PCR products cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA) were analyzed for TCR sequences using CEQ DTCS-Quick Start Kit according to the manufacturer's protocol (Beckman Coulter Inc., Fullerton, CA, USA).

^{51}Cr release cytotoxicity assay

BALB/3T3 fibroblast line (H-2^d), J774 macrophage line (H-2^d), p815 mastocytoma line (H-2^d), EL-4 lymphoma line (H-2^b), L929 fibroblast line (H-2^k) obtained from Dainippon Sumitomo Pharma (Osaka, Japan) and synovial cells of SKG mice (H-2^d) were used as target cells. Synovial cells (1×10^4) were seeded in 96-well flat-bottom plates with 40 U/well of IFN-γ for 2 days and radiolabeled with 2.5 μCi/well of $Na_2^{51}CrO_4$ (Daiichi Radioisotope Laboratories, Ltd, Tokyo, Japan) for 2 h. Other target cells (3×10^5) were radiolabeled with 20 μCi of $Na_2^{51}CrO_4$ for 2 h and seeded in 96-well round-bottom plates at 1×10^4 cells per well. Effector cells (4×10^5) were added in each well in triplicate and incubated for 8 h. Relative cytotoxicity was calculated as follows from the radioactivity released in the culture supernatant; percent specific lysis = 100(experimental − spontaneous)/(maximal − spontaneous) counts per minute. Maximal lysis and spontaneous release were determined from target cells incubated with surfactant ×7 (Flow Laboratories, ICN Biomedicals, Inc., Aurora, OH, USA) or without effector cells, respectively.

Adoptive transfer

Spleen T cells from SKG mice or (SKG × BALB/c)F_1 mice and each SKG T cell clones (1×10^7) were intravenously transferred to C.B-17 SCID mice (8 weeks) or BALB/c-nu/nu mice (6 weeks), respectively. Control dengue 2F7 and 3F2 clone were collected 10–14 days after *in vitro* stimulation with specific peptide-pulsed irradiated (33 Gray) BALB/c spleen cells and transferred as described above. Severity of arthritis was scored weekly as previously described (14).

Clinical assessment of arthritis

Joint swelling was monitored by inspection and scored as follows: 0, no joint swelling; 0.1, swelling of one finger joint; 0.5, mild swelling of wrist or ankle and 1.0, severe swelling of wrist or ankle. Scores for all fingers and toes, wrists and ankles were totalled for each mouse (14).

Histological assessment of interstitial pneumonitis

Interstitial pneumonitis was evaluated microscopically depending on diffusely affected area: −, normal histology; +, 10–30%; ++, 30–60%; +++, >60% of the sections of the lungs showed pneumonitis.

Histology and immunohistochemistry

Tissues were fixed in 10% neutral formalin, paraffin embedded and stained with Haematoxylin & Eosin (H&E). Joints were additionally decalcified for 3 weeks in 10% EDTA in PBS before staining. For immunohistochemistry of joints, deparaffinized sections were incubated with 20% normal rabbit serum (Dako, Hamburg, Germany) in PBS for 15 min to block non-specific binding, primary rat anti-Ly-6G mAb (Gr-1, RB6-8C5; BD PharMingen) with appropriate dilutions overnight at 4°C, biotinylated polyclonal rabbit anti-rat antibody (Dako) and HRP-conjugated streptavidin (Dako). The slides were developed using diaminobenzidene (Elite Kit; Vector, Burlingame, CA, USA) and counterstained with Mayer's hematoxylin.

For immunohistochemistry of lungs, tissues were fixed in 4% phosphate-buffered PFA (pH 7.4) and embedded in Tissue-Tek OCT compound (Ted Pella, Inc., Redding, CA, USA). Cryostat sections were stained with rat mAbs to mouse CD4 (H129.19), CD8a (53-6.7), CD45R/B220 (RA3-6B2), Ly-6G (RB6-8C5) (BD PharMingen) and F4/80 (Cl: A3-1) (CALTAG Laboratories, Burlingame, CA, USA) with appropriate dilutions followed by incubation with biotinylated secondary antibodies and HRP-conjugated streptavidin. The slides were developed as described above.

Southern blot analysis

The persistence of transferred clones in the recipients was assessed by Southern blot analysis. Two micrograms of total RNA of each tissue was treated with DNaseI and reverse transcribed using Superscript II (Invitrogen, Carlsbad, CA, USA). Nested PCRs were performed as described previously (24) to amplify TCR β chain of 35S or dengue 2F7 with the primers specific for V, J and C region. Ten microliters of the PCR products were separated on 2% agarose gel, transferred onto Hybond-N+ membranes (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. The membranes were prehybridized overnight with PerfectHyb (TOYOBO CO., Ltd, Osaka, Japan) at 54°C and hybridized with the third complementarity-determining region (CDR3)-specific probes labeled with ^{32}P-deoxyadenosine triphosphate for 3 h at 54°C. The membranes were washed in ×2 standard saline citrate (SSC) and 0.1% SDS at room temperature and ×0.2 SSC and 0.1% SDS at 37°C. RNA extracts of 35S and dengue 2F7 clones, diluted to 1% of concentration with RNA of L9 cells, were used as positive controls. The detection limits of 35S and dengue 2F7 were compared using the serial dilution of positive controls and both systems detected the RNA extract corresponding to the amount of one cell.

The sequences of PCR primers and probes are as follows; 35S: first PCR (BV8S3-1: 5′-ATA TGG TGC TGG CAA CCT TC-3′ and MCB1: 5′-AGG ATT GTG CCA GAA GGT AG-3′), second PCR (BV8S3-2: 5′-ACC AGA ACA ACG CAA GAA GAC T-3′ and MCB2: 5′-TTG TAG GCC TGA GGG TCC-3′), third PCR (BV8S3-3: 5′-TTC CTC CTG CTG GAA TTG GC-3′ and BJ1.5: 5′-TAG AAC AGA GAT CGA GTC CC-3′) and probe (5′-AGT GGG ACA GGG GGC AAC CA-3′). Dengue 2F7: first PCR (BV8S1-1: 5′-CCC AAA GTC CAA GAA GCA AG-3′ and MCB1), second PCR (BV8S1-2: 5′-GTA CAA GGC CTC CAG ACC AA-3′ and MCB2), third PCR (BV8S1-3: 5′-TGG CTT CCC TTT CTC AGA CA-3′ and BJ2.7: 5′-AAG GAG ACC TTG GGT GGA GT-3′) and probe (5′-TGC CAC CAA CGA CAA CTC CT-3′).

Results

Induction of arthritis and interstitial pneumonitis in SCID mice by the transfer of SKG splenic T cells

In our conventional housing environment, SKG mice started to develop arthritis around 2 months of age and

histologically evident mild interstitial pneumonitis around 6 months of age (14). To determine the role of T cells in SKG mouse autoimmunity, we transferred splenic T cells from 3-month-old arthritic SKG mice (without histologically evident pneumonitis or colitis) to T/B-cell-deficient C.B-17 SCID mice, which are histocompatible with SKG mice on the BALB/c background (14). Within 2 months after transfer, the recipient developed arthritis (14) and mild but histologically evident interstitial pneumonitis (Table 1, Fig. 1); they also developed mild colitis (data not shown). Similar cell transfer from non-arthritic heterozygotes of the SKG mutation failed to induce such lesions in the recipients. Age-matched SCID mice similarly maintained in our facility did not develop these lesions histologically (data not shown). The results thus indicate that SKG T cells are able to adoptively transfer arthritis and also have a potential to induce interstitial pneumonitis and colitis when transferred to SCID mice.

Establishment of T cell clones from arthritic joints

To analyze the mechanism of such T cell-mediated inflammatory tissue damage in multiple organs, we attempted to establish T cell clones from arthritic joints of SKG mice, as described in Materials and methods. Two T cell clones, designated 35S and 73S, were established in separate experiments. The clones were maintained and expanded with culture medium containing IL-2 and Con A (see Materials and methods). $CD8^+$ CTL clones specific for dengue virus NS3 protein were used as control.

Cytofluorometric analyses revealed that the 35S and 73S clones were $CD8^+$. Both expressed α and β chains of the TCR, and the expression level of the TCR on 35S was slightly lower than normal (Fig. 2). In response to *in vitro* PMA and ionomycin stimulation, 35S and 73S produced IFN-γ but no detectable amount of TNF-α, IL-4, IL-5, IL-6, IL-10 or IL-17 by ELISA (Table 2).

Clonality of each T cell line was confirmed by MHA (24) (data not shown) and sequence analysis of the TCR α and β chains with determination of the amino acid sequences of the TCRs (Table 3). Interestingly, these T cell clones shared in common the BV8S3 TCR Vβ subfamily; yet, the CDR3 sequences of the TCR β chains were different (26–29).

Table 1. Induction of arthritis, interstitial pneumonitis and colitis in SCID mice by the transfer of SKG splenic T cells

Spleen cell donor	Recipients	Arthritis	Interstitial pneumonitis	Colitis
SKG	1	++ (4.6)	++	+
	2	++ (4.0)	++	+
	3	++ (4.0)	+	+
	4	++ (3.0)	+	–
(SKG × BALB/c)F_1	1	–	–	–
	2	–	–	–
	3	–	–	–
	4	–	–	–

Cells (1×10^7) of T cells prepared from spleens of indicated mice were intravenously transferred to 8-week-old SCID mice. The severity of arthritis, interstital pneumonitis and colitis in these mice was histologically assessed 2 months later.

Autoreactivity of T cell clones

In ^{51}Cr release cytotoxicity assay to determine cytotoxic activity of the SKG clones against syngeneic synovial cells, 35S and 73S lysed SKG synovial cells prepared by crude collagenase digestion of inflamed synovium (44.0 and 16.3% of specific lysis, respectively, at a high 40:1 ratio), while control dengue 2F7 clone did not (Fig. 3A). 35S lysed not only syngeneic synovial cells but also MHC-matched cell lines, such as BALB/c-derived 3T3 cells, macrophage-like J774 cells and DBA/2 ($H\text{-}2^d$)-derived P815 cells, whereas the clone failed to lyse allogenic EL-4 ($H\text{-}2^b$) lymphoid or L929 ($H\text{-}2^k$) fibroblast cell line (Fig. 3B). Thus, 35S appears to recognize a ubiquitous self-peptide in an MHC-restricted manner. These

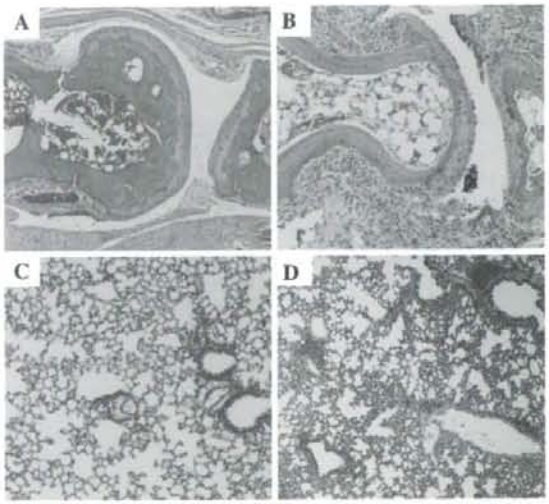

Fig. 1. Arthritis and pneumonitis in SCID mice transferred with T cells from SKG mice. Histology of a joint (A) and lung (C) of a SCID mouse T cell transferred from (SKG × BALB/c)F_1 mouse. Arthritis (B) and interstitial pneumonitis (D) in a SCID mouse T cell transferred from a SKG mouse. H&E staining (A and B, ×100; C and D, ×50).

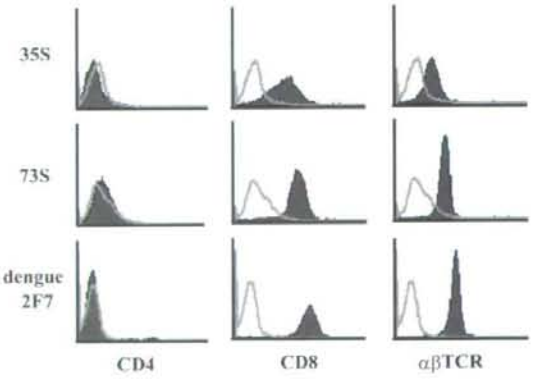

Fig. 2. Expression levels of CD4, CD8 and $\alpha\beta$ TCR on 35S, 73S and dengue 2F7 clones.

functional characteristics, together with cell surface and cytokine-secreting profiles, indicate that 35S and 73S are CTL and that they bear self-reactive specificity.

Induction of synovitis in BALB/c nude mice by adoptive transfer of T cell clones

To examine possible arthritogenicity of the T cell clones, they were transferred to BALB/c nude mice once, and the degree of joint swelling of each recipient mouse was assessed once a week for 12 months (Fig. 4). Transfer of 35S and 73S clones induced joint swelling with incidences of 57.1% (4 out of 7 mice) and 42.9% (3 out of 7 mice), respectively, during the observation period; synovitis was histologically evident in 71.4% (5 out of 7 mice) in each transfer (Table 4, Fig. 5). Once joint swelling started in one joint following cell transfer, it slowly progressed with remissions and exacerbations, leading to swelling of other joints in a symmetrical fashion (Figs 4 and 5A–D). Two mice showed progressive debilitation to death without an apparent cause, although one of them showed dermatitis; with debilitation, joint swelling somehow remitted in these mice.

Histologically, swollen joints showed marked synovial and peri-articular inflammation when examined 6–12 months after cell transfer (Fig. 5E and F). The inflammation accompanied a marked proliferation of synovial lining cells, infiltration of inflammatory cells into subsynovial tissue and joint cavity and active angiogenesis; pannus eroded the adjacent cartilage and bone (Fig. 5F). Gr-1-positive neutrophils were abundant among the infiltrating cells, as observed in the arthritic lesions of SKG mice (14, 15), whereas few T cells infiltrated into the inflammation sites (Fig. 5G and H).

In accordance with the appearance of multinuclear cells at the interface between proliferating synoviocytes and bone, many tartrate-resistant acid phosphatase-positive osteoclasts were observed in the inflamed joints (Fig. 6A–D). Safranin-O staining revealed a decrease in proteoglycan in the articular cartilage matrix of severely affected joints (Fig. 6E and F). Notably, Gr-1-positive cells, mainly neutrophils, also increased in the bone marrow (BM) of the affected recipients (Fig. 6G and H).

A high level of circulating rheumatoid factors was detected in one mouse out of seven recipients of the 35S clone and in none of the recipients of other clones (data not shown).

Some of the swollen joints following transfer of 35S $CD8^+$ clones exhibited higher expression levels of IL-17 mRNA assessed by quantitative reverse transcription (RT)–PCR than those from mice transferred with control $CD8^+$ clones (Supplementary Figure 1A, available at *International Immunology* Online), despite that 35S failed to produce IL-17 upon *in vitro* stimulation.

Taken together, the $CD8^+$ T cell clones prepared from arthritic lesions of SKG mice were able to induce arthritis in athymic nude recipients, leading to the destruction of the surrounding cartilage and the bone.

Table 2. Cytokine production (ng/ml) of T cell clones derived from SKG joints and control clones

	TNF-α	IFN-γ	IL-4	IL-5	IL-10	IL-6	IL-17
35S	0.02	180	0.03	<0.02	<0.04	0.2	<0.01
73S	0.02	80	0.03	<0.02	1.2	<0.05	<0.01
Dengue 2F7	0.2	10	ND	ND	<0.04	<0.05	<0.01
Dengue 3F2	0.02	20	ND	ND	<0.04	<0.05	<0.01

Culture supernatant of activated cells by PMA and ionomycin for 16 h were assayed by ELISA. ND, not done.

Table 3. CDR3 sequences of the TCR α and β chain used by the SKG T cell clones

TCR α chains	AV	V	N	J	AJ
35S	3S6	C A V T	S D	S G T Y Q R F	13
73S	3S1	C A A S M	R R	N S G T Y Q R F	13
Dengue 2F7	2S2/7	C A A		N Q G G R A L I F	15
Dengue 3F2	2S2/7	C A A	S G R D	Y A N K M I F	47
TCR β chains	**BV**	**V**	**N-D-N**	**J**	**BJ**
35S	8S3	C A S S G	T G G	N Q A P L F	1.5
73S	8S3	C A S S G	W G D	A E Q F F	2.1
Dengue 2F7	8S1	C A T	N D N	S Y E Q Y E	2.7
Dengue 3F2	8S2	C A S E	T R	E Q Y F	2.6

The amino acid sequences of the V, D and J regions of the TCR were determined according to the nucleotide sequences. AV and BV gene families were assigned according to Arden *et al.* (26). AJ genes were numbered according to Koop *et al.* (27). BJ genes were assigned according to Malissen *et al.* (28) and Gascoigne *et al.* (29).

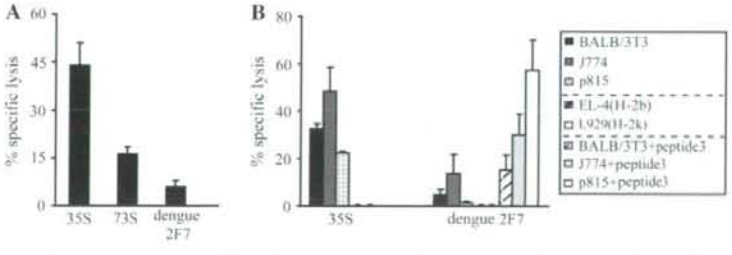

Fig. 3. *In vitro* self-reactivity of SKG T cell clones. (A) CTL activity of SKG T cell clones against SKG synovial cells. CTL clones specific for dengue virus NS3 protein, dengue 2F7, was used as control. IFN-γ-treated target cells were ^{51}Cr labeled in adherent condition and incubated with effector cells for 8 h (E:T ratio = 40). (B) CTL activity of SKG T cell clones against various types of cell lines (E:T ratio = 40). CTL activity of dengue 2F7 clone was also analyzed against $H\text{-}2^{d}$ cells pulsed with a specific peptide (E:T ratio = 10). All assays were conducted in triplicate with 8 h of incubation. The mean and standard deviation of three independent experiments are shown in each bar.

Induction of interstitial pneumonitis in BALB/c nude mice by the transfer of T cell clones

Notably, histologically evident severe alveolitis and diffuse interstitial pneumonitis also developed in all the recipients of 35S and 73S but not in those recipients of dengue 2F7 and 3F2 clones (Table 4 and Fig. 7A–D). Some recipients of 35S and 73S developed only pneumonitis without histologically evident synovitis. No histologically apparent inflammation was observed in other tissues/organs including the liver and the colon in any of these recipient mice (data not shown). The diffuse pulmonary lesions (Fig. 7A and B) comprised thickening of the alveolar walls, and perivascular and peribronchiolar infiltration by inflammatory cells (Fig. 7C and D). Immunohistochemical analysis of the 73S recipients 6 months after cell transfer revealed the infiltration of a large number of granulocytes as Gr-1$^+$ cells (Fig. 7E), macrophages as F4/80$^+$ cells (Fig. 7F) and B cells as B220$^+$ cells (Fig. 7G) into the alveolar walls and spaces and also the perivascular and peribronchiolar area where only a small number of CD8$^+$ T cells were detected, which might be transferred to CD8$^+$ clones or derived from nude mice (30) (Fig. 7H). CD4$^+$ T cells were occasionally found in the lesions and could be those derived from endogenous T cells that might develop extrathymically in aged nude mice (Fig. 7I) (30).

The pulmonary tissues with severe interstitial pneumonitis following CD8$^+$ clone transfer exhibited higher expression levels of IL-17 mRNA by quantitative RT-PCR compared with the mice transferred with control CD8$^+$ clones (Supplementary Figure 1B, available at *International Immunology* Online).

Thus, the SKG arthritogenic T cell clones are able to induce interstitial pneumonitis when transferred to athymic nude mice.

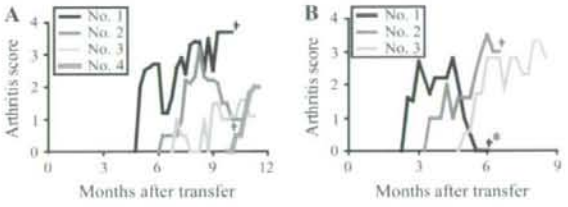

Fig. 4. Time course of joint swelling in the recipient mice of SKG T cell clones, 35S (A) and 73S (B). Score for all paws were totalized for each mouse. +, Sacrificed at the indicated time points; *, the mouse developed dermatitis at 5 months after transfer.

Detection of transferred clones in recipient mice

Since T cells were hardly detected by immunohistochemistry at the site of synovitis or pneumonitis 6 months after clone transfer (data not shown and see above), the persistence of transferred clones in the recipients was assessed by RT-PCR amplification of TCR β chain gene and Southern blot analysis of the products with a CDR3 sequence-specific probe. We adopted this method to avoid detecting nude mouse-derived oligoclonal endogenous T cells that may expand with aging (see above) (30–32). For example, a clone-specific TCR message of the 35S clone was detected in the majority of recipient spleens 1 month after transfer but not in the spleens examined 6 months later (Fig. 8). As shown in Fig. 9, the messages were

Table 4. Development of arthritis and interstitial pneumonitis in BALB/c nude mice transferred with T cell clones

Clone	Individual recipients	Macroscopically evident arthritis			Histological analysis	
		Onset (months)	Sacrifice (months)	Clinical score[a]	Synovitis[b]	Interstitial pneumonitis[c]
35S	1	5	10	3.7	++	+
	2	6	11	3.2	++	++
	3	7	11	1.6	++	+++
	4	10	12	2.0	++	+++
	5	–	9	0	+	±
	6	–	12	0	—	+++
	7	–	12	0	—	+++
73S	1	2.5	6	2.8	++	++
	2	3.5	6	3.5	++	+
	3	5	9	3.3	++	++
	4	–	8	0	+	++
	5	–	9	0	+	+++
	6	–	12	0	—	++
	7	–	12	0	—	++
Dengue 2F7	1	–	9	0	—	—
	2	–	9	0	—	—
	3	–	9	0	—	—
	4	–	9	0	—	—
	5	–	12	0	—	—
	6	–	12	0	—	—
	7	–	12	0	—	—
Dengue 3F2	1	–	12	0	—	—
	2	–	12	0	—	—

Six-week-old BALB/c nude mice were intravenously injected with 1×10^7 cells of each clone. The incidence of joint swelling of the recipient mice was examined weekly. Mice were sacrificed 6–12 months after cell transfer.

[a]Maximum clinical score of arthritis.

[b]–, Without change; +, microscopically observed synovitis without joint swelling; ++, macroscopically obvious joint swelling.

[c]–, Normal histology; +, 10–30%; ++, 30–60%; +++, >60% of the sections of the lungs showed pneumonitis (Fig. 7).

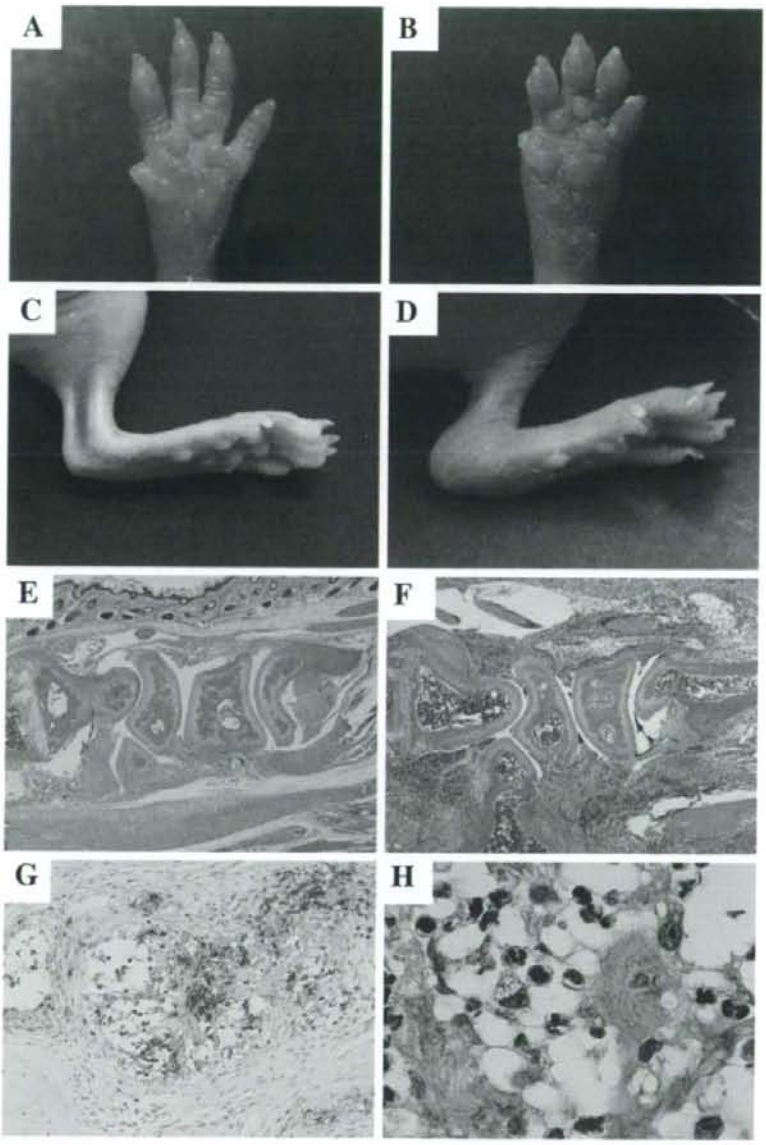

Fig. 5. Arthritis in athymic nude mice transferred with SKG T cell clones. (A–D) Macroscopic views of a forepaw (A) and a hind paw (C) of a recipient of control dengue 2F7 and a forepaw (B) and a hindpaw (D) of a recipient of 35S. (E–H) Histology of the joints of recipients of control dengue 2F7 (E) or 35S (F). Proliferation of the synovial lining cells, erosive destruction of cartilage and bone and infiltration of inflammatory cells is noted in a joint of a 35S recipient (F) (H&E staining, ×40). (G) Gr-1-positive cells were abundant among the infiltrating cells in a joint of 35S recipient mouse (×200). High-magnification view (×1000) of the synovial lesion in 35S transferred mouse, showing that most of the infiltrating cells are granulocytes or monocytes (H) (H&E staining). (A, C and E) 12 months after transfer. (B, D and F–H) 10 months after transfer.

detected in every tested tissue with high frequency for the first 3 months after cell transfer; the detection rate became lower with time; clone-specific TCR signals were not detected in most tissues examined at 6–11 months after transfer, irrespective of the swelling of the joints and the presence of interstitial pneumonitis by histological examination. These findings collectively indicate that the T cell clones initiate arthritis but the progression and persistence of the disease may not require the expansion of the clones even if a small number of them might persist in the joints and the lung.

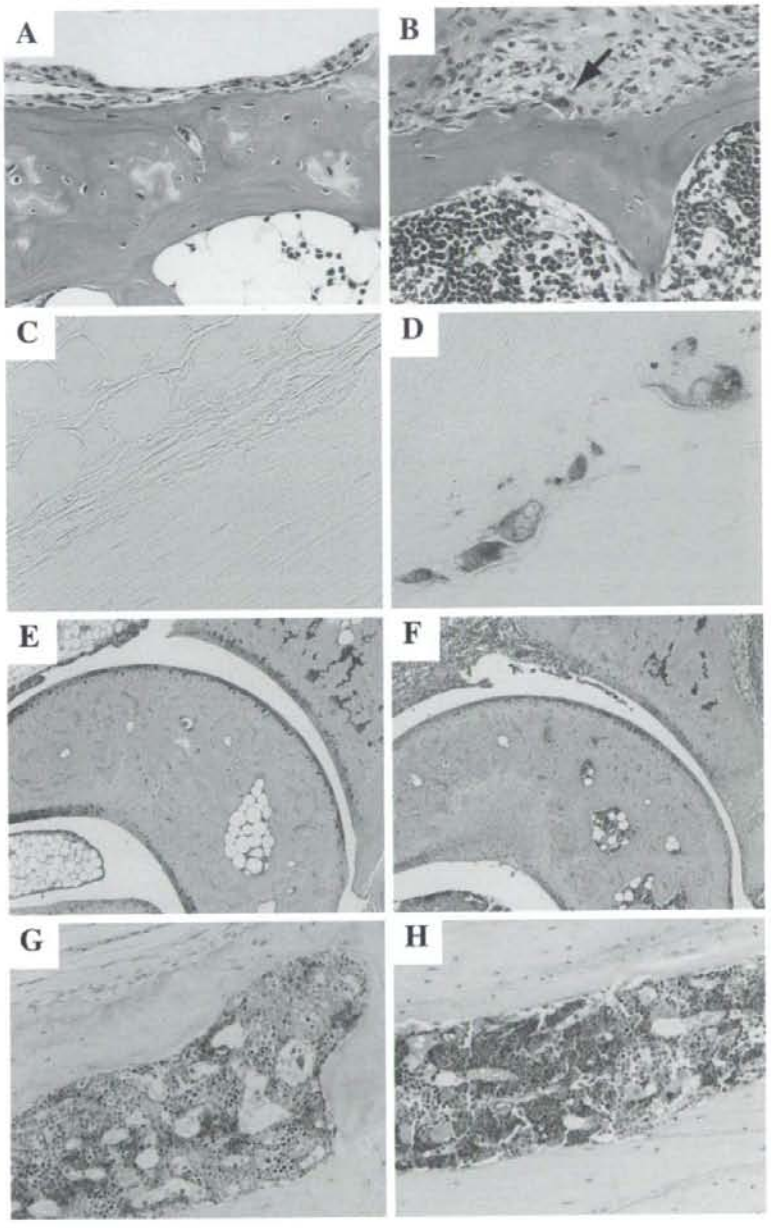

Fig. 6. Bone and cartilage destruction in athymic nude mice transferred with SKG T cell clones. High magnification of H&E-stained sections of a nude mouse recipient of dengue 2F7 (A) or 35S (B), showing bone erosion by pannus and BM activation (×400). Multinuclear cells (osteoclasts) (arrow) are also observed. Tartrate-resistant acid phosphatase-positive cells (osteoclasts) are detected in a 35S recipient (D) but not in a 2F7 recipient (C) (×400). By Safranin-O staining, proteoglycan stained red decreases in the articular cartilage matrix of a recipient of 35S (F) but not in a recipient of 2F7 (E) (×100). By immunohistochemistry, Gr-1-positive cells increase in the BM of a 35S recipient (H) but not in a 2F7 recipient (G) (×200). (A, C, E and G) 12 months after transfer; (B, D, F and H) 10 months after transfer.

Discussion

In this study, we have established two distinct CD8$^+$ T cell clones from arthritic lesions of SKG mice. Interestingly, both exhibited *in vitro* autoreactivity against not only synoviocytes but also a variety of MHC-matched cell lines and elicited both arthritis and interstitial pneumonitis when transferred to

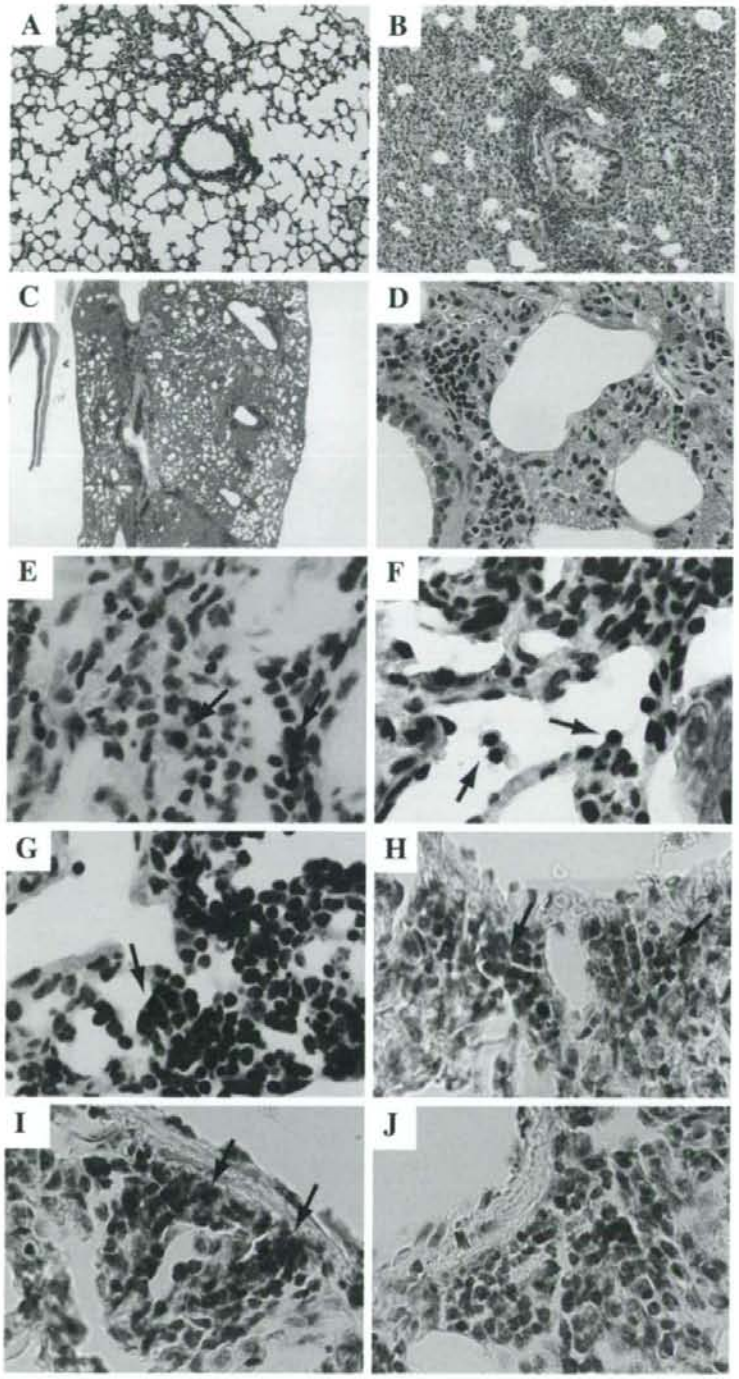

Fig. 7. Interstitial pneumonitis induced by the transfer of SKG T cell clones. (A–D) H&E-stained sections of the lungs of the recipients of control dengue 2F7 clone (A) or 73S clone (B–D) (A–B, ×100). Lower (C, ×10) and higher (D, ×400) magnification of the lung of 73S clone recipient show thickening of alveolar walls diffusely in the lung. (E–J) Serial sections of a lung of a 73S recipient mouse were stained for Ly-6G (Gr-1) (E), F4/80 (F), B220/CD45R (G), CD8a (H) or CD4 (I), with staining control (J) (×400). Typically positive cells in these stainings are arrowed. (A–J) 6 months after transfer.

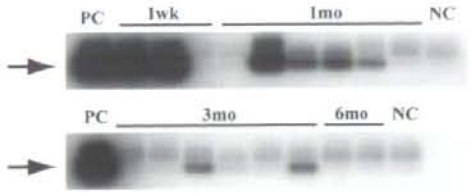

Fig. 8. Detection of a clone-specific TCR message of 35S clone in spleens by RT-PCR amplification and Southern blot analysis. After transfer of 1×10^7 clone cells to BALB/c nude mice, RNA was extracted from spleens at indicated days. PC, positive control (RNA from 35S clone diluted to 1%); NC, negative control (RNA from a 6-month-old non-treated BALB/c athymic nude mouse). The separate lanes represent individual mice.

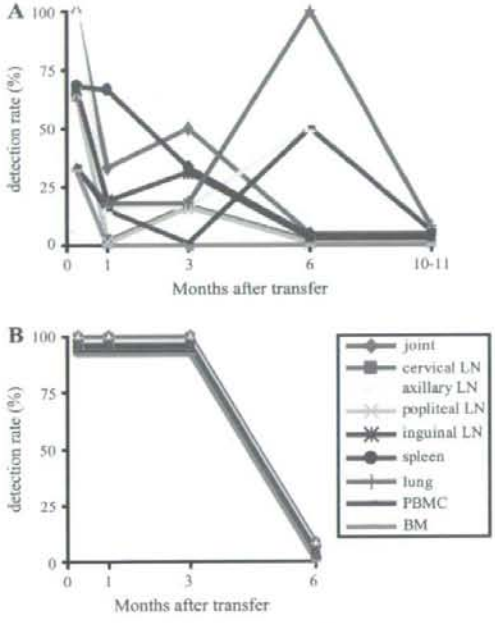

Fig. 9. Detection of TCR mRNA of the transferred clones in recipient BALB/c nude mice. 35S (A) or dengue 2F7 (B) in the recipients were detected by Southern blot analysis using primers and probes specific for TCR V and J region and CDR3 sequences of each clone. All mice with at least one positive signal out of four joints were considered to be positive. (A) $n = 3$ at 1 week; $n = 6$ at 1 month; $n = 6$ at 3 months; $n = 2$ at 6 months; $n = 2$ at 10–11 months. (B) $n = 2$ in every group. No signal was detected in control 6- or 11-month old BALB/c nude mice in each Southern blot analysis (data not shown).

histocompatible T cell-deficient mice. Furthermore, the arthritic and pulmonary lesions chronically progressed irrespective of the decline in the number of transferred T cell clones to hardly detectable levels in either lesion.

Our previous study showed that bulk $CD4^+$ T cells alone from arthritic SKG mice were able to transfer the disease to athymic nude mice, whereas bulk $CD8^+$ T cells alone were not and that abundant $CD4^+$ T cells and only a small number of $CD8^+$ T cells were found by immunohistochemistry in the arthritic subsynovial tissue of arthritic SKG mice (14). These apparently opposing results with $CD8^+$ T cell clones versus bulk $CD8^+$ T cells indicate that potentially arthritogenic $CD8^+$ T cells are present in SKG mice and may usually need $CD4^+$ T cell help for induction of arthritis; yet, they are potentially able to mediate arthritis without $CD4^+$ T cell help if they are strongly activated, clonally expanded to a large number or possibly selected for stronger self-reactivity during *in vitro* culture. It remains to be determined how $CD8^+$ clones elicit proliferative synovitis rather than cytotoxic killing of certain cellular elements in the joint. One possibility is that these $CD8^+$ clones, which exert *in vitro* killing activity at a high T cell/target cell ratio, might also be able to stimulate synoviocytes through secreting cytokines. It is of interest in this regard that the joints and the lungs with severe pneumonitis in some recipients of the $CD8^+$ clones showed active transcription of IL-17 mRNA (Supplementary Figure 1, available at *International Immunology* Online). Although the $CD8^+$ clones did not produce detectable amounts of IL-17 by *in vitro* stimulation, they might produce the cytokine in the joints or interact with nude mouse-derived α/β or γ/δ T cells and stimulate them to secrete IL-17 (33, 34). It is of note that a large number of $Gr\text{-}1^+$ mature neutrophils exuded into the joint fluid and infiltrated into the subsynovial tissue of the recipient nude mice, as in the arthritic lesions of SKG mice (14). BM of the clone recipients also showed an increase in the number of $Gr\text{-}1^+$ mature neutrophils. It remains to be determined how $CD8^+$ T cells mediate arthritis and pneumonitis in SKG mice by recruiting other cellular elements including neutrophils, how they increase neutrophils in the BM and whether IL-17, which is capable of recruiting neutrophils, is involved in these processes (35, 36).

It also needs further investigation whether IFN-γ secreted by the transferred $CD8^+$ clones or their killing activity could contribute to the development of synovitis. IFN-γ may activate synoviocytes directly or indirectly through activating macrophages, facilitating synoviocyte proliferation. It might up-regulate the expression of MHC class I in synovial cells, rendering them susceptible to cytotoxic activity of $CD8^+$ T cells. With these apparently opposing activities of arthritogenic $CD8^+$ T cells (i.e. killing versus proliferation of synoviocytes), they mediate proliferative synovitis rather than synoviocyte destruction presumably because synoviocytes might be more sensitive *in vivo* to the stimulatory effect than the cytotoxicity (see Discussion below).

The $CD8^+$ clones exhibited *in vitro* cytotoxic activity against not only syngeneic synovial cells but also a variety of MHC-matched lymphoid and non-lymphoid cell lines. Although their precise antigen specificities need to be determined, this finding suggests that these clones may recognize a ubiquitous self-antigen (for example, ubiquitous cellular protein such as hsp complexed with MHC or the MHC molecule itself) expressed in the joint and lung and other tissues, rather than a common self-antigen exclusively expressed in the joint and lung. If this is the case, how are the joint and the lung selectively affected by these T cell clones? For the following reasons, one could attribute this to unique characteristics of the synoviocytes, and possibly the alveolar macrophage, as the target of this autoimmunity. Compared with other tissue cells, the synoviocytes are

highly sensitive to pro-inflammatory cytokines, for example systemic overproduction of transgenic TNF-α or IL-1 almost exclusively produces chronic arthritis even in mice deficient of both T and B cells (37–39); similarly, systemic deficiency of the IL-1R antagonist, and resulting overproduction of IL-1, or systemic alteration of signal transduction via IL-6 receptor results in predominant development of arthritis with no inflammatory damage to other tissues (40, 41). These findings collectively indicate that synoviocytes are much more sensitive to the SKG self-reactive T cell clones (at least to those secreting pro-inflammatory cytokines) than other tissue cells, even if the common self-antigens recognized by the clones are ubiquitously expressed. In addition, synoviocytes are unique in that they are the target cells and also the mediators of autoimmunity, i.e. upon stimulation (e.g. by cytokines or via cell contact stimulation by self-reactive T cells), they proliferate and secrete pro-inflammatory cytokines (e.g. IL-1, IL-6 and TNF-α) and other inflammatory substances (matrix metalloproteinases and prostaglandins), mediating inflammation and tissue damage (42). It is likely that the cells composing the alveolar walls, in particular the alveolar macrophages, are sensitive and responsive to T cell self-reactivity in a similar manner as synoviocytes and that excessively and chronically activated macrophages might mediate alveolitis and interstitial inflammation. A similar mechanism might also be responsible for the development of colitis in SKG mice (Table 1).

We do not assert, however, that SKG arthritis and pneumonitis are solely mediated by T cells recognizing a ubiquitous common self-antigen. We have previously shown that SKG mice spontaneously produce IgG isotype auto-antibody specific for joint-rich type II collagen or IgG antibody cross-reactive with hsp-70 of Tuberculosis bacilli (14). This indicates that helper $CD4^+$ T cells that specifically react with these self-antigens may also be induced in SKG mice either primarily or secondarily to joint damage. Moreover, we have recently shown that some self-reactive T cells in SKG mice may not be arthritogenic but can polyclonally stimulate antigen-presenting cells in the spleen and lymph nodes to secrete IL-6 and other cytokines, which in turn facilitate differentiation of potentially arthritogenic self-reactive T cells to T_h17 effector T cells that mediate synovitis (19). In addition to our current approach to the characterization of antigen specificity of SKG autoimmune T cells by preparing T cell clones, efforts are being made to further characterize infiltrating T cells *in situ* at a single-cell level by amplifying their TCR message.

Tracing the fate of transferred T cell clones revealed that clone-specific TCR gene messages gradually diminished not only in the inflamed joints and the lungs but also in the regional lymph nodes and spleens of the recipients, becoming hardly detectable in 6–11 months; yet, inflammation in the joints and the lung continued to progress and severe arthritis and pneumonitis were apparent even 12 months after clone transfer. Thus, initial triggering of synovitis requires arthritogenic T cells; yet, synovitis apparently becomes less T cell dependent in a later phase, albeit it chronically progresses with the formation of pannus destroying adjacent cartilage and bone, as in human RA (2). This may correlate with the findings in humans that T cell-targeted mAb therapy is not much efficacious in the treatment of RA at a chronic stage (43). Further characterization of each stage of disease development in SKG mice will contribute to our understanding of the cellular and molecular basis of the T cell-dependent and -independent phases of disease progression in the joints and also in the lung in RA.

In conclusion, we have shown that $CD8^+$ T cell clones established from arthritogenic lesions of SKG mice are capable of mediating not only arthritis but also interstitial pneumonitis immunopathologically resembling ILD in RA. This provides a possible common pathogenetic basis between arthritis and ILD in RA. The etiology of RA is largely obscure at present (1, 2). Yet, there are recent findings that genetic polymorphism of the PTPN22-encoded lymphoid tyrosine phosphatase, which alters signal transduction at a TCR proximal step involving ZAP-70, contributes significantly (second only to MHC polymorphism) to the susceptibility to RA and other autoimmune diseases (22, 23, 44, 45). The polymorphism might be responsible for thymic generation of arthritogenic and other self-reactive T cells. Further elucidation of the mechanism by which such autoreactive T cells are generated and activated in SKG mice, and characterization of putative ubiquitous self-antigen recognized by self-reactive T cells capable of mediating arthritis and pneumonitis, would facilitate our understanding of the etiology and the pathogenetic mechanism of RA as a systemic autoimmune disease. This should help devising preventive or curative measures for the disease.

Supplementary data

Supplementary figure is available at *International Immunology* Online.

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Disclosures

The authors declare no conflicting interests.

Abbreviations

BM	bone marrow
CDR3	the third complementarity-determining region
H&E	haematoxylin & eosin
hsp	heat shock protein
ILD	interstitial lung disease
MHA	microplate hybridization assay
PMA	phorbol myristate acetate
RA	rheumatoid arthritis
RT	reverse transcription
SSC	standard saline citrate
TNF	tumor necrosis factor
ZAP-70	ζ-associated protein of 70 kDa

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Cell

Regulatory T Cells and Immune Tolerance

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Regulatory T cells (Tregs) play an indispensable role in maintaining immunological unresponsiveness to self-antigens and in suppressing excessive immune responses deleterious to the host. Tregs are produced in the thymus as a functionally mature subpopulation of T cells and can also be induced from naive T cells in the periphery. Recent research reveals the cellular and molecular basis of Treg development and function and implicates dysregulation of Tregs in immunological disease.

Introduction

The mammalian immune system protects the host from a broad range of pathogenic microorganisms while avoiding misguided or excessive immune reactions that would be deleterious to the host. Both protective and harmful immune responses are principally mediated by T and B cells, which possess enormous diversity in antigen recognition, high antigen specificity, potent effector activity, and long-lasting immunologic memory. Because of this potency, serious damage to the host may ensue if aberrant immune responses, such as autoimmunity or allergy, are triggered. A major challenge in immunology and medicine is to determine how unresponsiveness of the adaptive immune system to self-antigens (that is, immunological self-tolerance) is established and maintained, and how the quality and magnitude of adaptive immune responses to non-self-antigens are controlled so as to avoid damage to the host. Understanding the mechanisms of immunological self-tolerance will also provide insights into how weak immune responses, such as those against tumor antigens in cancer patients or against microbial antigens in chronic infection, can be augmented, or conversely, how strong immune responses such as graft rejection can be restrained.

There are two types of mechanisms, "recessive" and "dominant," for achieving self-tolerance and immune homeostasis. In the recessive mechanisms of self-tolerance, the fate of antigen-exposed self-reactive lymphocytes is determined in a cell-intrinsic manner. For example, some lymphocytes are programmed to die by apoptosis when exposed to self-antigen at an immature stage of their development in the central generative organs (the thymus for T cells and the bone marrow for B cells). Other lymphocytes may replace self-reactive T cell or B cell receptors (TCRs and BCRs, respectively) with nonreactive ones, a process called receptor editing. Those that have escaped clonal deletion and receptor editing may further mature but can be rendered anergic (functionally inactivated) upon exposure to self-antigen. In addition, their activation thresholds may be raised by the expression of inhibitory receptors or negative signaling molecules or they may not survive long because of activation-induced cell death. The cell-intrinsic control of cell fate or activation threshold also contributes to the inhibition of excessive immune responses to non-self-antigens.

In the dominant or cell-extrinsic mechanism, certain T cells actively keep in check the activation and expansion of aberrant or overreactive lymphocytes, in particular other types of T cells. Until recently, the physiological significance, and even the existence of T cell-mediated immune suppression, has been highly contentious. Yet there is now firm evidence that the normal immune system produces a population of T cells, called regulatory T cells (Tregs), that are specialized for immune suppression. Disruption in the development or function of Tregs is a primary cause of autoimmune and inflammatory diseases in humans and animals. Moreover, it is now emerging that every adaptive immune response involves recruitment and activation of not only effector T and B cells but also Tregs, and that the balance between the two populations is critical for the proper control of the quality and magnitude of adaptive immune responses and for establishing or breaching tolerance to self- and non-self-antigens.

In this Review, we discuss the roles of Tregs in self-tolerance and immune homeostasis, the cellular and molecular basis of their development and function, and how they might be exploited at the cellular and molecular levels to control a wide spectrum of physiological and pathological immune responses.

Thymus-Derived Tregs

It has been known for nearly 40 years that simple manipulation of the thymus and T cells, but not self-antigen, can produce autoimmune disease in normal animals (reviewed in Sakaguchi, 2000). Neonatal thymectomy at a critical period (around day 3 after birth) of selected strains of normal mice results in autoimmune damage of various organs (such as the thyroid, stomach, ovaries, and testes) and the appearance of tissue-specific autoantibodies in the circulation. Adult thymectomy of selected strains of normal rats followed by several rounds of sublethal X-irradiation produces autoimmune thyroiditis and type I diabetes. Importantly, inoculation of normal T cells, in particular $CD4^+$ T cells or $CD4^+CD8^-$ thymocytes, from untreated syngeneic animals inhibits the development of autoimmunity (Sakaguchi et al., 1982; Fowell and Mason, 1993). These results indicated that normal animals harbor not only potentially pathogenic self-reactive T cells but also T cells that suppress autoimmunity, with

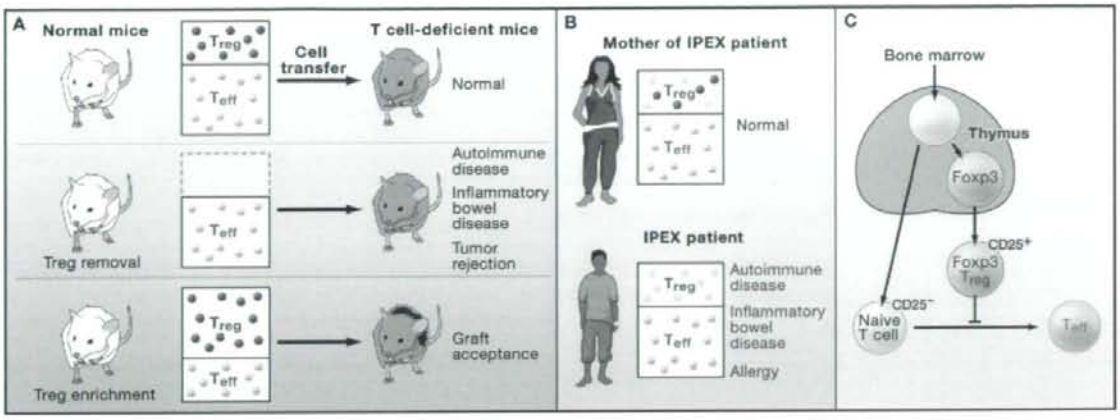

Figure 1. Effects of Treg Deficiency in Mice and Humans

(A) T cell suspensions prepared from normal mice can be depleted of $CD25^+CD4^+$ regulatory T cells (Treg) and transferred to syngeneic T cell-deficient mice (such as athymic nude mice). The recipient mice spontaneously develop autoimmune disease and inflammatory bowel disease and reject tumor cells. By contrast, when $CD25^+CD4^+$ Tregs are enriched from normal mice and transferred, the recipient nude mice accept allogeneic skin grafts.

(B) Male children are afflicted with IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome). Mothers bear heterozygous defects of the *FOXP3* gene and either have reduced numbers of normal Tregs or are mosaic for normal Tregs and functionally defective Tregs because of random inactivation of the X chromosome. Blue- and yellow-colored circles indicate intact Tregs and effector T cells (Teff), respectively. Dotted circles indicate dysfunctional Tregs (or the absence of Tregs).

(C) The normal thymus produces Foxp3-expressing natural Tregs. Tregs mainly suppress the development of effector T cells from naive T cells.

the latter dominantly controlling the former. A critical experiment to prove this interpretation was to identify autoimmune-suppressive T cells using a definitive phenotypic marker and to determine whether removal of the population from normal adult animals disrupts self-tolerance and leads to autoimmune disease. Indeed, when $CD4^+$ T cell suspensions from the spleens of normal mice or rats were depleted of cells bearing a particular marker profile ($CD5^{high}$ or $CD45RC^{low}$) ex vivo, and the remaining $CD4^+$ T cells were transferred to syngeneic T cell-deficient athymic nude mice or rats, the recipients spontaneously developed autoimmune disease in multiple organs within a few months (Sakaguchi et al., 1985; Powrie and Mason, 1990) (Figure 1A). Reconstitution of the depleted $CD4^+$ T cell subpopulation inhibited autoimmunity. Subsequent efforts to search for a more specific marker with which to delineate the putative autoimmune-preventive $CD4^+$ T cells revealed the CD25 molecule (the IL-2 receptor α chain) as a candidate (Sakaguchi et al., 1995). Transfer of T cell suspensions depleted of $CD25^+$ T cells indeed produces autoimmune disease in athymic nude mice, whereas cotransfer of a small number of $CD25^+CD4^+$ T cells clearly inhibits the development of autoimmunity.

Notably, depletion of naturally arising Tregs not only elicits autoimmunity but also augments immune responses to non-self-antigens. Treg depletion produces inflammatory bowel disease, which likely results from excessive immune responses to commensal bacteria in the intestine (Singh et al., 2001). Removal or reduction of $CD25^+CD4^+$ Tregs also provokes effective tumor immunity in otherwise nonresponding animals and augments microbial immunity in chronic infection, leading to eradication of tumors or microbes, respectively (reviewed in Wang and Wang, 2007; Belkaid and Rouse, 2005). Conversely, $CD25^+CD4^+$ T cells enriched from normal mice suppress allergy, establish tolerance to organ grafts, prevent graft-versus-host disease after bone marrow transplantation, and promote feto-maternal tolerance (reviewed in Sakaguchi, 2005) (Figure 1A).

$CD25^+CD4^+$ Tregs are also present in the periphery of humans (reviewed in Baecher-Allan et al., 2004). T cells that are reactive with a particular self-antigen targeted in autoimmune disease (such as glutamic acid decarboxylase in type I diabetes) or a tumor-associated antigen can be easily expanded in vitro from peripheral blood of normal healthy individuals when T cells are stimulated with self or tumor antigen after depletion of $CD25^+CD4^+$ Tregs (Danke et al., 2004).

With these findings, it is possible to formulate several key notions concerning dominant self-tolerance and immune regulation. First, the normal immune system generates $CD25^+CD4^+$ Tregs that are engaged in suppressing immune responses toward self, quasi-self (such as autologous tumor cells), and non-self (such as microbes and allografts). Second, the normal thymus produces potentially pathogenic self-reactive T cells as well as functionally mature Tregs; mature Tregs persist in the periphery and exert dominant control over the self-reactive T cells. Third, Treg deficiency in the periphery is sufficient to evoke chronic T cell-mediated autoimmunity and immunopathology.

Foxp3 Controls Treg Development and Function

Naturally occurring Tregs specifically express the transcription factor Foxp3 (forkhead box P3), a member of the forkhead/winged-helix family of transcription factors. Foxp3 is a master regulator of Treg development and function. The *Foxp3* gene was first identified as the defective gene in the

mouse strain Scurfy. Surfy is an X-linked recessive mutant that is lethal in hemizygous males within a month after birth, exhibiting hyperactivation of $CD4^+$ T cells and overproduction of proinflammatory cytokines (Brunkow et al., 2001). Mutations of the human gene *FOXP3* are the cause of the genetic disease IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), which is the human counterpart of Scurfy (reviewed in Ochs et al., 2005; Figure 1B).

Immunological and clinical similarities between IPEX in humans and autoimmunity/inflammation produced in rodents by Treg depletion prompted researchers to determine the possible role of Foxp3 in Treg development and function (Hori et al., 2003; Khattri et al., 2003; Fontenot et al., 2003). The studies revealed that $CD25^+CD4^+$ peripheral T cells and $CD25^+CD4^+CD8^-$ thymocytes in normal mice express Foxp3, whereas other thymocytes/T cells, either in a resting or activated state, do not. Ectopic retroviral transduction of the *Foxp3* gene in $CD25^-CD4^+$ T cells can convert them to $CD25^+CD4^+$ Treg-like cells that are able to suppress proliferation of other T cells in vitro and inhibit the development of autoimmune disease and inflammatory bowel disease in vivo (Hori et al., 2003). *Foxp3* transduction in naive T cells also upregulates the expression of CD25 and other Treg-associated cell-surface molecules, such as cytotoxic T cell-associated antigen-4 (CTLA-4) and glucocorticoid-induced TNF receptor family-related gene/protein (GITR), whereas it represses the production of IL-2, IFN-γ, and IL-4. Both Foxp3-deficient mice and Scurfy mice, whose Foxp3 protein lacks the forkhead domain, harbor few $CD25^+CD4^+$ Tregs, and inoculation of $CD25^+CD4^+$ T cells from normal mice prevents severe systemic inflammation in Scurfy mice (Fontenot et al., 2003). In bone marrow chimera with a mixture of cells from wild-type and *Foxp3*-deficient mice, *Foxp3*-deficient bone marrow cells failed to give rise to $CD25^+CD4^+$ Tregs, whereas *Foxp3*-intact bone marrow cells generated Tregs that suppressed disease development. Conversely, in transgenic mice that overexpress Foxp3, the number of $CD25^+CD4^+$ T cells is enhanced; $CD25^-CD4^+$ T cells and $CD8^+$ T cells expressed high levels of *Foxp3* and exerted suppression in vitro (Khattri et al., 2003). Recent analyses reveal that Foxp3-expressing T cells appear shortly after birth and show that the development of autoimmune/inflammatory disease follows their depletion (Kim et al., 2007; Lahl et al., 2007).

Thus, the transcription factor Foxp3 is critical for α/β TCR-positive T cells to differentiate to Tregs in the thymus (Figure 1C). High-level expression of Foxp3 is sufficient to confer suppressive activity to normal non-Treg cells. Further, genetic disruption of Foxp3 in humans and mice provides unequivocal evidence that dominant self-tolerance is operating in both species (Figure 1B). Foxp3 is currently the most reliable molecular marker for natural Tregs and provides clues with which to decipher the molecular and genetic basis of Treg development and function.

Interleukin 2 Maintains $Foxp3^+$ Tregs

Interleukin 2 (IL-2) is another molecule critical for the function of Tregs. The Treg marker CD25 is a component of the high-affinity IL-2 receptor (IL-2R) and is functionally essential for Treg development. IL-2 has long been thought to be a major cytokine for T cell proliferation and differentiation based on its effects on T cell growth in vitro. It has therefore been puzzling that mice lacking IL-2 do not exhibit serious defects in T cell differentiation and function. Instead, these mice spontaneously develop T cell-mediated fatal lymphoproliferative/inflammatory disease with autoimmune components (such as hemolytic anemia and lymphocytic infiltration into multiple organs) and hyperreactivity to commensal microbes (reviewed in Malek and Bayer, 2004). Mice deficient in CD25 or CD122 (another component of the IL-2R) also succumb to a similar set of ailments, generally called IL-2 deficiency syndrome. In humans, CD25 deficiency, which accompanies severe autoimmunity and allergy, is indistinguishable from IPEX (Caudy et al., 2007). Evidence suggests that the syndrome is due to deficiency or dysfunction of $Foxp3^+$ Tregs. First, the number of $Foxp3^+$ Tregs is reduced in mice lacking either CD25 or IL-2 (Antony et al., 2006), and autoimmunity in CD25-deficient mice can be prevented by inoculating them with wild-type $CD25^+CD4^+$ T cells (reviewed in Malek and Bayer, 2004). Second, T cell-specific deficiency of STAT5a and b, which mediate signaling from the IL-2R β chain to the nucleus, abrogates the development of $Foxp3^+$ Tregs, producing autoimmune/inflammatory disease (Burchill et al., 2007; Yao et al., 2007). Third, administration of a high dose of neutralizing anti-IL-2 monoclonal antibody to normal neonatal mice substantially reduces the number of $Foxp3^+CD25^+CD4^+$ T cells for a limited period and elicits autoimmune diseases similar to those produced by Treg depletion (Setoguchi et al., 2005). In addition, IL-2 is required for sustained expression of Foxp3 and CD25 in natural Tregs and enhances their suppressive function, at least in vitro (Fontenot et al., 2005; Shevach et al., 2006). Although other γ_c cytokines also contribute to Treg development and maintenance, they are unable to fully replace the roles of IL-2 or prevent IL-2 deficiency syndrome. For example, in contrast with mild reduction of $Foxp3^+$ cells in mice lacking either IL-2 or CD25, deficiency of CD122 (shared by IL-2R and IL-15R) or CD132 (shared by IL-2R, IL-4R, IL-7R, IL-9R, IL-21R, and IL-15R),or double deficiency of IL-2 and IL-15 lead to a profound reduction in the number of Tregs (Fontenot et al., 2005; Burchill et al., 2007). Yet, deficiency of IL-4, IL-7, or IL-15 alone does not alter the number of $Foxp3^+$ cells or produce autoimmunity. Thus, IL-2 is vital and irreplaceable for the development, survival, and function of $Foxp3^+$ natural Tregs.

It is well known that IL-2 has multiple targets, including $CD4^+$ and $CD8^+$ T cells, B cells, and natural killer cells, and exerts pleiotropic functions with apparently contradictory effects on immune responses. It facilitates differentiation of $CD4^+$ T cells to Th1 and Th2 cells and expands $CD8^+$ memory T cells and natural killer cells. On the other hand, IL-2 promotes apoptosis in antigen-activated T cells. IL-2 also maintains $Foxp3^+$ natural Tregs, expands them at high doses, and facilitates TGF-β-dependent differentiation of naive T cells to $Foxp3^+$ Tregs, but inhibits TGF-β/IL-6-dependent differentiation of naive T cells to inflammatory Th17 cells (Laurence et al., 2007). Thus, assuming that the main source of IL-2 is activated T cells, there is a negative feedback control of immune responses via IL-2; that is, IL-2 produced by activated nonregulatory T cells contributes

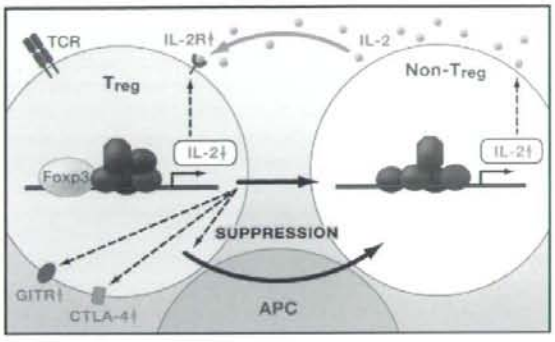

Figure 2. Key Roles of IL-2 in Immune Homeostasis

Interactions among Tregs, non-Treg cells, and antigen-presenting cells (APC) and feedback control of Treg function via IL-2. Foxp3, together with other transcription factors and coactivators/corepressors, represses the transcription of IL-2 in Tregs, rendering them highly dependent on exogenous IL-2 (mainly produced by activated non-Treg cells) for their maintenance and function. Foxp3 also activates the genes encoding Treg-associated molecules such as CD25, CTLA-4, and GITR and confers suppressive activity to Tregs, which directly suppress non-Treg cells or modulate the function of APC to activate non-Treg cells.

to the maintenance, expansion, and activation of natural Tregs, which in turn limits the expansion of nonregulatory T cells (Figure 2). Disruption of this IL-2-mediated feedback loop at any step promotes the development of autoimmune/inflammatory disease. Further, manipulation of this feedback loop is instrumental in tuning the intensity of Treg-mediated suppression, hence the strength of a variety of immune responses.

Control of Treg Function by Foxp3

How does Foxp3 orchestrate the cellular and molecular programs involved in Treg function? It has been shown that Foxp3, which appears to operate as a homo-oligomer, interacts with the transcription factors NFAT (nuclear factor of activated T cells), AML1 (acute myeloid leukemia-1)/Runx1 (runt-related transcription factor 1), the histone acetyl transferase (HAT)/histone deacetyl transferase (HDAC) complex, and possibly NF-κB (Figure 3A).

The activity of NFAT is controlled by calcium and the calcium-dependent protein phosphatase calcineurin. Upon activation, NFAT forms a complex with AP-1 and NF-kB and promotes the expression of *Il2*, *Il4*, *Ctla4*, and other genes in conventional T cells, thus contributing to the activation of T cells and their differentiation to effector T cells (reviewed in Rao et al., 1997). A recent study has shown that the interaction between Foxp3 and NFAT is dependent upon their cooperative binding to DNA (Wu et al., 2006). Amino acid substitutions in the forkhead domain of Foxp3 that disrupt the Foxp3-NFAT interaction impair the ability of Foxp3 to repress *Il2*, activate *Ctla4* and *Cd25*, and confer suppressive activity when expressed in normal T cells. Although four known types of NFAT may function redundantly, mice deficient in both NFATc2 and NFATc3 spontaneously develop severe lymphoadenopathy and selective activation of Th2 cells accompanying hyperproduction of IgE, suggesting that the deficiency might disrupt the function of Foxp3 and consequently impair Treg function (Ranger et al., 1998).

AML1/Runx1 is crucial for normal hematopoiesis including thymic T cell development (Taniuchi et al., 2002). It is expressed in conventional T cells and to a slightly lesser degree in natural Tregs (Ono et al., 2007). In contrast with NFAT, which is dephosphorylated by calcineulin and translocates from the cytoplasm to the nucleus upon T cell activation, AML1/Runx1 may constitutively bind to the *Il2* promoter at the site upstream to the binding sites of NFAT, AP-1, and NF-kB. Upon T cell activation, it may act as an organizing factor that facilitates the assembly of transcriptional activation complexes containing NFAT on the *Il2* promoter. Alternatively, AML1/Runx1, alone or together with its interacting partners such as p300 and CREB-binding protein, may cooperate with the NFAT transcription complex to activate the *Il2* promoter. In contrast, in Tregs, AML1/Runx1 physically binds to Foxp3 at its N-terminal region between the leucine zipper domain and the forkhead box (Figure 3A). Disrupting the binding between Foxp3 and AML1/Runx1 impairs the Foxp3-dependent suppression of IL-2 production and attenuates suppressive activity without affecting the binding of Foxp3 to NFAT. Knockdown of AML1/Runx1 in natural Tregs abrogates their suppressive activity. Further, Treg-specific disruption of the AML1/Runx1 gene in mice affects Foxp3 function and causes autoimmune disease similar to that produced by Treg depletion (A. Kitoh, M.O., and S.S., unpublished data).

Transcriptional control exerted by Foxp3 also involves HATs, such as TIP60, and HDACs, such as HDAC7 and HDAC9 (Li and Greene, 2007) (Figure 3A). Acetylation of Foxp3 by TIP60 enhances the binding of Foxp3 to the *Il2* promoter and augments the repression of *Il2* (Chen et al., 2006). It remains to be determined how the interaction of Foxp3 with coactivators and corepressors modifies the function of NFAT and AML1/Runx1 and controls the expression of Foxp3 target genes including *Il2*, *Cd25*, *Ctla4*, and *Gitr*.

Another approach to understanding how Foxp3 controls Treg function is to identify Foxp3 target genes genomewide. Transcriptome analysis has revealed several genes, such as those encoding G protein-coupled receptor 83 (Gpr83) or Extracellular matrix 1 (Ecm1), that are predominantly expressed in natural Tregs and Foxp3-transduced T cells. Other genes, such as those encoding Granzyme B and the transcription factor Helios, are highly specific to Tregs but are apparently expressed independently of Foxp3 (Fontenot et al., 2005; Sugimoto et al., 2006; Hill et al., 2007). Retroviral transduction of either group of genes in $CD25^{-}CD4^{+}$ T cells failed to confer suppressive activity in vitro (Sugimoto et al., 2006).

Recent searches for Foxp3 target genes have shown that Foxp3 directly or indirectly controls hundreds (~700) of genes and binds directly to ~10% of them (Marson et al., 2007; Zheng et al., 2007). Foxp3-binding genes include those encoding signal transduction molecules (such as *Zap70* and *Ptpn22*), transcription factors (such as *Crem*), cytokines (e.g., *Il2*), cell-surface molecules (such as *Il2ra*, *Ctla4*, and *FasL*), enzymes for cell metabolism (such as *Pde3b*), and intergenic microRNAs (such as *miR-155*). microRNAs do indeed appear to play an important role in Treg development. T cell-specific

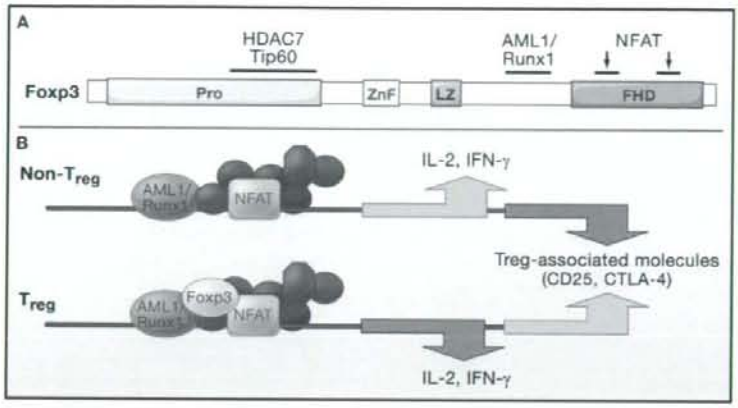

Figure 3. Control of Treg Function by Foxp3
(A) The structure of the transcription factor Foxp3. Bars indicate the binding sites for other transcription factors or chromatin-remodeling enzymes. Pro: Proline-rich region, ZnF: zinc finger domain, LZ: leucine zipper domain, FHD: forkhead box.
(B) The transcriptional complexes involving NFAT and AML1/Runx1 activate or repress the genes encoding cytokines and several cell-surface molecules in Treg and non-Tregs, depending on the presence of Foxp3.

depletion of Dicer, an RNase enzyme required for processing double-stranded RNA, hampers thymic development of Foxp3$^+$ T cells and elicits inflammatory bowel disease (Cobb et al., 2006). Altogether, these genomewide studies clearly show that Foxp3 functions as an activator as well as a repressor of transcription depending on the target.

Taken together, the characterization of Foxp3 interacting partners and Foxp3 target genes has revealed that Foxp3 interacts with transcription factors (such as NFAT and AML1/Runx1) that otherwise facilitate the activation of non-Treg cells and their differentiation into effector T cells. This suggests a model of Foxp3 function, in which Foxp3 overrides or hijacks the transcription machinery for effector T cells, thereby functionally converting them to Tregs (Figure 3B). Supporting this model, in mice genetically engineered to express a substantially lower amount of Foxp3 than normal, natural Tregs not only lose suppressive activity but also spontaneously differentiate to effector T cells that secrete IL-2, IFN-γ, and IL-4 (Wan and Flavell, 2007). Furthermore, Foxp3 may interact with RORγt, a key transcription factor for the differentiation of naive T cells to Th17 cells, and thereby inhibit Th17 differentiation (Bettelli et al., 2006, and see below). It remains an open question as to how the Foxp3 complex containing NFAT and AML1/Runx1 (together with HATs and HDACs) controls the genes that mediate suppression. In addition, the existence of genes that are highly Treg specific but Foxp3 independent suggests that there might be a higher level of transcriptional regulation upstream of Foxp3 or coregulation of Treg differentiation and function with other transcription factors.

The Antigen Recognition Repertoire of Tregs

A cardinal feature of Foxp3$^+$ natural Tregs is that unlike the majority of thymus-produced naive T cells, they are already functionally mature (that is, competent to prevent autoimmune disease and exert suppression in vitro) and "antigen-primed" in the thymus, before encountering antigen in the periphery (Sakaguchi et al., 1982; Itoh et al., 1999). Foxp3$^+$ thymocytes are detectable from a late CD4$^+$CD8$^+$ stage to the CD4- or CD8-single positive stage, constituting ~5% of mature CD4$^+$CD8$^-$ thymocytes and less than 1% of CD4$^-$CD8$^+$ thymocytes (Fontenot et al., 2005) (Figure 4A). Only a small fraction of Foxp3$^+$ thymocytes develop in mice deficient in the class I and II (MHC) histocompatibility complex, indicating that Foxp3$^+$ thymocytes or their Foxp3$^-$ precursors are subjected to positive and negative selection in the thymus through interactions with self-peptide/MHC ligands expressed by thymic stromal cells.

How then do thymocytes differentiate into functional Foxp3$^+$ Tregs through the thymic selection process? Evidence suggests that natural Tregs may possess TCRs with higher affinity for thymic MHC/self-peptide ligands than those of other T cells, and that such highly self-reactive T cells would be recruited to the Treg cell lineage in the course of thymic T cell selection. First, in double-transgenic mice expressing a transgene-encoded peptide (e.g., an ovalbumin peptide) in the thymic stromal cells at a high level, the majority of T cells expressing transgenic α and β chains specific for the peptide differentiate to CD25$^+$CD4$^+$ Tregs (Jordan et al., 2001; Kawahata et al., 2002). In contrast, Tregs fail to develop when double-transgenic mice express either a low-affinity transgenic TCR or a high concentration of the peptide, presumably due to insufficient positive selection or strong negative selection, respectively. This apparently high self-reactivity of Foxp3$^+$ cells could be a consequence of positive selection of highly self-reactive T cells to the Treg lineage or a resistance of differentiated Foxp3$^+$ cells to negative selection (van Santen et al., 2004). Second, in TCR transgenic mice, which spontaneously develop CD25$^+$CD4$^+$ Tregs in an equivalent number as in nontransgenic mice, Tregs predominantly express endogenous TCR α chains paired with the transgenic β chain. In contrast, the majority of CD25$^-$CD4$^+$ T cells express the TCR solely composed of transgenic α and β chains (Itoh et al., 1999). RAG deficiency, which blocks gene arrangement at the endogenous TCR α chain locus, abrogates the development of CD25$^+$CD4$^+$ Tregs in TCR transgenic mice, indicating that self-reactive TCRs utilizing endogenous α chains may contribute to the positive selection of thymocytes to Tregs. The lack of Tregs due to RAG deficiency exacerbates autoimmune disease in mice expressing autoimmune transgenic TCRs. Third, a mutation in the gene encoding LAT (linker of activated T cell), a TCR proximal signal transduction molecule, abolishes the generation of Foxp3$^+$ Tregs in the thymus and periphery while allowing the development of other T cells. This indicates that Treg development requires a strong signal

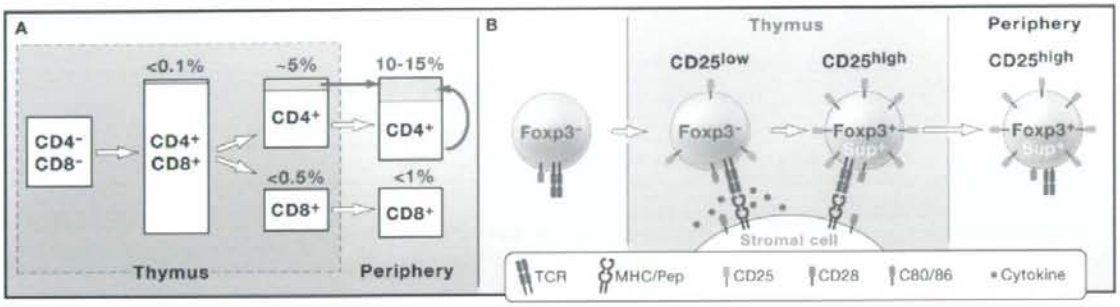

Figure 4. Thymic Development of Foxp3⁺ Cells

(A) The composition of Foxp3⁺ cells in each thymocyte subpopulation is shown as a percentage. In the periphery, CD4⁺ non-Treg cells can differentiate to Foxp3⁺ Tregs under certain conditions.

(B) Development of Foxp3⁺ Tregs in the thymus involves interaction with thymic stromal cells via various molecules. Foxp3⁻ thymocytes at a late CD4⁺CD8⁺ or an early CD4⁺CD8⁻ stage turn on a Treg differentiation program when they receive signals produced by the interaction between their TCRs and MHC/self-peptide complexes on thymic stromal cells, between their accessory molecules (e.g., CD28) and their ligands (e.g., CD80 and CD86), and/or via stromal cell-derived humoral factors (e.g., cytokines). Foxp3 expression following cell fate determination confers suppressive activity and stabilizes Treg function and phenotype (e.g., CD25 expression). Blue indicates that the thymocyte has a suppressive function (Sup+).

via the TCR in the thymus (Koonpaew et al., 2006). Furthermore, in TCRβ-fixed transgenic mice with a single copy of the TCRα locus or a "mini-TCRα" transgenic locus (which inhibits the appearance of T cells expressing double α chains), sequence analyses of TCR Vα segments of a particular Vα subfamily in CD25⁺ or CD25⁻CD4⁺ T cells have revealed distinct repertoires of the two populations, although the degree of overlap needs to be assessed more precisely (Hsieh et al., 2006; Pacholczyk et al., 2007).

In addition to the affinity of the interaction between the TCR and self-peptide/MHC, the intensity of the interaction between T cell accessory molecules and their ligands on thymic stromal cells contributes to the generation of natural Tregs. For example, deficiency of CD28, CD40, CD11a/CD18 (LFA1), or CD80 and CD86 (B7) results in a substantial reduction of CD25⁺CD4⁺ Tregs in the thymus and periphery (reviewed in Sakaguchi, 2005).

At the cellular level, both medullary thymic epithelial cells (mTECs) and dendritic cells in the thymus contribute to Treg generation as well as negative selection of naive T cells. Deficiency in the tumor necrosis factor receptor-associated factor 6 (TRAF6) or NF-κB-inducing kinase results in the absence of mature mTECs, hampering the development of Tregs (Akiyama et al., 2005; Kajiura et al., 2004). In humans, the cytokine TSLP (thymic stromal lymphopoietin) secreted by Hassal's corpuscles, which are derived from mTECs, appears to act on thymic dendritic cells to promote the differentiation of thymocytes to Tregs (Watanabe et al., 2005). A population of mTECs ectopically express a set of tissue-specific antigens (TSAs), such as insulin, under direct or indirect control of the gene *Aire* (autoimmune regulator) (reviewed in Mathis and Benoist, 2007). Interestingly, Aire-deficient mice spontaneously develop autoimmune diseases similar to those produced by Treg depletion. A recent study has suggested that Aire⁺ mTECs expressing TSAs may induce or facilitate the development of TSA-specific Tregs in normal thymus, and that a paucity of TSA-specific Tregs may contribute to the occurrence of autoimmune disease in Aire deficiency (Aschenbrenner et al., 2007).

Thus, the avidity of interaction between thymocytes and stromal cells is critical in determining the fate of developing thymocytes to the Treg lineage. Interestingly, recent studies suggest that Foxp3 per se is not required for this initial cell fate determination in the thymus (Lin et al., 2007; Gavin et al., 2007). In mice in which the Foxp3 encoding region is replaced by a green fluorescent protein (GFP) reporter, GFP-positive thymocytes, which do not express the Foxp3 protein, exhibit some elements of the Treg phenotype (including the expression of Treg-associated cell-surface molecules, such as CD25), although they are not suppressive in vitro. This indicates that the interaction of developing thymocytes with thymic stromal cells turns on a program of transcriptional regulation in parallel with or upstream of Foxp3, and that once the *Foxp3* gene is switched on, Foxp3 may stabilize and sustain the Treg phenotype and confer suppressive activity. Future efforts will examine how spatiotemporal interactions between thymocytes and stromal cells turn on the Treg differentiation program in developing thymocytes. Specifically, it needs to be determined whether the intensity of signaling via the TCR and accessory molecules alone is sufficient for cell fate determination to the Treg lineage or whether stromal cell-derived humoral factor(s) including cytokines are also required. It is also unclear how the interaction evokes Foxp3 expression in Treg-committed thymocytes (Figure 4B).

Peripheral Generation of Tregs from Naive T Cells

Naive T cells in the periphery can also acquire Foxp3 expression and consequently Treg function in several experimental settings, for example, in vitro antigenic stimulation of naive T cells in the presence of TGF-β (Chen et al., 2003; Apostolou and von Boehmer, 2004; Kretschmer et al., 2005). In TGF-β-dependent in vitro Treg induction, IL-6 hampers the differentiation of naive T cells to Foxp3⁺ cells (Bettelli et al.,

2006). Interestingly, TGF-β stimulation in the presence of IL-6 facilitates T cell differentiation to Th17 cells in mice (Veldhoen et al., 2006; Bettelli et al., 2006). IL-2 facilitates the differentiation of naive CD4+ T cells into Foxp3+ Tregs but inhibits their differentiation into Th17 cells (Laurence et al., 2007). Additionally, retinoic acid, which is secreted by a particular subset of dendritic cells in the gut-associated lymphoid tissue, inhibits IL-6-driven induction of Th17 cells. In the presence of TGF-β, retinoic acid facilitates the differentiation of naive T cells to Foxp3+ Tregs (Benson et al., 2007; Mucida et al., 2007; Sun et al., 2007; Coombes et al., 2007). Hence, orally administered protein antigens presented by retinoic acid-producing dendritic cells in the gut-associated lymphoid tissue may induce Foxp3+ Tregs. This could be a plausible mechanism of oral tolerance (Figure 5).

Yet, it remains to be determined whether Tregs induced from naive T cells in the periphery are functionally stable in vivo and to what extent they contribute to the peripheral pool of Foxp3+ Tregs (Figure 4A). For example, in mice the regulatory regions of the *Foxp3* gene are more widely demethylated in natural Tregs than in TGF-β-induced Tregs, suggesting functional instability of the latter (Floess et al., 2007). In addition, unlike mice, naive T cells in humans readily express Foxp3 upon TCR stimulation, although the expression is generally much lower and more transient than in natural Tregs (Yagi et al., 2004; Gavin et al., 2006). Assuming that Foxp3 can suppress possible effector programs in T cells, it is an intriguing question whether Foxp3 not only confers suppressive function to natural Tregs but also may serve as an intrinsic brake to the further activation of antigen-stimulated effector T cells in humans.

Besides Foxp3+ Tregs, there are other types of Treg cells that can be induced from naive T cells in the periphery; for instance, CD4+ T cells secreting IL-10 and TGF-β, called Tr1 cells, are produced in vitro by antigenic stimulation of naive T cells in the presence of IL-10 (Chen et al., 1994; Groux et al., 1997). Antigen-specific TGF-β-secreting T cells, called Th3 cells, were originally propagated from animals that became tolerant to orally administered protein antigen. At least some Th3 cells appear to be TGF-β-induced Foxp3+ Tregs. Tr1 cells do not express Foxp3, yet their properties in vitro are very similar to those of Foxp3+ Tregs. For example, they exhibit diminished proliferation in response to antigenic stimulation, exert cell contact-dependent suppression, scarcely produce IL-2, and display an activated cell-surface phenotype (Vieira et al., 2004). It is likely that Tr1 and natural Foxp3+ Tregs may share some common suppressive mechanims in vitro, although long-term stability of Tr1 and Th3 cells needs to be further assessed in vivo.

A variety of other T cell subpopulations including $CD8^+$, $CD4^-CD8^-$, and γ/δ T cells have also been reported to exhibit an immunosuppressive activity (reviewed in Shevach, 2006). There is so far little evidence that they play crucial roles in natural self-tolerance. CD8+ suppressor T cells were widely and intensively studied in the 1970s but have defied precise molecular characterization. These cells may now need to be recharacterized with currently available molecular tools. It is noteworthy that antigen immunization with strong adjuvant

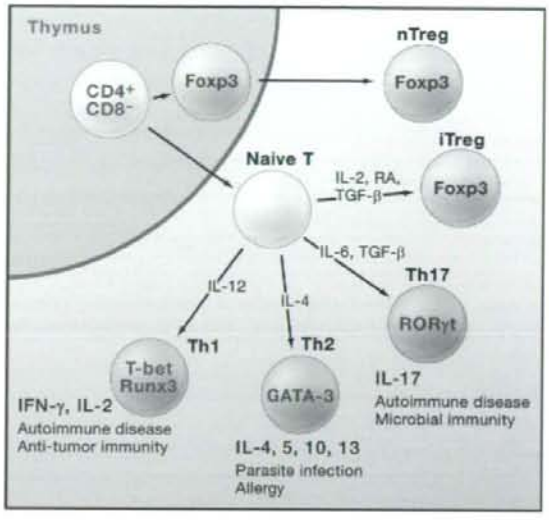

Figure 5. Differentiation of Naive CD4+ T Cells into Tregs or Effector T Cells

Cytokines and transcription factors that promote the differentiation of naive T cells into Tregs or effector T cells are shown. The transcription factors T-bet and Runx3, GATA3, or RORγt are required for the differentiation of naive T cells into Th1, Th2, or Th17 cells, respectively. nTreg, natural Treg; iTreg, induced Treg; RA, retinoic acid.

expands not only $CD4^+Foxp3^+$ T cells but also $CD8^+Foxp3^+$ T cells from a number that is negligible in unimmunized mice (Haribhai et al., 2007). These cells could plausibly correspond to some of the antigen-induced CD8+ suppressor T cells previously described (Cantor et al., 1976).

Trafficking and Localization of Tregs

Where are Foxp3+ natural Tregs found in the body? Dendritic cells in the draining lymph nodes present tissue-specific self-antigens (Scheinecker et al., 2002), and Foxp3+ Tregs specific for tissue self-antigens are enriched in the regional lymph nodes (Samy et al., 2005). This indicates that antigen-specific Tregs migrate to and become activated in regional lymph nodes where tissue-specific self-antigens or microbial antigens are presented. They also migrate into inflamed tissues, infectious sites, and tumors (Belkaid et al., 2002). In addition to TCRs that recognize specific antigens, Foxp3+ Tregs express a spectrum of homing receptors, including adhesion molecules and chemokine receptors (Huehn and Hamann, 2005). In the peripheral lymph nodes, the majority (~80%) of Foxp3+ Tregs express CCR7 at high levels. Foxp3+ Tregs are slightly higher than $Foxp3^-$ T cells in the expression of CCR4, CCR6, CXCR4, and CXCR5. A fraction (~30%) of Foxp3+ Tregs express CD103 (integrin $\alpha_E\beta_7$), which interacts with its ligand E-cadherin expressed by epithelial cells; a substantial fraction (~50%) highly express CD62L (L-selection), which interacts with the vascular addressins CD34, GlyCAM-1, and MAdCAM-1 expressed by the endothelium in lymph nodes and mucosal lymphoid tissue (reviewed in Huehn and Hamann, 2005). The expression of these homing receptors

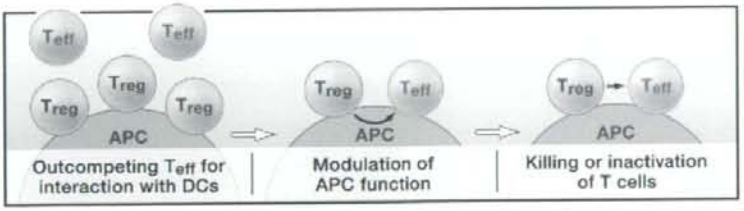

Figure 6. Possible Mechanisms of Treg-Mediated Suppression

More than one mechanism of Treg-mediated suppression may operate for the control of a particular immune response in a synergistic and sequential manner. Antigen-activated Tregs are recruited to antigen-presenting cells (APCs), especially dendritic cells (DCs), and out-compete antigen-specific naive T cells regarding interactions with dendritic cells mainly because of the high expression of adhesion molecules (such as LFA-1) by Tregs. Tregs then modulate dendritic cell function. For example, Tregs promote the downregulation of dendritic cell CD80 and CD86 by a CTLA-4-dependent mechanism. Some Tregs may further differentiate to kill or inactivate responder T cells by secreting granzyme/perforin or immunosuppressive cytokines (such as IL-10).

in Tregs controls their trafficking and localization and consequently the compartmentalization of Treg-controlled immune responses.

The expression patterns of homing receptors correlate with the functional heterogeneity of Tregs. CCR7$^+$CD62L^{high} Tregs, which migrate to the secondary lymphoid tissues, have an enhanced capacity to prevent the development of autoimmune diabetes in a mouse model, suggesting suppression of self-reactive T cells in the regional lymph nodes (Szanya et al., 2002). Foxp3$^+$ Tregs that express CD103 are memory-like and more suppressive than CD103$^-$ Tregs in vitro and in vivo. In a murine colitis model, Foxp3$^+$ Tregs that express CD103 are able to control colitis in the colonic mucosa (Uhlig et al., 2006). Similarly, CCR5$^+$ Tregs preferentially migrate to cutaneous lesions of *Leishmania major* infection (Yurchenko et al., 2006).

The expression patterns of homing receptors are basically similar to those of effector T cells migrating to the same tissue site. CCR4$^+$ Tregs as well as CCR4$^+$ effector T cells accumulate in inflamed skin following local antigen inoculation (Sather et al., 2007). CCR6$^+$ Tregs and CCR6$^+$ Th17 cells are both elevated in inflamed joints in an animal model of rheumatoid arthritis (Hirota et al., 2007). Additionally, retinoic acid secreted by intestinal CD103$^+$ dendritic cells induces gut-homing integrin $\alpha_4\beta_7$ in Tregs and non-Treg cells.

Treg-specific deficiency of a particular homing receptor or its blockade compromises Treg function, for example, in the control of tissue-localized inflammation, local maintenance of allograft tolerance, and fetomaternal tolerance (Lee et al., 2005; Yurchenko et al., 2006; Schneider et al., 2007; Sather et al., 2007; Kallikourdis et al., 2007). Thus, migration receptors on Tregs are highly informative in assessing Treg behavior in vivo and can be exploited to control immune responses through altering Treg trafficking and localization.

Treg Activation, Proliferation, and Differentiation

Upon antigen exposure in the regional lymph nodes, Foxp3$^+$ Tregs become activated and exert suppression at a much lower concentration of antigen than naive T cells. In TCR transgenic mice, the concentration of peptide required to activate the peptide-specific Tregs to exert suppression in vitro has been estimated to be 10- to 100-fold lower than the concentration needed for activating naive T cells with the same antigen specificity (Takahashi et al., 1998). This finding suggests that natural Tregs can be activated even by immature dendritic cells whose expression levels of CD80/86 and self-peptide/MHC are too low to activate naive self-reactive T cells. This might enable natural Tregs to exert dominant and tonic suppression of self-reactive T cells. It could also contribute to the prevention of autoimmunity stemming from molecular mimicry (the antigenic crossreaction between a self-molecule and a microbial substance) because natural Tregs could be more easily activated than self-reactive T cells by such a substance (Stephens et al., 2005).

Notably, unlike their hypoproliferation upon antigenic stimulation in vitro, a sizable fraction of Foxp3$^+$ natural Tregs are continuously proliferating in vivo presumably through the recognition of self-antigen and commensal microbes (Fisson et al., 2003; Setoguchi et al., 2005). Natural Tregs can also expand clonally in vivo and in vitro following antigenic stimulation and retain their suppressive function after expansion (Klein et al., 2003; Yamazaki et al., 2003; Fehérvári and Sakaguchi, 2004; Yamaguchi et al., 2007).

Various stimuli via accessory molecules expressed by Foxp3$^+$ Tregs also lead to their expansion in vivo and in vitro. Stimulation of GITR, which is constitutively expressed in Tregs at a higher level than in other T cells, facilitates vigorous proliferation of Tregs in the presence of IL-2 in vitro (McHugh et al., 2002; Shimizu et al., 2002). In addition, Tregs can expand following stimulation via Toll-like receptors (TLRs), independent of TCR recognition of a specific antigen. Tregs respond to ligands for TLR2, 4, 5, and 8, including bacterial flagellin that acts through TLR5 (reviewed in Sutmuller et al., 2006). A similar effect is observed with 60 kDa heat shock protein, a self-molecule, with Pam3Cys via mouse TLR2, or with lipopolysaccharide via TLR4.

Irrespective of active proliferation the number of Foxp3$^+$ natural Tregs is fairly constant in normal animals (10%–15% of CD4$^+$ T cells), indicating that cell death helps to maintain Treg homeostasis. Indeed, following antigenic stimulation Tregs downregulate Bcl-2, an antiapoptotic protein (Yamaguchi et al., 2007). Further, among Foxp3$^+$CD4$^+$ T cells in humans, antigen-stimulated naive Foxp3^{low} cells differentiate into effector/memory-like Foxp3^{high} cells, which vigorously proliferate and then die (M. Miyara and S.S., unpublished data).

Thus, Foxp3$^+$ Tregs that have migrated to lymphoid and nonlymphoid tissues become activated, proliferate, exert suppressive activity, and then die. However, it remains to be determined whether some Tregs further differentiate into long-lived memory-type cells.

Multiple Modes of Treg-Mediated Suppression?

A key issue of current research on Tregs is to understand the mechanism of Treg-mediated suppression. Foxp3$^+$CD25$^+$CD4$^+$ natural Tregs suppress the proliferation of naive T cells and

their differentiation to effector T cells in vivo. They can also suppress effector activities of differentiated CD4+ and CD8+ T cells and the function of natural killer cells, natural killer T cells, B cells, macrophages, osteoclasts, and dendritic cells (reviewed in von Boehmer, 2005; Shevach, 2006; Miyara and Sakaguchi, 2007; Tang and Bluestone, 2008). In vitro Tregs suppress the proliferation and cytokine production (in particular of IL-2) of responder T cells when the two populations are cocultured and stimulated by antigen in the presence of antigen-presenting cells (APC) (Takahashi et al., 1998; Thornton and Shevach, 1998). Once activated by a particular antigen, Tregs can suppress responder T cells irrespective of whether they share antigen specificity with the Treg (Takahashi et al., 1998).

Several mechanisms of Treg-mediated suppression have been proposed, and these include secretion by the Treg of immunosuppressive cytokines, cell-contact-dependent suppression, and functional modification or killing of APC (Figure 6). For example, IL-10 and TGF-β contribute to suppression of inflammatory bowel disease induced in mice by Treg depletion (Read et al., 2000). Tregs coexpressing Foxp3 and IL-10 at a single-cell level are indeed found in lamina propria of the intestine but not in the spleen (Uhlig et al., 2006; Maynard et al., 2007). TGF-β may act as a direct mediator of suppression, condition responder T cells to be sensitive to suppression, and/or maintain Foxp3 expression and suppressive activity in Tregs (reviewed in von Boehmer, 2005). A recent study has shown that Foxp3+ natural Tregs predominantly produce immunosuppressive IL-35, a new member of the IL-12 family (Collison et al., 2007).

Alternatively, absorption of cytokines by Tregs may induce apoptosis in responder T cells (Pandiyan et al., 2007). Tregs might also kill responder T cells or APC through cell-to-cell contact by a granzyme- or perforin-dependent mechanism, or through delivery of a negative signal to responder T cells. Possible negative signals include upregulation of intracellular cyclic AMP, which leads to inhibition of T cell proliferation and IL-2 production, or the generation of pericellular adenosine catalyzed by CD39 (ectonucleoside triphosphate diphosphohydrolase 1) and CD73 (ecto-5'-nucleotidase) expressed by Tregs (reviewed in Tang and Bluestone, 2008). Activated Tregs may also downmodulate CD80/86 expression on APC or stimulate dendritic cells to form the enzyme indoleamine 2, 3-dioxygenase, which catabolizes the essential amino acid tryptophan to kynurenines that are toxic to T cells; both appear to be dependent on the expression of CTLA-4 by Tregs.

Taken together, these findings suggest a model of Treg-mediated suppression: (1) upon antigenic stimulation, antigen-specific Tregs, which are highly mobile, are swiftly recruited via chemokines to dendritic cells presenting the antigen and out-compete antigen-specific naive T cells in aggregating around the dendritic cells; (2) antigen-activated Tregs contacting dendritic cells then downmodulate dendritic cell function, thereby hindering the activation of other T cells that are recruited to the dendritic cells; (3) Tregs may then further differentiate to secrete granzyme/perforin, IL-10, or other immunosupressive cytokines (such as IL-35) depending on the strength and duration of antigenic stimulation and the local milieu of cytokines and other substances (Figure 6). Consistent with this model, intravital imaging of Tregs and responder T cells in a lymph node by two-photon microscopy suggests that activated Tregs hamper stable contacts between responder T cells and antigen-presenting dendritic cells (Tang et al., 2006; Tadokoro et al., 2006). Assuming that in vivo activation of a naive T cell is a process that requires contact with an antigen-presenting dendritic cell for several hours (Celli et al., 2007), Treg-mediated interference may suffice to render abortive the activation of responder T cells, thereby suppressing an immune response.

Deficiencies of some Treg-expressing molecules (such as LAG3, granzymes, and IL-35) can impair Treg suppression in vitro by affecting a particular mode of suppression but fail to cause autoimmunity in vivo because other modes may effectively compensate for the deficiencies. Among the various molecules involved in suppression in vivo or in vitro, CTLA-4 is critically important for the following reasons. Foxp3+ Tregs constitutively express CTLA-4, Foxp3 directly controls the expression of CTLA-4, and CTLA-4 blockade abrogates suppression. Further, not only germline deletion but also Treg-specific conditional deficiency of CTLA-4 induces fatal autoimmunity/inflammation in mice (Salomon et al., 2000; Read et al., 2000; Takahashi et al., 2000; K. Wing and S.S., unpublished data). Additional study is required to elucidate the molecular basis of suppression mediated by Tregs. The fate of responder T cells that are suppressed by Tregs is also unclear, that is, whether they remain nonactivated, die by apoptosis, or become anergic.

Tregs: A Clinical Perspective

To what extent are Treg anomalies responsible for immunological diseases in humans and how might Tregs be exploited to control physiological and pathological immune responses?

Polymorphisms of several genes including *Ctla4*, *Il2*, *Cd25*, and *Ptpn22* significantly contribute to genetic susceptibility to common autoimmune diseases, such as type I diabetes (Wellcome Trust Case Control Consortium, 2007). Deficiency of these genes, in particular *Ctla4*, *Il2*, and *Cd25*, produces severe autoimmunity in mice presumably through an effect on Treg development and function. Similarly, CTLA-4 blockade or IL-2 neutralization for a limited period elicits T cell-mediated autoimmune disease in otherwise normal mice. It is therefore possible that the polymorphisms of these genes may alter Treg development or function and thereby render the host susceptible to autoimmune disease (Yamanouchi et al., 2007). Environmental factors might also affect Tregs and thereby contribute to the development of autoimmunity. Tregs display greater proliferation and higher metabolic activity than non-Tregs under physiological conditions. As a consequence, they are more sensitive to ionizing radiation, radiomimetic drugs (such as cyclophosphamide), and deficiency of certain vitamins such as folic acid (Fisson et al., 2003; Brode et al., 2006; Setoguchi et al., 2005; Yamaguchi et al., 2007).

Although Treg depletion can evoke autoimmunity, it can also provoke and enhance tumor immunity in rodents (Shimizu et al., 1999) (Figure 1). In vitro T cell responses against tumor-associated antigens are enhanced by stimulating T

cells from cancer patients (or even normal individuals) with tumor antigen after the depletion of natural Tregs (Danke et al., 2004; Nishikawa et al., 2005). In addition, Foxp3+ Tregs are abundant in tumors. Thus, natural Tregs that promote self-tolerance may act to impede immune surveillance against cancers in normal individuals and suppress potential responsiveness to autologous tumors in cancer patients. Targeting Tregs is a promising approach for cancer immunotherapy. Such approaches could include local depletion of Tregs in the tumor mass, attenuation of Treg function at the time of therapeutic vaccination with tumor antigen, and ex vivo expansion of tumor-infiltrating lymphocytes after the depletion of Tregs.

Similar to tumor immunity, depletion or reduction of natural Tregs enhances immune responses to pathogenic microbes (reviewed by Belkaid and Rouse, 2005). Yet, complete eradication of microbes via Treg depletion might hamper the development of effective immunological memory for the microbes, thus impairing the secondary immune responses upon reinfection (Belkaid et al., 2002). Hence, Tregs control the quality and tune the magnitude of antimicrobial immune responses to protect the host from pathogenic microbes while avoiding collateral immunopathology or inappropriate responses to commensal microbes.

Foxp3+ natural Tregs retain their suppressive function after expansion in vivo and in vitro. By exploiting this stable suppressive activity and proliferative capacity, strategies that clonally expand antigen-specific natural Tregs while inhibiting the activation and expansion of effector T cells will help to induce transplantation tolerance and suppress graft rejection. Furthermore, in the presence of Tregs that actively maintain graft tolerance, naive T cells could be newly recruited to the graft site and could differentiate into graft-specific Tregs, thereby augmenting graft tolerance (Waldmann et al., 2006). The same principle could be applied to the treatment of autoimmune disease, as well as allergy, and inflammatory diseases and might be used to augment feto-maternal tolerance in pregnancy.

For the purpose of attenuating or enhancing Treg-mediated immune suppression in various clinical settings, it is necessary to find a specific molecular marker that can selectively and reliably differentiate between Tregs and effector T cells. Biologicals, such as monoclonal antibodies, and small molecules that have differential effects on Tregs and effector T cells may represent the next generation of therapeutic reagents to selectively suppress or enhance immune responses by controlling the balance between Tregs and effector T cells. Further elucidation of the cellular and molecular processes underlying the development and function of Tregs will help to establish new strategies for the treatment and prevention of immunological diseases and for the control of a wide spectrum of physiological immune responses.

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