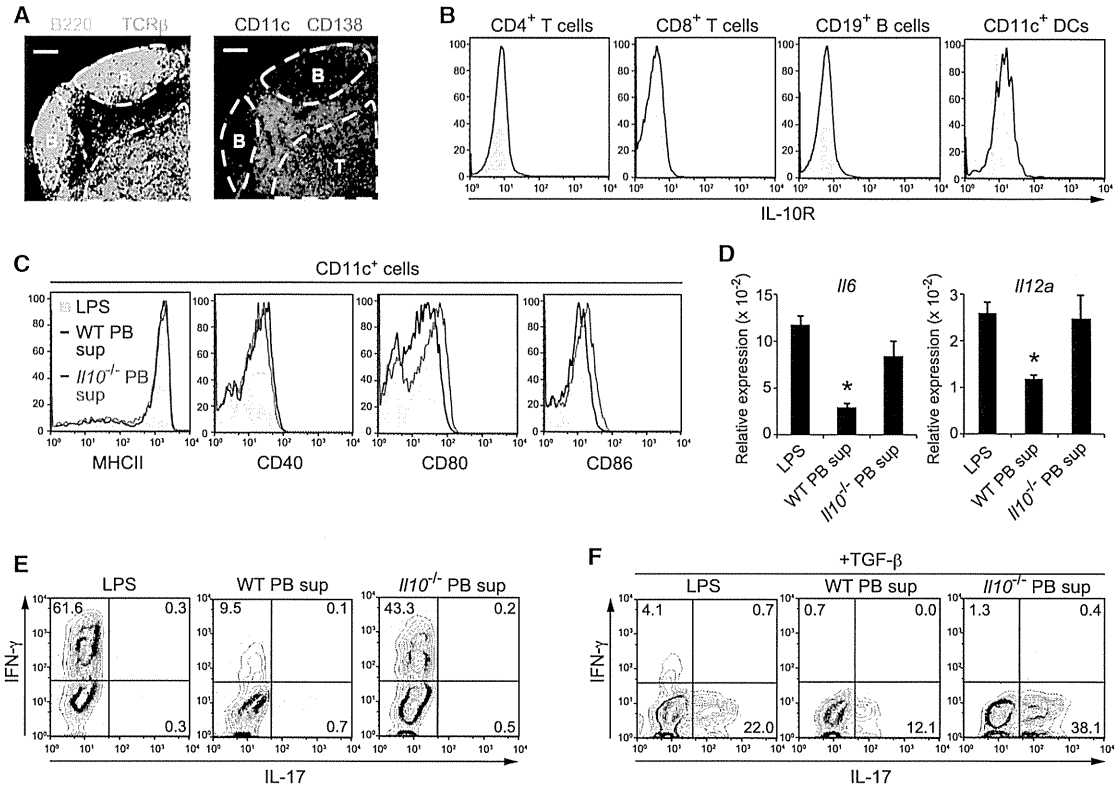


Figure 5. Plasmablasts Inhibit Dendritic Cell Function to Generate Autoreactive T Cells

(A) Histological analysis of dLNs harvested from wild-type mice 14 days after MOG_{35-55} immunization. Sections were stained with B220 and TCR-β Abs (left) or with CD11c and CD138 Abs (right). Original magnification, ×10; scale bars represent 100 μm.

(B) Flow cytometry of IL-10R expression by cells harvested from dLNs of wild-type mice 14 days after MOG_{35-55} immunization. Cells were stained with an IL-10R mAb (open histogram) or isotype control (shaded histogram).

(C) Flow cytometry of $CD11c^+$ cells harvested from dLNs of wild-type mice 14 days after MOG_{35-55} immunization followed by stimulation with LPS alone (shaded histogram) or supernatants from wild-type plasmablasts (PB) (WT PB sup; black histogram) and *Il10*$^{-/-}$ plasmablasts (*Il10*$^{-/-}$ PB sup; red histogram) activated with LPS and then anti-IgM.

(D) Quantitative RT-PCR analysis of *Il6* and *Il12a* transcripts in $CD11c^+$ cells harvested from dLNs of wild-type mice 14 days after MOG_{35-55} immunization, stimulated with LPS or WT and *Il10*$^{-/-}$ PB sup, normalized to the expression of *Gapdh*. Data are presented as mean ± SD. *$p < 0.05$ versus DC treated with *Il10*$^{-/-}$ PB sup (Student's t test).

(E and F) Cytokine profiles of TCR^{MOG}-expressing naive $CD4^+$ T cells cocultured with dLN $CD11c^+$ cells stimulated with LPS or WT and *Il10*$^{-/-}$ PB sup in the absence (E) or presence (F) of TGF-β together with MOG_{35-55} for 72 hr. Percentages of IFN-γ^+ and/or IL-17^+ cells are shown.

Results represent one of three similar experiments. See also Figure S5.

of activated T cells (NFAT), which is activated by Ca^{2+} and the calmodulin-dependent phosphatase calcineurin, is also vital for BCR-induced IL-10 production (Matsumoto et al., 2011). Retroviral expression of a constitutively active form of calcineurin A (caCN) markedly increased BCR-induced IL-10 production in an IRF4-dependent manner (Figure 4G), suggesting that NFAT-dependent IL-10 production requires IRF4. Moreover, we found that IRF4 has a B cell regulatory role in vivo because B-cell-specific *Irf4*-deficient chimeric mice lacking $CD138^+CD44^{hi}$ cells in the dLNs became susceptible to EAE (Figures 4H and S4). Together, these data indicate that IRF4 is essential for B cell IL-10 production to suppress EAE.

Plasmablast-Derived IL-10 Inhibits Dendritic Cell Function to Generate Pathogenic T Cells

We next elucidated the mechanisms by which plasmablasts suppress EAE. Immunohistochemical analysis of the dLNs in EAE-induced mice revealed that $CD138^+$ plasmablasts were mainly colocalized with $CD11c^+$ dendritic cells (DCs) in the extrafollicular region between T cell zones and B cell follicles (Figure 5A). Given that DCs, but not T and B cells, expressed detectable amounts of IL-10 receptor (IL-10R) (Figure 5B), we next examined whether DC function is affected by plasmablast-derived IL-10. When DCs were stimulated with supernatants derived from wild-type plasmablasts activated with LPS and then anti-IgM, the expression of MHCII, CD40, CD80, and CD86 was unchanged, but *Il6* and *Il12* mRNA was significantly decreased. This effect was not observed with *Il10*$^{-/-}$ plasmablasts (Figures 5C and 5D). Consistent with these results, Th1 cell differentiation of MOG-specific T cells was markedly prevented by supernatants from wild-type, but not *Il10*$^{-/-}$, plasmablasts when cocultured with DCs (Figure 5E). Very similar results were obtained with TGF-β-mediated Th17 cell generation (Figure 5F). Furthermore, we also observed equivalent results

when DCs were cocultured with Blimp1-GFP$^+$ plasmablasts (Figure S5). Thus, these results suggest that IL-10-producing plasmablasts inhibit DC functions to generate autoreactive T cells. This does not exclude the possibility that other cell types will be affected in vivo by plasmablast IL-10.

Human Plasmablasts Are IL-10-Producing B Cells

Our findings that plasmablasts represent the IL-10-producing B cells in mice led us to test whether this also applies to humans. B cells were isolated from peripheral blood of healthy donors and cultured with CpG (a TLR9 agonist) and/or cytokine cocktails including IL-2, IL-6, and interferon-alpha (IFN-α), which are known to provide conditions for effective plasmablast differentiation (Jego et al., 2003; Joo et al., 2012). Indeed, we detected CD27^{hi}CD38$^+$ putative plasmablasts after culture with CpG, while concomitant treatment with CpG and cytokine cocktails induced a greater frequency of an additional population of CD27^{int}CD38$^+$ cells as well as CD27^{hi}CD38$^+$ cells (Figure 6A). In particular, IFN-α was considerably effective for CD27^{int}CD38$^+$ differentiation and essentially the same results were obtained with IFN-β instead of IFN-α (Figure S6A). We found that IL-10 production was greatly induced in culture with a mixture of CpG and cytokine cocktails (Figure 6B). Both CD27^{hi}CD38$^+$ and CD27^{int}CD38$^+$ populations had a progressive loss of CD20, CD180, and Pax5 (Figures 6C, S6B, and S6C). Inversely, they had higher expression of IRF4, Blimp1, and XBP1 proteins and their transcripts (Figures 6C and S6C) and showed morphological maturation into plasma cells, as displayed by larger size with abundant cytoplasm, eccentric nuclei, and perinuclear haloes (Figure 6D). Consistent with these observations, both CD27^{hi}CD38$^+$ and CD27^{int}CD38$^+$ cells substantially secreted IgM (Figure 6E). Given the lack of a human mature plasma cell marker CD138 (Figure S6B), CD27^{int}CD38$^+$ cells as well as CD27^{hi}CD38$^+$ cells can be considered as plasmablasts whereas the CD27^{hi} cells apparently are more mature than CD27^{int} cells in view of their phenotypes. To determine which populations produce IL-10, we purified four fractions based on CD27 and CD38 expression after culture. ELISA assay showed that CD27^{int}CD38$^+$ plasmablasts selectively secreted IL-10 (Figure 6F). As a further test of this finding, we conducted IL-10 secretion assay by using IL-10 capture and detection antibodies, which allow us to detect live IL-10-secreting cells and found that the majority of IL-10$^+$ B cells consisted of CD27^{int}CD38$^+$ cell fraction (Figure 6G). Of note, this IL-10$^+$CD27^{int}CD38$^+$ population substantially secreted IgM, as assessed by ELISPOT assay (Figure 6H), suggesting that IL-10-producing B cells are Ig-secreting CD27^{int}CD38$^+$ plasmablasts.

We next addressed the issue of why CD27^{int}, but not CD27^{hi}, plasmablasts produce IL-10. Given that freshly prepared peripheral blood B cells consist of three major populations, i.e., CD24^{lo}CD27$^-$CD38$^-$ (naive mature), CD24^{hi}CD27$^-$CD38^{lo} (naive immature), and CD24^{hi}CD27$^+$CD38$^-$ (memory) cells (Figure 6I), the origin of each might be different. To test this hypothesis, they were sorted and then cultured. Memory B cells were predominantly differentiated into CD27^{hi}CD38$^+$ plasmablasts, whereas naive immature B cells and mature B cells, albeit to a lesser degree, became CD27^{int}CD38$^+$ plasmablasts (Figure 6I). Naive B-cell-derived CD27^{int} plasmablasts produced considerably more IL-10 (Figures 6J, S6D, and S6E). Collectively, these findings establish that human plasmablasts that arise from naive and especially immature B cells, but not memory B cells, are the major IL-10-producing B cells.

DISCUSSION

Our findings identify plasmablasts as the IL-10-producing B cells that can suppress autoimmunity. This was the case for EAE, where they were developed in the dLNs under the control of Blimp1 and IRF4 and disease progression was enhanced by their deletion. Furthermore, human plasmablasts also preferentially secreted IL-10, and these cells were derived from naive but not memory B cells.

It was previously thought that splenic B cells secrete the IL-10 that limits EAE. Instead, we found that CD138$^+$ plasmablasts in the dLNs were the major producers of this cytokine during EAE. This was the case when assessed by *Il10*$^{Venus/+}$ reporter mice or quantitative RT-PCR. In accordance with previous reports using several other IL-10 reporter lines injected with LPS or infected with *Salmonella* (Madan et al., 2009; Maseda et al., 2012; Shen et al., 2014), we also observed Venus expression in splenic CD138$^+$ cells in *Il10*$^{Venus/+}$ mice. However, amounts were very low and the frequency of positive cells was unaffected by EAE induction. By contrast, IL-10$^+$ plasmablasts in the dLNs were newly generated within extrafollicular foci, implying negative feedback regulation to protect excessive inflammation.

Our finding of severe EAE pathogenesis in the absence of plasmablasts due to B-cell-specific deletion of Blimp1 or IRF4 supports the idea that plasmablasts possess regulatory activity in vivo. This regulatory function is dependent on the dLNs and independent of the spleen. On the other hand, results from adoptive transfer studies were interpreted to mean that splenic B cells, especially the CD1d^{hi}CD5$^+$ B cell population, could suppress EAE through some unknown mechanism (Matsushita et al., 2008). Although we also observed that adoptive transfer of splenic B cells normalized EAE, plasmablast generation in the dLNs was required. CD1d^{hi}CD5$^+$ B cells extensively differentiate into plasmablasts in culture (Maseda et al., 2012) and their adoptive transfer from mice lacking IL-21R, CD40, and MHCII, which are indispensable for plasma cell differentiation (McHeyzer-Williams et al., 2012), into *Cd19*$^{-/-}$ mice does not resolve EAE development (Yoshizaki et al., 2012). Therefore, this population might serve as plasmablast precursors in an adoptive transfer setting.

The finding of in vitro BCR-dependent IL-10 production specifically in Blimp1$^+$ cells provides further evidence for the importance of plasmablasts and can explain the previously demonstrated need for TLR signaling for BCR-mediated IL-10 expression (Matsumoto et al., 2011). This idea is also supported by the observation of impaired IL-10 secretion in the absence of IRF4, which resulted in defective plasmablast differentiation (Figure S3A). Thus, IRF4 is required for IL-10 expression along with plasmablast differentiation in vitro and in vivo. Importantly, TLR and BCR signals induce the expression of IRF4 (De Silva et al., 2012) and therefore operate upstream of both plasmablast differentiation and IL-10 production. We detected deposition of IRF4 at the CNS9 region upstream enhancer in the *Il10* locus. This is in agreement with published studies that demonstrated

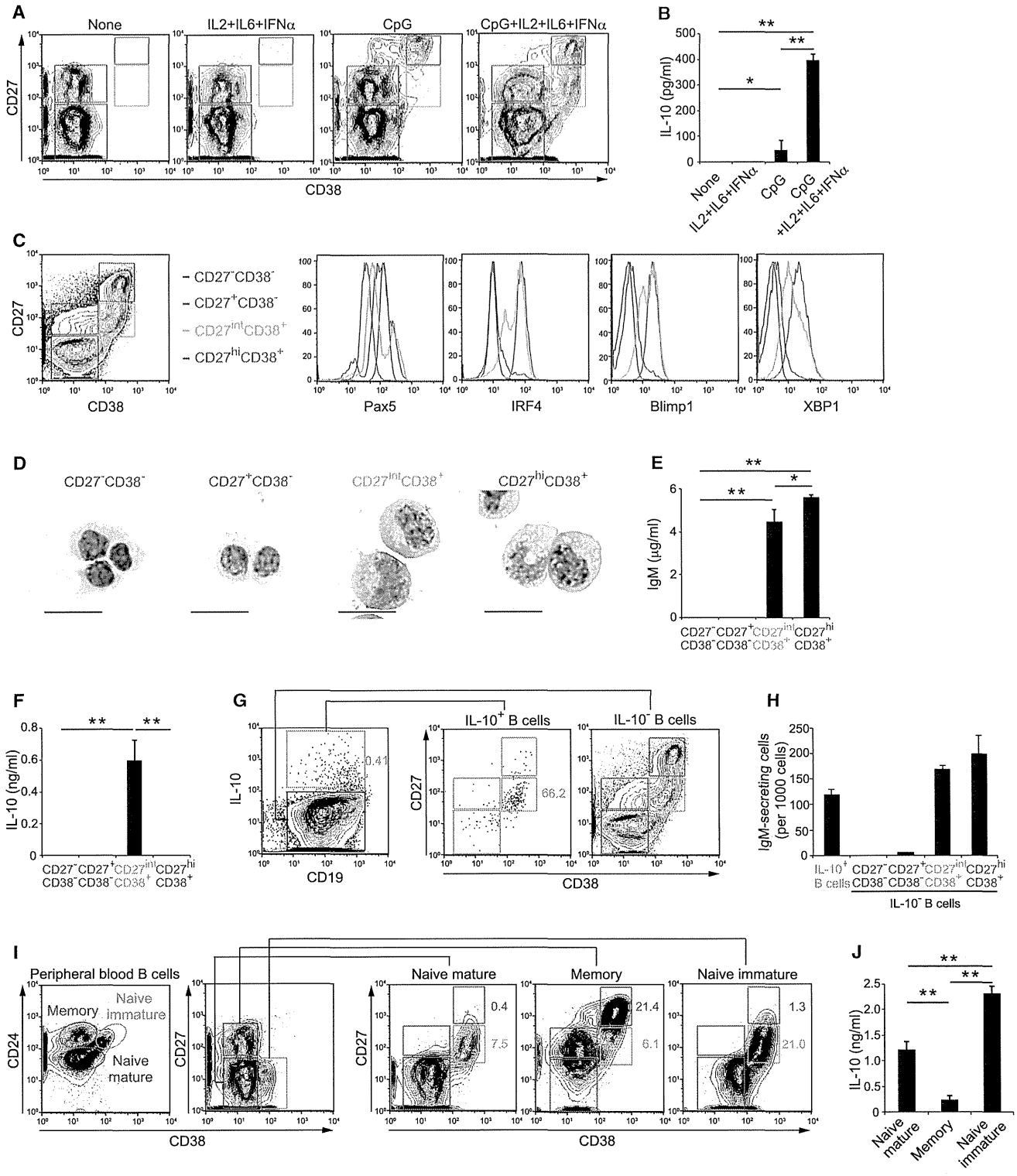


Figure 6. Human Plasmablasts Are IL-10-Producing B Cells

(A) Flow cytometry of B cells isolated from healthy blood donors and cultured with IL-2, IL-6 plus IFN-α (IL2, IL6, IFNα), and/or CpG for 96 hr. Four populations—$CD27^{-}CD38^{-}$ (red), $CD27^{+}CD38^{-}$ (blue), $CD27^{int}CD38^{+}$ (green), and $CD27^{hi}CD38^{+}$ (purple) cells—are gated.

(B) ELISA of IL-10 secreted by B cells isolated from peripheral blood of healthy donors and cultured with IL-2, IL-6 plus IFN-α, and/or CpG for 96 hr.

(C) Flow cytometry of B cell populations indicated in the left panel.

(legend continued on next page)

IRF4 binding to the same element in various types of cells (Cretney et al., 2011; Lee et al., 2009; Li et al., 2012). NFAT bound to the same region in Th2 cells, which was essential for IL-10 transcription (Lee et al., 2009). Taking into account our previous finding that B-cell-mediated IL-10 production requires NFAT activation (Matsumoto et al., 2011), it seems likely that IRF4 serves as an NFAT transcription partner to produce IL-10 in plasmablasts.

Unexpectedly, Blimp1-deficient B cells secreted IL-10 in our in vitro experiments despite impaired $CD138^+$ cell differentiation. Considering that the initiation of plasma cell differentiation takes place in vitro in the absence of Blimp1 (Kallies et al., 2007), it seems possible that IL-10 production is initiated already in the early preplasmablastic stage of plasma cell development, which is independent of Blimp1 (Kallies et al., 2007). However, we could detect little Venus-positive $CD138^-$ B cell population in mice during EAE (data not shown). Given that GFP^+CD138^- cells in *Prdm1*$^{gfp/+}$ mice were effectively generated in vitro (Figure 4A), but not in vivo (Figure 1B), it seems likely that no or few preplasmablasts as is detected in culture exist in vivo.

We now have evidence that naive B-cell-derived plasmablasts represent the most significant IL-10 producers in humans. Although activation of human peripheral blood B cells with CpG caused $CD27^{hi}CD38^+$ plasmablast generation, our results establish that additional treatment with cytokines including IL-2, IL-6, and, especially, IFN-α drove the differentiation of $CD27^{int}CD38^+$ plasmablasts that predominantly secrete IL-10. Given that IFN-α enhances $CD38^+$ expression on naive B cells (Giordani et al., 2009) and can induce plasma cell differentiation (Jego et al., 2003), IFN receptor signals seem likely to be key for IL-10-producing plasmablast generation. Indeed, patients with SLE have high serum IFN-α concentrations (Kim et al., 1987) and increased $CD27^{int}CD38^+$ cells in peripheral blood (Arce et al., 2001), suggesting that $IL\text{-}10^+$ plasmablast expansion might be the result of the inflammatory conditions. Furthermore, the treatment with IFN-β, another type I IFN approved for MS therapy, enhances B cell IL-10 secretion after BCR and CD40 ligation (Ramgolam et al., 2011). Although the precise mechanism by which IFN-β suppresses MS remains unclear, one of the possible explanations is that IFN-β might promote generation of IL-10-producing plasmablasts. Noteworthy, in clinical trials, MS patients who received Atacicept, a transmembrane activator and calcium-modulating cyclophilin-ligand interactor (TACI)-Ig fusion protein to deplete antibody-secreting cells, had exacerbated inflammatory symptoms (Hartung and Kieseier, 2010). This would be consistent with an inhibitory function for human plasmablasts. We have provided evidence that IL-10-producing plasmablasts effectively stem from naive immature B cells. This might support a recent study that human IL-10-competent B cells were enriched in immature $CD24^{hi}CD38^{hi}$ B cells after culture with CD40 stimulation (Blair et al., 2010). We found that memory B-cell-derived plasmablasts failed to secrete IL-10, suggesting that the immediate precursor of developing plasmablasts would dictate the balance between cells that promote autoimmunity by antibody production or have regulatory capacity that protects from overt pathology.

In conclusion, our findings have identified plasmablasts in the dLNs as the IL-10-producing B cells that suppress autoimmunity. We also established a phenotype for human plasmablasts that predominantly secreted IL-10. Our study might lead to better understanding of the nature of autoimmune diseases and provide a basis for exploring new therapeutic strategies.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice were purchased from CLEA Japan. *Bcl6*$^{yfp/yfp}$ (Kitano et al., 2011), *Il10*$^{Venus/+}$ (Atarashi et al., 2011), *Irf4*$^{-/-}$ (Mittrücker et al., 1997; Suzuki et al., 2004), *Mb1*$^{Cre/+}$ (Hobeika et al., 2006), μMT (Kitamura et al., 1991), and *Prdm1*$^{gfp/+}$ (Kallies et al., 2004) mice have been described previously. *Il10*$^{-/-}$, *Prdm1*$^{f/f}$, *Sell*$^{-/-}$, and TCR^{MOG} transgenic mice were purchased from the Jackson Laboratory. We generated *Prdm1*$^{f/f}$*Mb1*$^{Cre/+}$ mice by crossing of *Prdm1*$^{f/f}$ mice with *Mb1*$^{Cre/+}$ mice. Mice were bred and maintained under specific-pathogen-free conditions and used at 6 to 12 weeks of age. Animal care and experiments were conducted according to the guidelines established by the animal committee of Osaka University.

Generation of Mixed Bone Marrow Chimeras

Mixed bone marrow chimeras were produced as described previously (Fillatreau et al., 2002). In brief, recipient wild-type mice received 800 cGy of X-ray irradiation. One day later, the recipients were reconstituted with a mixed inoculum of 80% μMT bone marrow cells supplemented with 20% bone marrow cells from *Irf4*$^{-/-}$ or *Sell*$^{-/-}$ mice. Control groups received 80% μMT and 20% wild-type bone marrow cells or 80% wild-type and 20% bone marrow cells from *Irf4*$^{-/-}$ or *Sell*$^{-/-}$ mice. Chimeric mice were left to fully reconstitute their lymphoid system for at least 12 weeks before EAE induction.

Induction and Assessment of EAE

EAE was induced by subcutaneous immunization with 200 μg of MOG_{35-55} (MBL) emulsified in complete Freund's adjuvant (CFA) containing 500 μg of

(D) May-Grünwald-Giemsa staining of sorted B cell populations after culture with IL-2, IL-6, IFN-α plus CpG for 96 hr. Original magnification, ×400; scale bars represent 20 μm.

(E and F) ELISA of IgM (E) and IL-10 (F) secreted by the indicated B cell populations after culture with IL-2, IL-6, IFN-α plus CpG for 96 hr and then cultured for an additional 24 hr.

(G) Flow cytometry of B cells cultured with IL-2, IL-6, IFN-α plus CpG for 96 hr and labeled with IL-10 capture and detection antibodies to detect $IL\text{-}10^+$ B cells. Percentage of $IL\text{-}10^+$ B cells and $IL\text{-}10^+CD27^{int}CD38^+$ cells are shown.

(H) ELISPOT of IgM secreted by the indicated B cell populations after culture with IL-2, IL-6, IFN-α plus CpG for 96 hr followed by an additional 24 hr culture.

(I) Flow cytometry of three B cell populations freshly isolated from peripheral blood of healthy donors and then cultured with IL-2, IL-6, IFN-α plus CpG for 96 hr. Three major populations such as $CD24^{lo}CD27^-CD38^-$ (naive mature; red), $CD24^{hi}CD27^-CD38^{lo}$ (naive immature; pink), and $CD24^{hi}CD27^+CD38^-$ (memory; blue) cells in peripheral blood B cells before culture are gated (left two panels). Percentages of $CD27^{int}CD38^+$ and $CD27^{hi}CD38^+$ cells after culture are shown (right three panels).

(J) ELISA of IL-10 secreted by naive mature, naive immature, and memory B cells isolated from healthy blood donors and cultured with IL-2, IL-6, IFN-α plus CpG for 96 hr.

Data shown are representative of three independent experiments. See also Figure S6.

(B, E, F, H, and J) Data are presented as mean ± SD. *$p < 0.05$, **$p < 0.001$ (Student's t test).

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.

ACKNOWLEDGMENTS

We thank T. Okada for $Bcl6^{yfp/yfp}$ mice; E. Hobeika and M. Reth for $Mb1^{Cre/+}$ mice; T. Matsuyama and K. Yui for $Irf4^{-/-}$ mice; Y. Nakatsuji and T. Okuno for discussion; and P.W. Kincade for critical reading and editing of the manuscript. This work was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (T.K., M.M., and Y.B.), Japan Science and Technology Agency and Core Research for Evolutional Science and Technology (T.K.), and Daiichi Sankyo Foundation of Life Science (Y.B.), and by grants and fellowships from the National Health and Medical Research Council of Australia (A.K. and S.L.N.), the Australia Research Council (A.K. and S.L.N.), and the Sylvia and Charles Viertel Foundation (A.K.) and was made possible through Victorian State Government Operational Infrastructure Support and Australian Government NHMRC IRIIS.

Received: June 3, 2014
Accepted: October 28, 2014
Published: December 4, 2014

REFERENCES

Arce, E., Jackson, D.G., Gill, M.A., Bennett, L.B., Banchereau, J., and Pascual, V. (2001). Increased frequency of pre-germinal center B cells and plasma cell precursors in the blood of children with systemic lupus erythematosus. J. Immunol. *167*, 2361–2369.

Atarashi, K., Tanoue, T., Shima, T., Imaoka, A., Kuwahara, T., Momose, Y., Cheng, G., Yamasaki, S., Saito, T., Ohba, Y., et al. (2011). Induction of colonic regulatory T cells by indigenous *Clostridium* species. Science *331*, 337–341.

Barr, T.A., Shen, P., Brown, S., Lampropoulou, V., Roch, T., Lawrie, S., Fan, B., O'Connor, R.A., Anderton, S.M., Bar-Or, A., et al. (2012). B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells. J. Exp. Med. *209*, 1001–1010.

Blair, P.A., Noreña, L.Y., Flores-Borja, F., Rawlings, D.J., Isenberg, D.A., Ehrenstein, M.R., and Mauri, C. (2010). $CD19^+CD24^{hi}CD38^{hi}$ B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic lupus erythematosus patients. Immunity *32*, 129–140.

Cretney, E., Xin, A., Shi, W., Minnich, M., Masson, F., Miasari, M., Belz, G.T., Smyth, G.K., Busslinger, M., Nutt, S.L., and Kallies, A. (2011). The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. Nat. Immunol. *12*, 304–311.

De Silva, N.S., Simonetti, G., Heise, N., and Klein, U. (2012). The diverse roles of IRF4 in late germinal center B-cell differentiation. Immunol. Rev. *247*, 73–92.

Duddy, M., Niino, M., Adatia, F., Hebert, S., Freedman, M., Atkins, H., Kim, H.J., and Bar-Or, A. (2007). Distinct effector cytokine profiles of memory and naive human B cell subsets and implication in multiple sclerosis. J. Immunol. *178*, 6092–6099.

Evans, J.G., Chavez-Rueda, K.A., Eddaoudi, A., Meyer-Bahlburg, A., Rawlings, D.J., Ehrenstein, M.R., and Mauri, C. (2007). Novel suppressive function of transitional 2 B cells in experimental arthritis. J. Immunol. *178*, 7868–7878.

Fillatreau, S., Sweenie, C.H., McGeachy, M.J., Gray, D., and Anderton, S.M. (2002). B cells regulate autoimmunity by provision of IL-10. Nat. Immunol. *3*, 944–950.

Giordani, L., Sanchez, M., Libri, I., Quaranta, M.G., Mattioli, B., and Viora, M. (2009). IFN-α amplifies human naive B cell TLR-9-mediated activation and Ig production. J. Leukoc. Biol. *86*, 261–271.

Gürcan, H.M., Keskin, D.B., Stern, J.N., Nitzberg, M.A., Shekhani, H., and Ahmed, A.R. (2009). A review of the current use of rituximab in autoimmune diseases. Int. Immunopharmacol. *9*, 10–25.

Hartung, H.P., and Kieseier, B.C. (2010). Atacicept: targeting B cells in multiple sclerosis. Ther. Adv. Neurol. Disord. *3*, 205–216.

Hobeika, E., Thiemann, S., Storch, B., Jumaa, H., Nielsen, P.J., Pelanda, R., and Reth, M. (2006). Testing gene function early in the B cell lineage in mb1-cre mice. Proc. Natl. Acad. Sci. USA *103*, 13789–13794.

Jego, G., Palucka, A.K., Blanck, J.P., Chalouni, C., Pascual, V., and Banchereau, J. (2003). Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6. Immunity *19*, 225–234.

Joo, H., Coquery, C., Xue, Y., Gayet, I., Dillon, S.R., Punaro, M., Zurawski, G., Banchereau, J., Pascual, V., and Oh, S. (2012). Serum from patients with SLE instructs monocytes to promote IgG and IgA plasmablast differentiation. J. Exp. Med. *209*, 1335–1348.

Kallies, A., and Nutt, S.L. (2007). Terminal differentiation of lymphocytes depends on Blimp-1. Curr. Opin. Immunol. *19*, 156–162.

Kallies, A., Hasbold, J., Tarlinton, D.M., Dietrich, W., Corcoran, L.M., Hodgkin, P.D., and Nutt, S.L. (2004). Plasma cell ontogeny defined by quantitative changes in blimp-1 expression. J. Exp. Med. *200*, 967–977.

Kallies, A., Hasbold, J., Fairfax, K., Pridans, C., Emslie, D., McKenzie, B.S., Lew, A.M., Corcoran, L.M., Hodgkin, P.D., Tarlinton, D.M., and Nutt, S.L. (2007). Initiation of plasma-cell differentiation is independent of the transcription factor Blimp-1. Immunity *26*, 555–566.

Kim, T., Kanayama, Y., Negoro, N., Okamura, M., Takeda, T., and Inoue, T. (1987). Serum levels of interferons in patients with systemic lupus erythematosus. Clin. Exp. Immunol. *70*, 562–569.

Kitamura, D., Roes, J., Kühn, R., and Rajewsky, K. (1991). A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. Nature *350*, 423–426.

Kitano, M., Moriyama, S., Ando, Y., Hikida, M., Mori, Y., Kurosaki, T., and Okada, T. (2011). Bcl6 protein expression shapes pre-germinal center B cell dynamics and follicular helper T cell heterogeneity. Immunity *34*, 961–972.

Klein, U., and Dalla-Favera, R. (2008). Germinal centres: role in B-cell physiology and malignancy. Nat. Rev. Immunol. *8*, 22–33.

Lampropoulou, V., Hoehlig, K., Roch, T., Neves, P., Calderón Gómez, E., Sweenie, C.H., Hao, Y., Freitas, A.A., Steinhoff, U., Anderton, S.M., and Fillatreau, S. (2008). TLR-activated B cells suppress T cell-mediated autoimmunity. J. Immunol. *180*, 4763–4773.

Lee, C.G., Kang, K.H., So, J.S., Kwon, H.K., Son, J.S., Song, M.K., Sahoo, A., Yi, H.J., Hwang, K.C., Matsuyama, T., et al. (2009). A distal cis-regulatory element, CNS-9, controls NFAT1 and IRF4-mediated IL-10 gene activation in T helper cells. Mol. Immunol. *46*, 613–621.

Li, P., Spolski, R., Liao, W., Wang, L., Murphy, T.L., Murphy, K.M., and Leonard, W.J. (2012). BATF-JUN is critical for IRF4-mediated transcription in T cells. Nature *490*, 543–546.

Madan, R., Demircik, F., Surianarayanan, S., Allen, J.L., Divanovic, S., Trompette, A., Yogev, N., Gu, Y., Khodoun, M., Hildeman, D., et al. (2009). Nonredundant roles for B cell-derived IL-10 in immune counter-regulation. J. Immunol. *183*, 2312–2320.

Maseda, D., Smith, S.H., DiLillo, D.J., Bryant, J.M., Candando, K.M., Weaver, C.T., and Tedder, T.F. (2012). Regulatory B10 cells differentiate into antibody-secreting cells after transient IL-10 production in vivo. J. Immunol. *188*, 1036–1048.

Matsumoto, M., Fujii, Y., Baba, A., Hikida, M., Kurosaki, T., and Baba, Y. (2011). The calcium sensors STIM1 and STIM2 control B cell regulatory function through interleukin-10 production. Immunity *34*, 703–714.

Matsushita, T., Fujimoto, M., Hasegawa, M., Komura, K., Takehara, K., Tedder, T.F., and Sato, S. (2006). Inhibitory role of CD19 in the progression of experimental autoimmune encephalomyelitis by regulating cytokine response. Am. J. Pathol. *168*, 812–821.

Matsushita, T., Yanaba, K., Bouaziz, J.D., Fujimoto, M., and Tedder, T.F. (2008). Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. J. Clin. Invest. *118*, 3420–3430.

Mauri, C. (2010). Regulation of immunity and autoimmunity by B cells. Curr. Opin. Immunol. *22*, 761–767.

Mauri, C., and Bosma, A. (2012). Immune regulatory function of B cells. Annu. Rev. Immunol. *30*, 221–241.

Mauri, C., Gray, D., Mushtaq, N., and Londei, M. (2003). Prevention of arthritis by interleukin 10-producing B cells. J. Exp. Med. *197*, 489–501.

McHeyzer-Williams, M., Okitsu, S., Wang, N., and McHeyzer-Williams, L. (2012). Molecular programming of B cell memory. Nat. Rev. Immunol. *12*, 24–34.

Mittrücker, H.W., Matsuyama, T., Grossman, A., Kündig, T.M., Potter, J., Shahinian, A., Wakeham, A., Patterson, B., Ohashi, P.S., and Mak, T.W. (1997). Requirement for the transcription factor LSIRF/IRF4 for mature B and T lymphocyte function. Science *275*, 540–543.

Oracki, S.A., Walker, J.A., Hibbs, M.L., Corcoran, L.M., and Tarlinton, D.M. (2010). Plasma cell development and survival. Immunol. Rev. *237*, 140–159.

Ramgolam, V.S., Sha, Y., Marcus, K.L., Choudhary, N., Troiani, L., Chopra, M., and Markovic-Plese, S. (2011). B cells as a therapeutic target for IFN-β in relapsing-remitting multiple sclerosis. J. Immunol. *186*, 4518–4526.

Shapiro-Shelef, M., Lin, K.I., McHeyzer-Williams, L.J., Liao, J., McHeyzer-Williams, M.G., and Calame, K. (2003). Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. Immunity *19*, 607–620.

Shen, P., Roch, T., Lampropoulou, V., O'Connor, R.A., Stervbo, U., Hilgenberg, E., Ries, S., Dang, V.D., Jaimes, Y., Daridon, C., et al. (2014). IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. Nature *507*, 366–370.

Suzuki, S., Honma, K., Matsuyama, T., Suzuki, K., Toriyama, K., Akitoyo, I., Yamamoto, K., Suematsu, T., Nakamura, M., Yui, K., and Kumatori, A. (2004). Critical roles of interferon regulatory factor 4 in $CD11b^{high}CD8\alpha^{-}$ dendritic cell development. Proc. Natl. Acad. Sci. USA *101*, 8981–8986.

Todd, S.K., Pepper, R.J., Draibe, J., Tanna, A., Pusey, C.D., Mauri, C., and Salama, A.D. (2014). Regulatory B cells are numerically but not functionally deficient in anti-neutrophil cytoplasm antibody-associated vasculitis. Rheumatology (Oxford) *53*, 1693–1703.

Watanabe, R., Ishiura, N., Nakashima, H., Kuwano, Y., Okochi, H., Tamaki, K., Sato, S., Tedder, T.F., and Fujimoto, M. (2010). Regulatory B cells (B10 cells) have a suppressive role in murine lupus: CD19 and B10 cell deficiency exacerbates systemic autoimmunity. J. Immunol. *184*, 4801–4809.

Yanaba, K., Bouaziz, J.D., Haas, K.M., Poe, J.C., Fujimoto, M., and Tedder, T.F. (2008). A regulatory B cell subset with a unique $CD1d^{hi}CD5^{+}$ phenotype controls T cell-dependent inflammatory responses. Immunity *28*, 639–650.

Yoshizaki, A., Miyagaki, T., DiLillo, D.J., Matsushita, T., Horikawa, M., Kountikov, E.I., Spolski, R., Poe, J.C., Leonard, W.J., and Tedder, T.F. (2012). Regulatory B cells control T-cell autoimmunity through IL-21-dependent cognate interactions. Nature *491*, 264–268.

Available online at www.sciencedirect.com

ScienceDirect

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.

Addresses
Experimental Immunology, WPI Immunology Frontier Research Center, Osaka University, Osaka 565-0871, Japan

Corresponding author: Sakaguchi, Shimon (shimon@ifrec.osaka-u.ac.jp)

Current Opinion in Immunology 2014, **27**:1–7

This review comes from a themed issue on **Tumour immunology**

Edited by **Philip K Darcy** and **David S Ritchie**

For a complete overview see the Issue and the Editorial

Available online 14th January 2014

0952-7915/$ – see front matter,

http://dx.doi.org/10.1016/j.coi.2013.12.005

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.

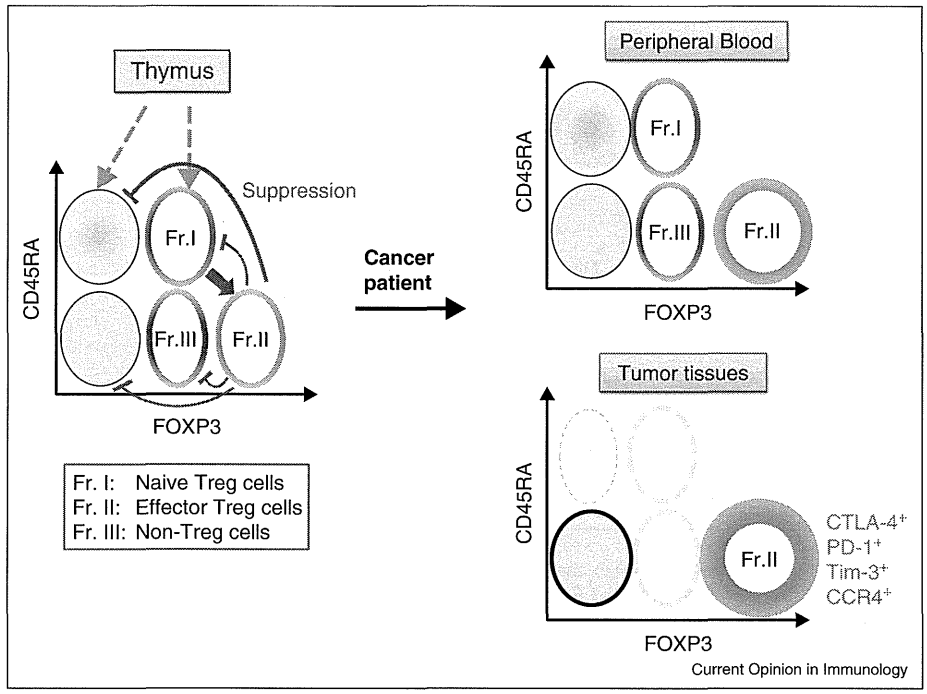
This review focuses on our current understanding of the roles of FOXP3$^+$ Treg cells in tumor immunity in humans and discusses a perspective for numerical or functional manipulation of Treg cells as a key strategy in cancer immunotherapy.

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



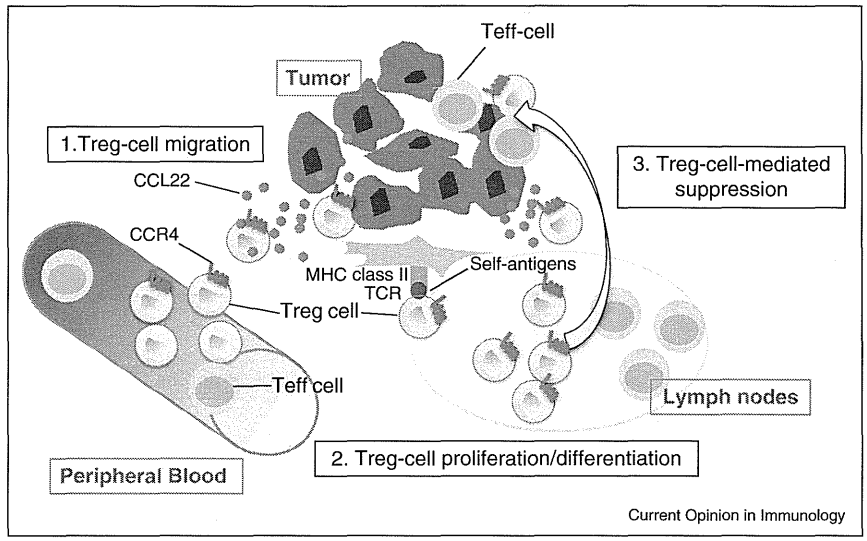
(Left) FOXP3$^+$CD4$^+$ T cells are dissected into three subpopulations by the expression levels of FOXP3 and the cell surface molecule CD45RA: FOXP3^{lo}CD45RA$^+$ cells (Fr. I), designated naive or resting Treg cells, which differentiate into FOXP3^{hi}CD45RA$^-$ cells (Fr. II), designated eTreg cells. 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.

Acknowledgement

We thank Dr. J.B. Wing for critical reading of this manuscript.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T: **A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma.** *Science* 1991, **254**:1643-1647.

2. Kawakami Y, Rosenberg SA: **Human tumor antigens recognized by T-cells.** *Immunol Res* 1997, **16**:313-339.

3. 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.

4. Boon T, Coulie PG, Van den Eynde BJ, van der Bruggen P: **Human T cell responses against melanoma.** *Annu Rev Immunol* 2006, **24**:175-208.

5. • Rosenberg SA, Yang JC, Restifo NP: **Cancer immunotherapy: moving beyond current vaccines.** *Nat Med* 2004, **10**: 909-915.

A comprehensive review on cancer vaccine trials and discusses problems of cancer vaccines.

6. Gnjatic S, Nishikawa H, Jungbluth AA, Gure AO, Ritter G, Jager E, Knuth A, Chen Y-T, Old LJ: **NY-ESO-1: review of an immunogenic tumor antigen.** *Adv Cancer Res* 2006, **95**:1-30.

7. Dougan M, Dranoff G: **Immune therapy for cancer.** *Annu Rev Immunol* 2009, **27**:83-117.

8. Zou W: **Regulatory T cells, tumour immunity and immunotherapy.** *Nat Rev Immunol* 2006, **6**:295-307.

9. Nishikawa H, Sakaguchi S: **Regulatory T cells in tumor immunity.** *Int J Cancer* 2010, **127**:759-767.

10. Sharma P, Wagner K, Wolchok JD, Allison JP: **Novel cancer immunotherapy agents with survival benefit: recent successes and next steps.** *Nat Rev Cancer* 2011, **11**:805-812.

11. Wing K, Sakaguchi S: **Regulatory T cells exert checks and balances on self tolerance and autoimmunity.** *Nat Immunol* 2010, **11**:7-13.

12. Shimizu J, Yamazaki S, Sakaguchi S: **Induction of tumor immunity by removing CD25$^+$CD4$^+$ T cells: a common basis between tumor immunity and autoimmunity.** *J Immunol* 1999, **163**:5211-5218.

13. Sakaguchi S, Miyara M, Costantino CM, Hafler DA: **FOXP3$^+$ regulatory T cells in the human immune system.** *Nat Rev Immunol* 2010, **10**:490-500.

14. •• Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, Parizot C, Taflin C, Heike T, Valeyre D *et al.*: **Functional delineation and differentiation dynamics of human CD4$^+$ T cells expressing the FoxP3 transcription factor.** *Immunity* 2009, **30**:899-911.

This study provides a classification of human FOXP3$^+$ Treg cells into subpopulations by their phenotypes, functions, and developmental cell fates. The classification also delineates a non-Treg cell fraction within the FOXP3$^+$ T-cell population and reflects adaptive changes of Treg cells in autoimmune and inflammatory diseases.

15. • Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow 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.

This study describes a clear correlation between the frequency of Treg cells in tumor tissues and poor prognosis in ovarian cancer patients. In additon, the authors showed a role of the CCR4–CCL22 axis for Treg-cell infiltration into tumors.

16. • Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, Jungbluth AA, Frosina D, Gnjatic S, Ambrosone C *et al.*: **Intraepithelial CD8$^+$ tumor-infiltrating lymphocytes and a high CD8$^+$/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer.** *Proc Natl Acad Sci U S A* 2005, **102**:18538-18543.

This study describes the importance of assessing the ratio of effector $CD8^+$ T cells to Treg cells in tumor tissues for evaluating the prognosis of cancer patients.

17. Alvaro T, Lejeune M, Salvado MT, Bosch R, Garcia JF, Jaen J, Banham AH, Roncador G, Montalban C, Piris MA: **Outcome in Hodgkin's lymphoma can be predicted from the presence of accompanying cytotoxic and regulatory T cells.** *Clin Cancer Res* 2005, **11**:1467-1473.

18. Badoual C, Hans S, Rodriguez J, Peyrard S, Klein C, Agueznay Nel H, Mosseri V, Laccourreye O, Bruneval P, Fridman WH *et al.*: **Prognostic value of tumor-infiltrating $CD4^+$ T-cell subpopulations in head and neck cancers.** *Clin Cancer Res* 2006, **12**:465-472.

19. Salama P, Phillips M, Grieu F, Morris M, Zeps N, Joseph D, Platell C, Iacopetta B: **Tumor-infiltrating $FOXP3^+$ T regulatory cells show strong prognostic significance in colorectal cancer.** *J Clin Oncol* 2009, **27**:186-192.

20. •• Sugiyama D, Nishikawa H, Maeda Y, Nishioka M, Tanemura A, Katayama I, Ezoe S, Kanakura Y, Sato E, Fukumori Y *et al.*: **Anti-CCR4 mAb selectively depletes effector-type $FoxP3^+CD4^+$ regulatory T cells, evoking antitumor immune responses in humans.** *Proc Natl Acad Sci U S A* 2013, **110**:17945-17950.

This study shows that specific depletion of the effector-type $FOXP3^+$ subpopulation can evoke potent tumor-antigen-specific immune responses *in vivo* and *in vitro*. It also suggests the possiblity of evoking anti-tumor immunity without serious autoimmunity via targeting effector-type $FOXP3^+$ Treg cells, rather than whole $FOXP3^+$ T cells.

21. Faget J, Biota C, Bachelot T, Gobert M, Treilleux I, Goutagny N, Durand I, Leon-Goddard S, Blay JY, Caux C *et al.*: **Early detection of tumor cells by innate immune cells leads to Treg recruitment through CCL22 production by tumor cells.** *Cancer Res* 2011, **71**:6143-6152.

22. Facciabene A, Peng X, Hagemann IS, Balint K, Barchetti A, Wang LP, Gimotty PA, Gilks CB, Lal P, Zhang L *et al.*: **Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and Treg cells.** *Nature* 2011, **475**:226-230.

23. Redjimi N, Raffin C, Raimbaud I, Pignon P, Matsuzaki J, Odunsi K, Valmori D, Ayyoub M: **$CXCR3^+$ T regulatory cells selectively accumulate in human ovarian carcinomas to limit type I immunity.** *Cancer Res* 2012, **72**:4351-4360.

24. Wang HY, Lee DA, Peng G, Guo Z, Li Y, Kiniwa Y, Shevach EM, Wang RF: **Tumor-specific human $CD4^+$ regulatory T cells and their ligands: implications for immunotherapy.** *Immunity* 2004, **20**:107-118.

25. Vence L, Palucka AK, Fay JW, Ito T, Liu YJ, Banchereau J, Ueno H: **Circulating tumor antigen-specific regulatory T cells in patients with metastatic melanoma.** *Proc Natl Acad Sci U S A* 2007, **104**:20884-20889.

26. Lehe C, Ghebeh H, Al-Sulaiman A, Al Qudaihi G, Al-Hussein K, Almohareb F, Chaudhri N, Alsharif F, Al-Zahrani H, Tbakhi A *et al.*: **The Wilms' tumor antigen is a novel target for human $CD4^+$ regulatory T cells: implications for immunotherapy.** *Cancer Res* 2008, **68**:6350-6359.

27. •• Bonertz A, Weitz J, Pietsch DHK, Rahbari NN, Schlude C, Ge YZ, Juenger S, Vlodavsky I, Khazaie K, Jaeger D *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.

This study describes the presence of tumor antigen-specific Treg cells in cancer patients, but not in healthy individuals, raising the possibility that cancer vaccines activate both effector T cells and Treg cells that recognize the same tumor antigens.

28. Fourcade J, Sun Z, Kudela P, Janjic B, Kirkwood JM, El-Hafnawy T, Zarour HM: **Human tumor antigen-specific helper and regulatory T cells share common epitope specificity but exhibit distinct T cell repertoire.** *J Immunol* 2010, **184**:6709-6718.

29. Nishikawa H, Kato T, Tawara I, Saito K, Ikeda H, Kuribayashi K, Allen PM, Schreiber RD, Sakaguchi S, Old LJ *et al.*: **Definition of target antigens for naturally occurring $CD4^+CD25^+$ regulatory T cells.** *J Exp Med* 2005, **201**:681-686.

30. Jordan MS, Boesteanu A, Reed AJ, Petrone AL, Holenbeck AE, Lerman MA, Naji A, Caton AJ: **Thymic selection of $CD4^+CD25^+$ regulatory T cells induced by an agonist self-peptide.** *Nat Immunol* 2001, **2**:301-306.

31. Sakaguchi S: **Naturally arising $CD4^+$ regulatory T cells for immunologic self-tolerance and negative control of immune responses.** *Annu Rev Immunol* 2004, **22**:531-562.

32. Francois V, Ottaviani S, Renkvist N, Stockis J, Schuler G, Thielemans K, Colau D, Marchand M, Boon T, Lucas S *et al.*: **The $CD4^+$ T-cell response of melanoma patients to a MAGE-A3 peptide vaccine involves potential regulatory T cells.** *Cancer Res* 2009, **69**:4335-4345.

33. Chen Y-T, Scanlan MJ, Sahin U, Tureci O, Gure AO, Tsang S, Williamson B, Stockert E, Pfreundschuh M, Old LJ: **A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening.** *Proc Natl Acad Sci U S A* 1997, **94**:1914-1918.

34. • Danke NA, Koelle DM, Yee C, Beheray S, Kwok WW: **Autoreactive T cells in healthy individuals.** *J Immunol* 2004, **172**:5967-5972.

This study shows that $CD4^+$ T cells specific for authentic self-antigens and tumor-associated self-antigens are present in the peripheral blood of healthy inidiviuals, but suppressed not to expand by Treg cells. Such self-reactive $CD4^+$ T cells become detectable when they are stimulated with self-antigens after removing Treg cells.

35. • Nishikawa H, Jager E, Ritter G, Old LJ, Gnjatic S: **$CD4^+CD25^+$ regulatory T cells control the induction of antigen-specific $CD4^+$ helper T cell responses in cancer patients.** *Blood* 2005, **106**:1008-1011.

See annotation to Ref. [34•].

36. Nishikawa H, Qian F, Tsuji T, Ritter G, Old LJ, Gnjatic S, Odunsi K: **Influence of $CD4^+CD25^+$ regulatory T cells on low/high-avidity $CD4^+$ T cells following peptide vaccination.** *J Immunol* 2006, **176**:6340-6346.

37. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M: **Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases.** *J Immunol* 1995, **155**:1151-1164.

38. Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T, Nakayama E: **Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor α) monoclonal antibody.** *Cancer Res* 1999, **59**:3128-3133.

39. Attia P, Maker AV, Haworth LR, Rogers-Freezer L, Rosenberg SA: **Inability of a fusion protein of IL-2 and diphtheria toxin (Denileukin Diftitox, DAB389IL-2, ONTAK) to eliminate regulatory T lymphocytes in patients with melanoma.** *J Immunother* 2005, **28**:582-592.

40. Dannull J, Su Z, Rizzieri D, Yang BK, Coleman D, Yancey D, Zhang A, Dahm P, Chao N, Gilboa E *et al.*: **Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells.** *J Clin Invest* 2005, **115**:3623-3633.

41. Jacobs JF, Punt CJ, Lesterhuis WJ, Sutmuller RP, Brouwer HM, Scharenborg NM, Klasen IS, Hilbrands LB, Figdor CG, de Vries IJ *et al.*: **Dendritic cell vaccination in combination with anti-CD25 monoclonal antibody treatment: a phase I/II study in metastatic melanoma patients.** *Clin Cancer Res* 2010, **16**:5067-5078.

42. Rech AJ, Mick R, Martin S, Recio A, Aqui NA, Powell DJ Jr, Colligon TA, Trosko JA, Leinbach LI, Pletcher CH *et al.*: **CD25 blockade depletes and selectively reprograms regulatory T cells in concert with immunotherapy in cancer patients.** *Sci Transl Med* 2012, **4**:134ra162.

43. Williams MA, Bevan MJ: **Effector and memory CTL differentiation.** *Annu Rev Immunol* 2007, **25**:171-192.

44. Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S: **Stimulation of $CD25^+CD4^+$ regulatory T cells through GITR breaks immunological self-tolerance.** *Nat Immunol* 2002, **3**:135-142.

45. Ko K, Yamazaki S, Nakamura K, Nishioka T, Hirota K, Yamaguchi T, Shimizu J, Nomura T, Chiba T, Sakaguchi S:

Treatment of advanced tumors with agonistic anti-GITR mAb and its effects on tumor-infiltrating Foxp3$^+$CD25$^+$CD4$^+$ regulatory T cells. *J Exp Med* 2005, **202**:885-891.

46. Mitsui J, Nishikawa H, Muraoka D, Wang L, Noguchi T, Sato E, Kondo S, Allison JP, Sakaguchi S, Old LJ *et al.*: **Two distinct mechanisms of augmented antitumor activity by modulation of immunostimulatory/inhibitory signals.** *Clin Cancer Res* 2010, **16**:2781-2791.

47. Valzasina B, Guiducci C, Dislich H, Killeen N, Weinberg AD, Colombo MP: **Triggering of OX40 (CD134) on CD4$^+$CD25$^+$ T cells blocks their inhibitory activity: a novel regulatory role for OX40 and its comparison with GITR.** *Blood* 2005, **105**:2845-2851.

48. Piconese S, Valzasina B, Colombo MP: **OX40 triggering blocks suppression by regulatory T cells and facilitates tumor rejection.** *J Exp Med* 2008, **205**:825-839.

49. •• Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC *et al.*: **Improved survival with ipilimumab in patients with metastatic melanoma.** *N Engl J Med* 2010, **363**:711-723.
This study decribes the first success of phase III clnical trials of immune-checkpoint blockade (anti-CTLA-4 mAb, Ipilimumab).

50. Robert C, Thomas L, Bondarenko I, O'Day S, M D J, Garbe C, Lebbe C, Baurain JF, Testori A, Grob JJ *et al.*: **Ipilimumab plus dacarbazine for previously untreated metastatic melanoma.** *N Engl J Med* 2011, **364**:2517-2526.

51. Leach DR, Krummel MF, Allison JP: **Enhancement of antitumor immunity by CTLA-4 blockade.** *Science* 1996, **271**:1734-1736.

52. Quezada SA, Peggs KS, Curran MA, Allison JP: **CTLA4 blockade and GM-CSF combination immunotherapy alters the intratumor balance of effector and regulatory T cells.** *J Clin Invest* 2006, **116**:1935-1945.

53. • Bulliard Y, Jolicoeur R, Windman M, Rue SM, Ettenberg S, Knee DA, Wilson NS, Dranoff G, Brogdon JL: **Activating Fcγ receptors contribute to the antitumor activities of immunoregulatory receptor-targeting antibodies.** *J Exp Med* 2013, **210**:1685-1693.
This study shows that a main anti-tumor effect of immune-checkpoint blockade can be depletion of FOXP3$^+$ Treg cells in tumor tissues rather than direct augmentation of effector T-cell function.

54. • Mark JS, John JE, Michael Q, Karla AH, Timothy C, Mohan S, Alan JK: **Anti-CTLA-4 antibodies of IgG2a isotype enhance antitumor activity through reduction of intratumoral regualtory T cells.** *Cancer Immunol Res* 2013, **1**:32-42.
See annotaion to Ref. [53•].

55. • Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, Roddie C, Henry JY, Yagita H, Wolchok JD *et al.*: **Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma.** *J Exp Med* 2013, **210**:1695-1710.
See annotaion to Ref. [53•].

56. Hodi FS, Butler M, Oble DA, Seiden MV, Haluska FG, Kruse A, Macrae S, Nelson M, Canning C, Lowy I *et al.*: **Immunologic and clinical effects of antibody blockade of cytotoxic T lymphocyte-associated antigen 4 in previously vaccinated cancer patients.** *Proc Natl Acad Sci U S A* 2008, **105**:3005-3010.

57. Liakou CI, Kamat A, Tang DN, Chen H, Sun J, Troncoso P, Logothetis C, Sharma P: **CTLA-4 blockade increases IFNγ-producing CD4$^+$ICOS^{hi} cells to shift the ratio of effector to regulatory T cells in cancer patients.** *Proc Natl Acad Sci U S A* 2008, **105**:14987-14992.

58. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S: **CTLA-4 control over Foxp3$^+$ regulatory T cell function.** *Science* 2008, **322**:271-275.

59. Ise W, Kohyama M, Nutsch KM, Lee HM, Suri A, Unanue ER, Murphy TL, Murphy KM: **CTLA-4 suppresses the pathogenicity of self antigen-specific T cells by cell-intrinsic and cell-extrinsic mechanisms.** *Nat Immunol* 2010, **11**:129-135.

60. Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsi K *et al.*: **Safety and activity of anti-PD-L1 antibody in patients with advanced cancer.** *N Engl J Med* 2012, **366**:2455-2465.

61. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB *et al.*: **Safety, activity, and immune correlates of anti-PD-1 antibody in cancer.** *N Engl J Med* 2012, **366**:2443-2454.

62. Hamid O, Robert C, Daud A, Hodi FS, Hwu WJ, Kefford R, Wolchok JD, Hersey P, Joseph RW, Weber JS *et al.*: **Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma.** *N Engl J Med* 2013, **369**:134-144.

63. Kryczek I, Liu R, Wang G, Wu K, Shu X, Szeliga W, Vatan L, Finlayson E, Huang E, Simeone D *et al.*: **FOXP3 defines regulatory T cells in human tumor and autoimmune disease.** *Cancer Res* 2009, **69**:3995-4000.

64. Berger R, Rotem-Yehudar R, Slama G, Landes S, Kneller A, Leiba M, Koren-Michowitz M, Shimoni A, Nagler A: **Phase I safety and pharmacokinetic study of CT-011, a humanized antibody interacting with PD-1, in patients with advanced hematologic malignancies.** *Clin Cancer Res* 2008, **14**:3044-3051.

65. Beyer M, Kochanek M, Darabi K, Popov A, Jensen M, Endl E, Knolle PA, Thomas RK, von Bergwelt-Baildon M, Debey S *et al.*: **Reduced frequencies and suppressive function of CD4$^+$CD25^{hi} regulatory T cells in patients with chronic lymphocytic leukemia after therapy with fludarabine.** *Blood* 2005, **106**:2018-2025.

66. Walter S, Weinschenk T, Stenzl A, Zdrojowy R, Pluzanska A, Szczylik C, Staehler M, Brugger W, Dietrich PY, Mendrzyk R *et al.*: **Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival.** *Nat Med* 2011, **17**:1094-1100.

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.

REFERENCES AND NOTES

1. A.-L. Hsu, C. T. Murphy, C. Kenyon, *Science* **300**, 1142–1145 (2003).
2. M. Fujimoto *et al.*, *J. Biol. Chem.* **280**, 34908–34916 (2005).
3. G. McColl *et al.*, *Cell Metab.* **12**, 260–272 (2010).
4. N. Kourtis, V. Nikoletopoulou, N. Tavernarakis, *Nature* **490**, 213–218 (2012).
5. V. Prahlad, T. Cornelius, R. I. Morimoto, *Science* **320**, 811–814 (2008).
6. Y. M. Hajdu-Cronin, W. J. Chen, P. W. Sternberg, *Genetics* **168**, 1937–1949 (2004).
7. P. E. Kroeger, R. I. Morimoto, *Mol. Cell. Biol.* **14**, 7592–7603 (1994).
8. N. D. Trinklein, J. I. Murray, S. J. Hartman, D. Botstein, R. M. Myers, *Mol. Biol. Cell* **15**, 1254–1261 (2004).
9. H. Terami *et al.*, *J. Cell Biol.* **146**, 193–202 (1999).
10. K. Ono, S. Ono, *Mol. Biol. Cell* **15**, 2782–2793 (2004).
11. T. Obinata, K. Ono, S. Ono, *J. Cell Sci.* **123**, 1557–1566 (2010).
12. Z. Balklava, S. Pant, H. Fares, B. D. Grant, *Nat. Cell Biol.* **9**, 1066–1073 (2007).
13. T. Kuwahara *et al.*, *Hum. Mol. Genet.* **17**, 2997–3009 (2008).
14. L. A. Herndon *et al.*, *Nature* **419**, 808–814 (2002).
15. W. Greene, S.-J. Gao, *PLOS Pathog.* **5**, e1000512 (2009).
16. H. Fares, I. Greenwald, *Nat. Genet.* **28**, 64–68 (2001).
17. A. Patton *et al.*, *Curr. Biol.* **15**, 1045–1050 (2005).
18. J. F. Casella, M. D. Flanagan, S. Lin, *Nature* **293**, 302–305 (1981).
19. I. Spector, N. R. Shochet, Y. Kashman, A. Groweiss, *Science* **219**, 493–495 (1983).
20. L. Whitesell, S. Lindquist, *Expert Opin. Ther. Targets* **13**, 469–478 (2009).
21. C. H. Nguyen *et al.*, *Biochem. J.* **452**, 321–329 (2013).
22. D. R. Ciocca, A. P. Arrigo, S. K. Calderwood, *Arch. Toxicol.* **87**, 19–48 (2013).

ACKNOWLEDGMENTS

Bioinformatic analysis is included in the supplementary materials. The following funding sources supported this research: Howard Hughes Medical Institute, National Center for Research Resources (5P41RR011823-17), National Institute of General Medical Sciences (8 P41 GM103533-17), and National Institute on Aging (R01AG027463-04). N.A.B. was funded by a postdoctoral fellowship to the Salk Center for Nutritional Genomics from the Leona M. & Harry B. Helmsley Charitable Trust; P.M.D. was funded by George E. Hewitt Foundation for Medical Research and National Institute of Aging (1K99AG042495-01A1). We thank the laboratory of G. Lithgow for generously sharing the HSP-16 antibody and J. Durieux for helping design figure illustrations. Some of the nematode strains used in this work were provided by the *Caenorhabditis* Genetics Center (University of Minnesota), which is supported by the NIH–Office of Research Infrastructure Programs (P40 OD010440).

SUPPLEMENTARY MATERIALS

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

10 March 2014; accepted 13 August 2014
10.1126/science.1253168

AUTOIMMUNITY

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

Yoshinaga Ito,[1] Motomu Hashimoto,[1,2,3,4]* Keiji Hirota,[2]* Naganari Ohkura,[2,5] Hiromasa Morikawa,[2] Hiroyoshi Nishikawa,[2] Atsushi Tanaka,[2,5] Moritoshi Furu,[3,6] Hiromu Ito,[3,6] Takao Fujii,[3,4] Takashi Nomura,[1] Sayuri Yamazaki,[7] Akimichi Morita,[7] Dario A. A. Vignali,[8,9] John W. Kappler,[10,11] Shuichi Matsuda,[6] Tsuneyo Mimori,[4] Noriko Sakaguchi,[2] Shimon Sakaguchi[1,2,12]†

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

[1]Department of Experimental Pathology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan. [2]Department of Experimental Immunology, Immunology Frontier Research Center, Osaka University, Suita 565-0871, Japan. [3]Department of the Control for Rheumatic Diseases, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan. [4]Department of Rheumatology and Clinical Immunology, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan. [5]Department of Frontier Research in Tumor Immunology, Center of Medical Innovation and Translational Research, Osaka University, Osaka 565-0871, Japan. [6]Department of Orthopaedic Surgery, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan. [7]Department of Geriatric and Environmental Dermatology, Graduate School of Medical Sciences, Nagoya City University, Nagoya 467-8601, Japan. [8]Department of Immunology, St. Jude Children's Research Hospital, Memphis, TN 38105, USA. [9]Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA. [10]Integrated Department of Immunology, National Jewish Health, Denver, CO 80206, USA. [11]Howard Hughes Medical Institute, National Jewish Health, Denver, CO 80206, USA. [12]Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, Tokyo 102-0075, Japan. *These authors contributed equally to this work. †Corresponding author. E-mail: shimon@ifrec.osaka-u.ac.jp

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 $V\beta6^+$ $CD4^+$ 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%)

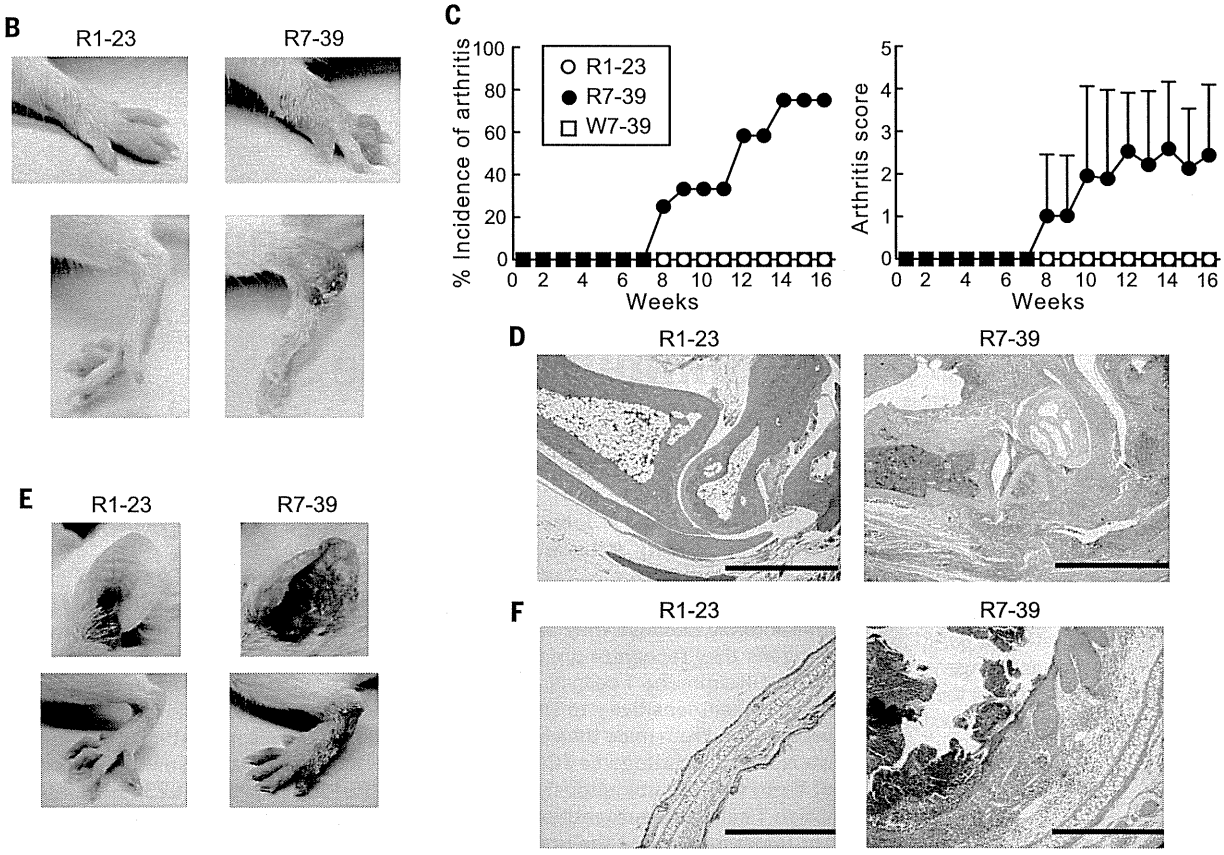


Fig. 1. Arthritis-inducing activity of two TCRs individually expressed in retrogenic mice. (**A**) Amino acid sequences and frequencies of two arthritogenic TCRs (7-39 and 6-39) and the nonarthritogenic 1-23 TCR. These three TCRs were obtained from three different mice. CDR, complementarity-determining 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 *TCRβ*$^{-/-}$ 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).

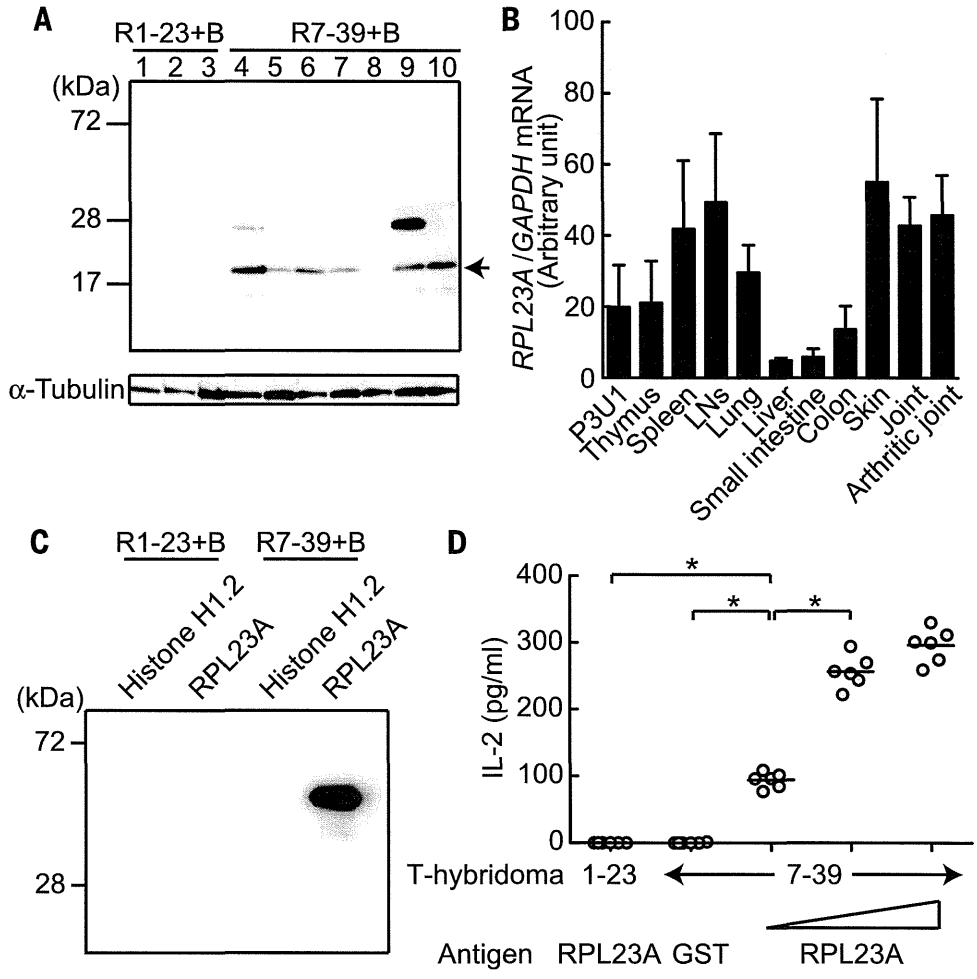


Fig. 2. Identification of the self antigen recognized by arthritogenic 7-39 TCRs. (**A**) Immunoblot analysis by sera from B cell–reconstituted R7-39 mice (n = 7) and B cell–reconstituted R1-23 mice (n = 3). Arrow indicates the commonly recognized protein. (**B**) Quantitative real-time polymerase chain 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

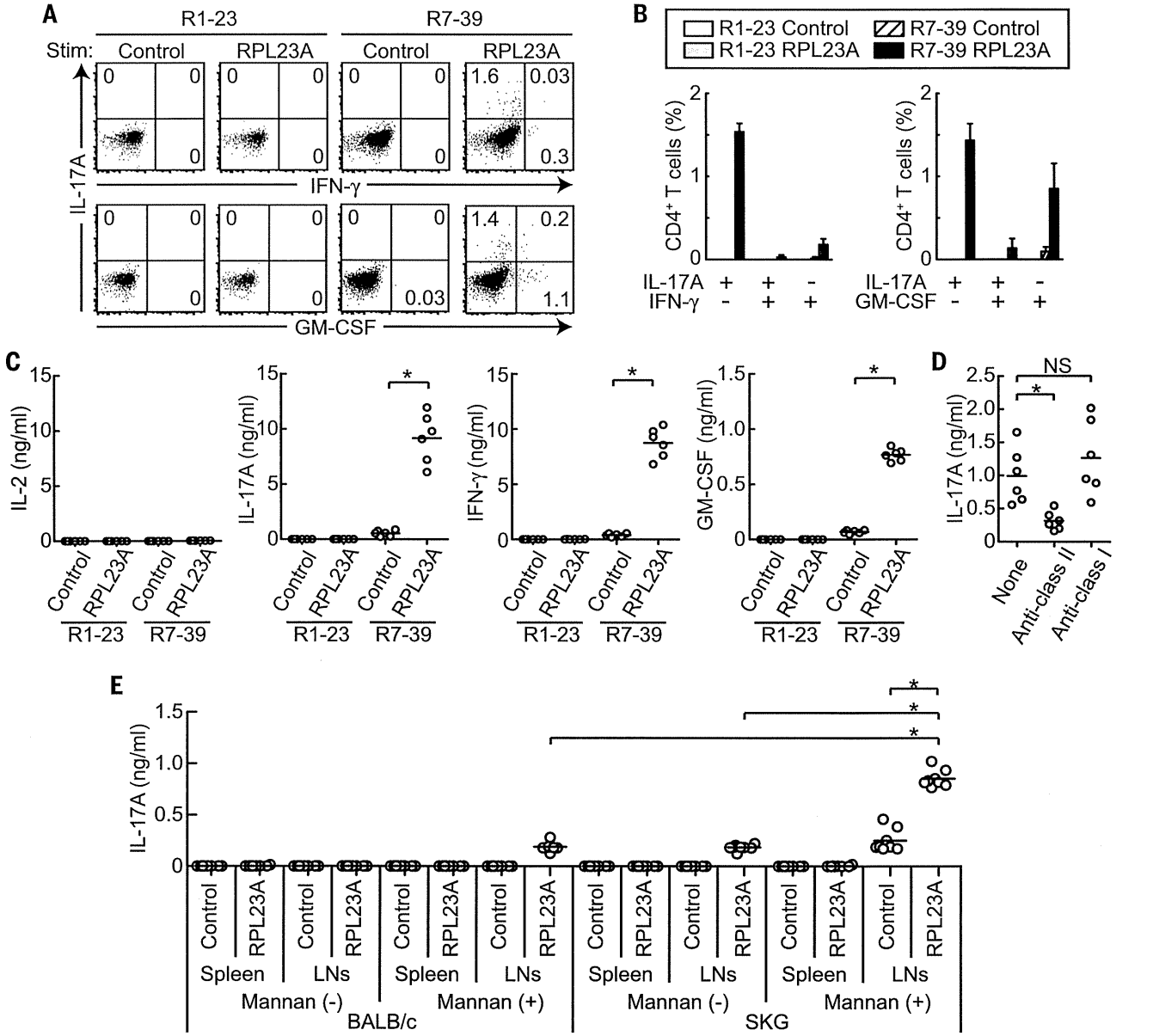


Fig. 3. RPL23A-reactive T_H cells in R7-39 mice. (**A**) Cytokine production by $CD4^+$ T cells from regional lymph nodes of R7-39 or R1-23 mice after in vitro stimulation with recombinant RPL23A or control glutathione *S*-transferase (GST) protein. Stim, stimulation. Data are representative of three 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

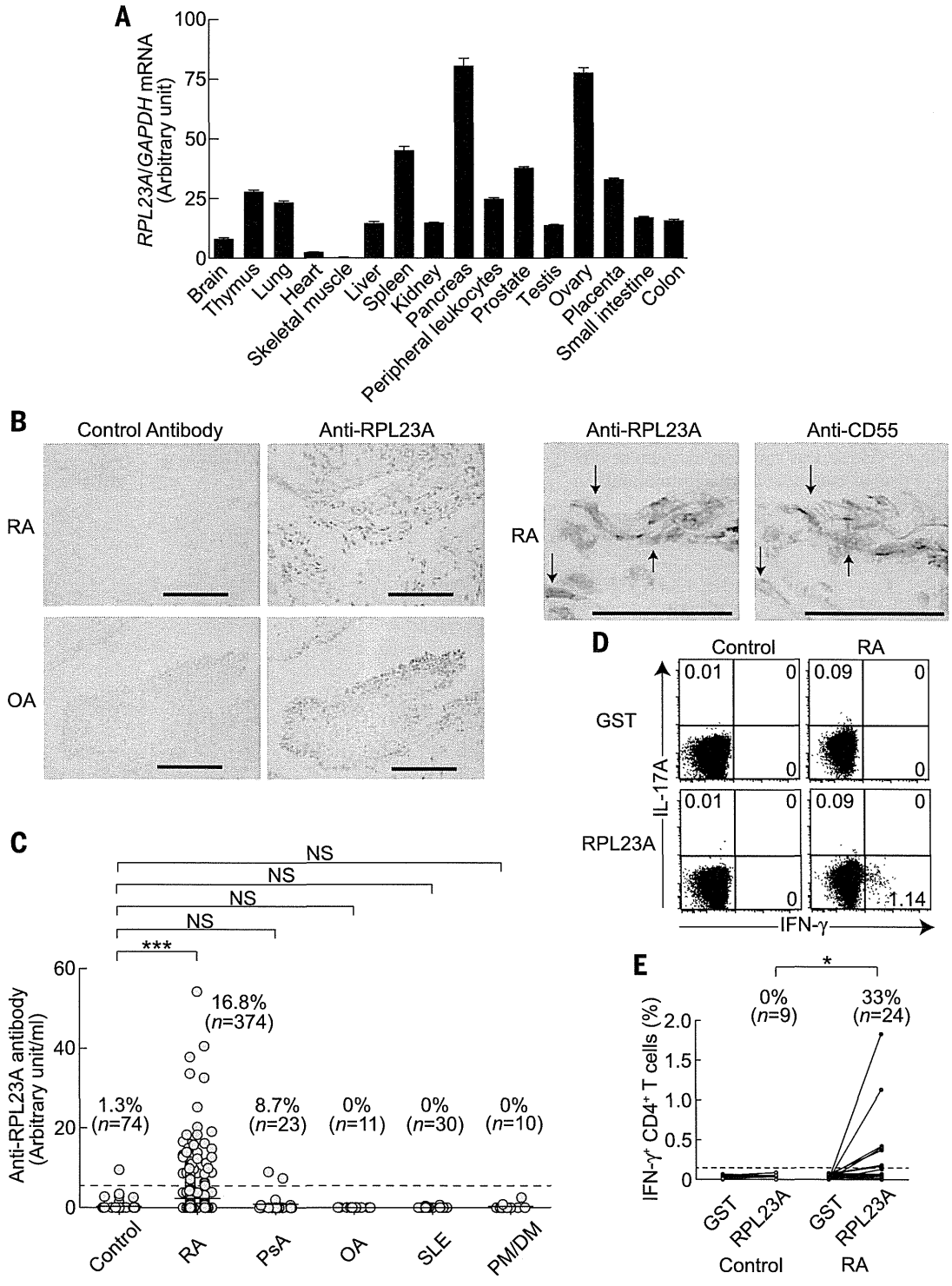


Fig. 4. Anti-RPL23A humoral and cellular immune responses in RA patients. (**A**) qPCR analysis of RPL23A gene expression in various tissues from healthy human subjects (n = 3). Results are shown as means ± SD and represent two independent experiments. (**B**) Immunohistochemical staining of synovial 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).