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Graded Attenuation of TCR Signaling Elicits Distinct Autoimmune Diseases by Altering Thymic T Cell Selection and Regulatory T Cell Function

Satoshi Tanaka,* Shinji Maeda,* Motomu Hashimoto,* Chihiro Fujimori,* Yoshinaga Ito,* Shin Teradaira,* Keiji Hirota,* Hiroyuki Yoshitomi,* Tomoya Katakai,† Akira Shimizu,† Takashi Nomura,* Noriko Sakaguchi,* and Shimon Sakaguchi* †,‡,§

Mice with a mutation of the ζ-associated protein of 70 kDa gene (skg mutation) are genetically prone to develop autoimmune arthritis, depending on the environment. In a set of mice with the mutation, the amount of ζ-associated protein of 70 kDa protein as well as its tyrosine phosphorylation upon TCR stimulation decreased from +/+, skg/+, skg/skg, to skg/− mice in a stepwise manner. The reduction resulted in graded alterations of thymic positive and negative selection of self-reactive T cells and Foxp3+ natural regulatory T cells (Tregs) and their respective functions. Consequently, skg/− mice spontaneously developed autoimmune arthritis even in a microbially clean environment, whereas skg/skg mice required stimulation through innate immunity for disease manifestation. After Treg depletion, organ-specific autoimmune diseases, especially autoimmune gastritis, predominantly developed in +/+, at a lesser incidence in skg/+, but not in skg/skg BALB/c mice, which suffered from other autoimmune diseases, especially autoimmune arthritis. In correlation with this change, gastritis-mediating TCR transgenic T cells were positively selected in +/+, less in skg/+, but not in skg/skg BALB/c mice. Similarly, on the genetic background of diabetes-prone NOD mice, diabetes spontaneously developed in +/+, at a lesser incidence in skg/+, but not in skg/skg mice, which instead succumbed to arthritis. Thus, the graded attenuation of TCR signaling alters the repertoire and the function of autoimmune T cells and natural Tregs in a progressive manner. It also changes the dependency of disease development on environmental stimuli. These findings collectively provide a model of how genetic anomaly of T cell signaling contributes to the development of autoimmune disease. The Journal of Immunology, 2010, 185: 2295–2305.

The strength of the signal originating from the TCRs interacting with self-peptide/MHC ligands expressed on thymic stromal cells is a key determinant of the fate of developing T cells. They are subjected to apoptosis because of overly low self-reactivity, positively selected for low self-reactivity, negatively selected for high self-reactivity, or driven to the Foxp3-expressing regulatory T cell (Treg) lineage (1–5). However, it remains to be determined whether genetic alteration of TCR signaling in developing T cells causes autoimmune disease by changing the sensitivity of self-reactive T cells to thymic selection or their effector function, or by impairing the development and function of natural Tregs, or by both.

There is accumulating evidence that genetically determined anomaly or variation in T cell signaling predisposes the host to autoimmunity. For example, the SKG strain of mice bears a mutation in the carboxyl-terminal Src homology 2 domain of ζ-associated protein of 70 kDa (ZAP-70), a highly T cell-restricted signaling molecule (6). They are genetically prone to develop T cell-mediated autoimmune arthritis, which immunopathologically resembles rheumatoid arthritis in humans (7). Other mutations of the ZAP-70 gene or mutations of other genes encoding TCR proximal signaling molecules, such as linker for activation of T cells, also impair the T cell selection in the thymus and T cell differentiation and function in the periphery to various extents, leading to autoimmunity/inflammation, immunodeficiency, or both (8–14). In humans, ZAP-70 deficiency affects thymic positive selection of CD8+ T cells and the function of CD4+ T cells (15–18). Additionally, a single-nucleotide polymorphism in lymphoid tyrosine phosphatase, which is encoded by the PTPN22 gene and interacts with ZAP-70 and other TCR proximal signaling molecules (19), is significantly associated with the occurrence of type 1 diabetes (T1D), rheumatoid arthritis, systemic lupus erythematosus, and autoimmune thyroid diseases (20–22). These findings collectively indicate that genetic anomalies or variations in TCR proximal signaling in T cells contribute to the occurrence of a variety of autoimmune diseases, including organ-specific and systemic ones. Furthermore, assuming that both environmental and genetic factors are required for the development of a common autoimmune disease, it is likely that such potentially autoimmune-inducing genetic anomalies or variations interact with environmental stimuli to elicit overt autoimmune disease.
In this report, we have analyzed how the degree of TCR signaling anomaly due to allelic increment of SKG/ZAP-70 gene mutation affects thymic positive and negative selection of self-reactive T cells as well as Foxp3+ natural Tregs and their respective functions, thereby leading to actual development of autoimmune disease. By comparing autoimmune-inducing capacity of T cells from wild-type (+/+), heterozygous (skg/+), or homozygous (skg/skg) mice, or mice expressing the skg allele and ZAP-70 null allele (skg−/−), we show that the increasing severity of the skg-induced impairment of T cell signaling correspondingly changes genetic predisposition to multiple autoimmune diseases through altering the TCR repertoire of self-reactive T cells. The impaired TCR signaling renders the host susceptible to some autoimmune diseases and, importantly, resistant to others. Furthermore, in contrast to SKG mice, which hardly develop arthritis in a microbiologically clean environment (23), we show that autoimmune disease spontaneously develops even in a specific pathogen-free (SPF) environment when signal impairment is severe. These results provide insights into how general T cell hyporesponsiveness due to genetic defect in TCR proximal signaling paradoxically produces T cell-mediated autoimmune disease, how the specificity and severity of autoimmune disease is genetically controlled via T cell signaling in a gene dosage-dependent fashion, and how environmental factors interact with genetic anomalies or variations, influencing the manifestation of autoimmune disease.

Materials and Methods

Mice

SKG mice, D011.10 or T26 TCR transgenic mice, and ZAP-70−/− deficient mice, all of which are on the BALB/c background, were previously described (24, 25). BALB/c mice and BALB/c nude mice were purchased from Japan SLC (Shizuoka, Japan); NOD mice and NOD SCID mice were from CLEA Japan (Tokyo, Japan). All experiments were conducted according to the institutional guidelines for animal welfare.

Flow cytometric analysis

The mAbs used for flow cytometric analysis were: anti-CD16/CD32, PE or FITC-anti-CD4, PE or biotinylated anti-CD8a, biotinylated anti-TCR Vβ14, streptavidin-PE, and PE-anti-phospho-ZAP–70 (Y319); BD Biosciences, San Jose, CA; FITC-anti-D011.10 TCR (KJ1.26; Caltag Laboratories, Burlingame, CA); streptavidin-PE–Cy5 (Dako, Glostrup, Denmark); allophycocyanin-anti–Foxp3 (FJK-16s; eBioscience, Franklin Lakes, NJ); and biotinylated anti-human nerve growth factor receptor (hNGFR) (Thermo Fisher Scientific, Waltham, MA). For analysis of phosphorylated ZAP-70, cells were fixed and permeabilized with the BD Phos Flow system (BD Biosciences) and then blocked with 10% mouse sera, followed by incubation with the specific Ab. All stained cells were analyzed by the Epics-XL analyzer (Beckman Coulter, Brea, CA).

Cell preparation

CD8+ and CD24+ cells were depleted from thymocyte suspensions by panning, incubated with FITC-anti-CD4, and CD4+ cells were sorted by an Epics AUTRA (Beckman Coulter), with resulting purity of >98%. For in vivo cell transfer, CD25+ cells were depleted by cytotoxic treatment with anti-CD25 (7D4) and rabbit complement (Cedarlane Laboratories, Burlington, Ontario, Canada) as previously described (26). For in vitro experiments, CD25+ T cells were depleted by cell sorter.

Proliferation assay

CD4+ T cells (2.5 × 10⁴) were incubated in round-bottom 96-well plates with 5.0 × 10⁴ irradiated splenocytes and graded concentrations of anti-CD3e (2C11) (BD Biosciences) in RPMI 1640 medium supplemented with 10% FCS, 50 µM 2-ME, and 10 mM HEPES buffer, and [H]thymidine deoxyribose incorporation was measured for the last 6 h of a 72-h incubation (27). For stimulation of ZAP-70 transfectants, incubation with OKT3 (1 µg/ml) was followed by ligation with anti-mouse IgG (10 µg/ml).

Neonatal thymectomy

Neonatal thymectomy (NTx) on day 3 after birth was performed as previously described, and the absence of thymic tissue was confirmed when they were sacrificed (28).

Cell transfer

Whole splenocytes (2 × 10⁶) were i.v. transferred from nondiabetic mice with NOD background to NOD,SCID mice. Blood glucose level of the recipients was monitored every week for 16 wk, and when the value was >500 mg/ml or was >200 mg/ml at 2 wk consecutively, the recipients were considered as diabetic and sacrificed.

ELISA

ELISA for antigastic parietal cell Ab was assessed, as described previously (26), with a standard positive serum from a BALB/c CD25+ cells transferred nude mouse and negative sera from normal BALB/c mice.

Histology

Every organ was fixed in 10% formalin and embedded in paraffin; the sections were stained with H&E. Joints were decalcified before embedding. Histological grading of gastritis was previously described (28).

Grading of arthritis

Macroscopic grading of joint swelling was previously described (6).

Statistical analysis

Welch’s t test or Fisher’s exact test was used for statistical analysis.

Results

Development of arthritis is dependent on genotype and environment

To investigate the possible contribution of altered TCR signaling ability to autoimmunity, we have made a series of mice having a varying number of skg allele and/or ZAP-70 null allele: +/-, skg/+, skg/skg, and skg−/- mice. These mice were assessed for spontaneous development of arthritis in our SPF condition over a 9-mo period. Notably, only skg−/- mice developed arthritis, which started around 8 wk of age in small joints and progressed to larger joints (wrists and ankles) in ∼90% of mice (Fig. 1A). We previously showed that the injection of zymosan to adult SKG mice evoked arthritis even in an SPF condition (23). In the present experiments, the treatment not only elicited severe arthritis in skg/skg mice, but also enhanced the disease in skg−/- mice with earlier onsets and in more severe forms than in the untreated skg−/- mice (Fig. 1B). Some (∼10%) of skg/+ and +/- mice transiently showed mild joint swelling. Unlike zymosan-evoked arthritis in skg/skg mice, T cells could not adoptively transfer the arthritis to skg/+ or +/- mice to syngeneic nude mice, indicating that they were not T cell-mediated autoimmune disease (Ref. 23 and data not shown). We previously showed that SKG arthritis was mediated predominantly by Th17 cells (29, 30). Th17 cells were increased in ratio and number in regional lymph nodes as well as spleens of untreated skg/skg and skg−/- mice, but not of skg/+ or +/- mice (Fig. 1C). Additionally, zymosan treatment expanded Th17 cells in skg/skg and skg−/- mice but not in skg/+ or +/- mice (Ref. 28 and data not shown).

These results indicate that the severity and the onset of arthritis as well as the dependency of disease development on environmental stimulation are distinct among skg−/-, skg/skg, skg+/+, or +/- mice. That is, environmental stimulation increases the penetrance of skg genetic effect as observed in skg/skg mice, and disease develops without (or with less) environmental stimulation when the genetic defect is as severe as in skg−/- mice.

Increasing skg dosage progressively alters thymic production of conventional T cells and Tregs

To determine the underlying mechanism of distinct disease severities among these mice, we first compared intracellular levels of ZAP-70 in thymocytes and T cells. In CD4+ and CD48+ (CD4 single positive [SP]) thymocytes and peripheral CD4+ T cells, the amount of ZAP-70 protein was clearly reduced in
a stepwise fashion from +/+, skg/+, skg/skg, to skg/− mice (Fig. 2A). The total numbers of thymocytes were comparable among these strains (legend for Fig. 2A). When compared with +/+ or skg/+ mice, however, the percentages of CD4+ or CD8+ T cells in the thymus and lymph nodes decreased significantly (p < 0.001) in skg/skg or skg/− mice, with more reduction have been found in the latter (Fig. 2B). Furthermore, CD4SP thymocytes in skg/skg or skg/− mice were composed of TCRβ high and TCRβ low cells; the latter were CD5 low and CD69 low, indicating that they have not been subjected to thymic selection (Fig. 2C, Supplemental Fig. 1). The percentages of Foxp3+ cells among TCRβ high CD4SP thymocytes were comparable among these strains, although the percentages among whole CD4SP were lower in skg/skg or skg/− mice, especially in the latter because of the presence of TCRβ low immature thymocytes in the CD4SP fraction (Fig. 2C, 2D). Therefore, in proportion with the reduction in the number of CD4SP thymocytes in skg/skg or skg/− mice, the number of Foxp3+ thymocytes was reduced in these mice. However, the ratios of Foxp3+ cells in their lymph nodes and spleens substantially increased, but the total numbers were comparable to +/+ or skg/+ mice because T lymphopenia in skg/skg and skg/− mice seemed to offset the increase in ratio. Additionally, peripheral Foxp3+ and Foxp3− T cells in the latter two strains exhibited a more activated phenotype (e.g., increase in the proportion of CD25 high, CD45RBlow, CD103 high, CD69 high, and/or CD62L low cells) and were more proliferative (i.e., Ki67+) compared with those in +/+ or skg/+ mice (Supplemental Fig. 2). T cells expressing CCR6, which is expressed by Th17 cells and Tregs recruited to the joint inflammation in SKG mice, also increased in skg/skg and skg/− mice (30). The relative increase in Foxp3+ cells and their activated phenotype in skg/skg and skg/− mice could be attributed to systemic inflammation in these strains (Fig. 1).

Regarding the mechanism of reduction in the amount of ZAP-70 protein in a gene dosage-dependent fashion, the transcription level of the ZAP-70 gene was comparable between skg homozygotes and heterozygotes and was reduced in skg/− mice (Supplemental Fig. 3). When SKG or normal ZAP-70 protein was expressed in Jurkat cells and stimulated via TCR, the former exhibited less tyrosine phosphorylation and also more rapid degradation than the normal ZAP-70 (Supplemental Fig. 3). Together with the result that the amount of ZAP-70 was much more profoundly reduced in skg/skg T cells than in skg/+ T cells (Fig. 2A), these findings collectively indicate that the mutation reduces TCR signaling by impairing tyrosine phosphorylation and facilitating the degradation of the mutated ZAP-70 protein.

Thus, the dosage of skg mutation progressively reduces TCR signaling, thereby altering thymic production of conventional T cells and Tregs.

Increasing skg dosage progressively alters TCR repertoire and function

Next, to assess possible effects of skg mutation on the TCR repertoire of developing T cells, we analyzed the usage of TCR Vβ subfamilies by Foxp3+ or Foxp3− TCRβ high CD4SP thymocytes (Fig. 3A). Those Vβ subfamilies (e.g., Vβ3, Vβ5, and Vβ11) reactive with endogenous superantigen encoded by Mtv-8 and Mtv-9, and hence deleted in +/+ mice with the BALB/c background (31), were significantly increased in both Foxp3+ and Foxp3− CD4SP thymocytes with an incremental increase from skg/+ skg/skg, to skg/− mice. The ratios of nondeleted Vβ subfamilies in skg/+ skg/skg, to skg/− mice could be attributed to systemic inflammation in these strains (Fig. 1).

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subfamilies, such as V β6, V β8.1, V β8.2, and V β10, have decreased by compensation. The degree of this resistance to deletion was more evident in Foxp3 + cells than in Foxp3 -/- cells, indicating that the former bear higher self-reactivity than the latter in mice with or without skg mutation. Similar results in self-reactivity were obtained with splenic Foxp3 + or Foxp3 -/- CD4+ T cells (data not shown). Moreover, when DO11.10 (DO) OVA peptide-specific TCR transgenic mice with skg/skg genotype were compared with DO +/+ mice, the former developed a higher number of T cells utilizing endogenous TCR α-chains, especially in Foxp3 + cells, paired with the transgenic TCR β-chain (Supplemental Fig. 4).

Functionally, proliferations of skg/- and skg/skg CD25 - CD4 + T cells were impaired in anti–CD3-stimulated proliferation assay in vitro, with more severe impairment of the former, whereas skg/+ and +/+ T cells were normal (Fig. 3B). The difference in cell proliferation was not due to the presence of different numbers of activated or memory type T cells in skg/skg and skg/- strains (Supplemental Fig. 2) since depletion of CD44 high cells from CD4 + T cell preparations did not alter the pattern of the differences (data not shown). CD4SP thymocytes also exhibited a similar pattern of differences in in vitro cell proliferation (data not shown). In Treg suppression assay in vitro, CD25 high CD4 + T cells from skg/skg or skg/- mice, especially from the latter, were significantly less suppressive in anti–CD3-stimulated coculture with normal CD25 - CD4 + T cells (Fig. 3C). Despite this in vitro hyporesponsiveness to polyclonal TCR stimulation (including allogeneic stimulation [Supplemental Fig. 5]), skg/skg T cells were more proliferative than +/- mice upon stimulation with self-Ags as revealed by in vivo homeostatic

**FIGURE 2.** The effects of skg mutation on ZAP-70 expression and T cell development. A, Thymocytes and spleen cells of 8-wk-old BALB/c mice with +/-, skg/+ , skg/skg, or skg/- - genotype were stained for CD4, CD8, TCR β, and ZAP-70. ZAP-70 expression levels in CD4 +CD8 - and CD4 + CD8 - thymocytes, and CD4 + TCR β - spleen cells are shown as histograms. The total number of thymocytes in each mouse was: +/-, 2.9 × 10⁶; skg/+, 2.2 × 10⁶; skg/skg, 2.8 × 10⁶; and skg/-, 3.5 × 10⁶. A representative of three independent experiments is shown. B, Percentages of CD4 + or CD8 - T cells in the thymus and lymph nodes of 8-wk-old mice are shown (n = 3–5). C, TCR β and Foxp3 staining of CD4SP thymocytes of the mice shown in A. A representative of three independent experiments is shown. D, Percentages of Foxp3 + cells among CD4SP thymocytes or TCR β high CD4SP thymocytes (n = 4–5). E, Percentages of Foxp3 + cells among lymph node CD4 + T cells of the mice shown in A (n = 3–5).
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**FIGURE 3.** The effects of skg mutation on TCR repertoire and function of conventional T cells and Tregs. A. Foxp3+ or Foxp3− CD4SP thymocytes from the strains of mice shown in A were assessed for the proportion of thymocytes expressing particular Vβ TCR subfamilies (n = 3–5 each). In Foxp3+ cells, there were statistically significant differences (p < 0.05) for the percentages of Vβ3+ cells between +/+ and skg/skg or skg−/−, skg+/+ and skg/skg or skg−/−; for Vβ5.1, 5.2+ cells and Vβ11+ cells, between +/+ and skg/skg or skg−/−, skg+/+ and skg/skg or skg−/−, +/+ and skg−/+ for Vβ3+, Vβ5.1, 5.2+, and Vβ11+ cells, and also between skg/skg and skg−/− for Vβ5.1, 5.2+ cells. B. In vitro 3-d proliferation of CD25+CD4+ T cells from the strains shown in A was assessed in triplicate in the presence of graded doses of anti-CD3 and X-irradiated splenic non-T cells. A representative result of three independent experiments is shown. C. In vitro Treg-mediated suppression assay utilizing BALB/c CD25+CD4+ T cells cocultured with graded numbers of CD25+CD4+ T cells from the strains shown in A. A representative result of three independent experiments is shown.

Disease-causing or -suppressing activity of ZAP-70−mutated self-reactive T cells and Tregs

Based on the results in Fig. 2, which showed mutation-induced alteration in the TCR repertoire as well as in vitro proliferative responses upon TCR stimulation when they mature. It also changes TCR repertoire of Foxp3+ and Foxp3− T cells progressively toward increased self-reactivity, and it impairs suppressive function of Foxp3+ natural Tregs.

Alteration of the spectrum and severity of autoimmune disease in Treg-depleted mice with different skg genotypes

We then examined whether the capacity of T cells with skg mutation to induce a variety of autoimmune diseases was determined in the thymus, and whether the dose of the mutation contributes to their capacity. We transferred the same number of CD25−CD4SP thymocytes prepared from skg/skg, skg−/−, or +/+ BALB/c mice to BALB/c nude mice. The survival of mice that received skg/skg CD25+ T cells was significantly lower in comparison with other groups (Fig. 5A); the surviving nude mice in each group developed autoimmune disease in a similar spectrum of organs/tissues (Fig. 5B). However, only the recipients of skg/skg thymocytes developed arthritis, and their incidence of gastritis was significantly lower compared with the recipients of +/+ or skg−/+ thymocytes.

It is known that BALB/c mice spontaneously develop gastritis and oophoritis following NTx on day 3 after birth presumably because NTx abrogates thymic production of natural Tregs from the beginning of their ontogeny (29, 32, 33). In ontogeny, Foxp3+ cells were detectable around day 3 after birth, and the ratio of Foxp3+ cells to CD4+ T cells was higher at an early age (around 1 wk after birth) in SKG mice, although total numbers of Foxp3+ proliferation and in vitro autologous MLR (Supplemental Fig. 5).
cells slightly reduced because of T lymphopenia (Supplemental Fig. 6; also, see above). skg/skg NTx mice harbored slightly less numbers of CD4+ T cells than did +/+ NTx or skg/+ NTx mice, and they had comparable numbers of Foxp3+ cells in the spleens (data not shown). The three groups equally survived the 3 mo of observation. As shown in Fig. 5C, NTx elicited arthritis in ∼40% of skg/skg mice in the SPF environment. Furthermore, compared with +/+ NTx mice, skg/skg NTx mice developed autoimmune disease in a wider spectrum of organs although the degree of joint swelling, histological severity of gastritis, and titers of anti-parietal cell autoantibodies were assessed 3 mo after transfer. F. Incidences of autoimmune diseases induced in the group shown in E. In B, grade 2 macroscopically evident gastritis and histologically evident oophoritis with complete loss of oocytes were counted (48). In E, closed or shaded circles indicate grade 2 and 1 gastritis, respectively.

FIGURE 4. Development of arthritis and other autoimmune diseases in SKG mice after Treg depletion. A, Enhanced development of arthritis in BALB/c nude mice transferred with CD25+ T cell-depleted or nondepleted CD4+ T cells (1×10⁷) from SKG mice with no joint swelling. B, Incidences of various histologically evident autoimmune diseases in the groups of mice shown in A. C, Representative histology of each disease shown in B. H&E staining (original magnification ×100). Scale bars, 200 µm. D and E, SKG or BALB/c CD25+ T cells were cotransferred with graded numbers of SKG or BALB/c CD4+ CD25+ T cells to BALB/c nude mice and the degree of joint swelling, histological severity of gastritis, and titers of anti-parietal cell autoantibodies were assessed 3 mo after transfer. F, Incidences of autoimmune diseases induced in the group shown in E. In B, grade 2 macroscopically evident gastritis and histologically evident oophoritis with complete loss of oocytes were counted (48). In E, closed or shaded circles indicate grade 2 and 1 gastritis, respectively.

Thus, the depletion of Foxp3+ natural Tregs in skg/skg mice by NTx or in skg/skg thymocyte transfer to nude mice enhances arthritis and also de novo produces some autoimmune diseases, while it reduces other autoimmune diseases (e.g., gastritis) that are frequent in Treg-depleted +/+ BALB/c mice (26, 29). Furthermore, this change in the incidence and the spectrum of autoimmune disease is dependent on the dosage of the mutation.

Modulation of autoimmunity in diabetes-prone NOD mice by skg mutation

To further assess the effect of the skg mutation on autoimmunity development, we introduced the mutation into the NOD background by backcrossing SKG mice to NOD mice more than eight times. Similar to SKG mice on the BALB/c background, NOD skg/skg mice had T lymphopenia of both CD4+ and CD8+ T cells (∼30% of NOD +/+ mice), as well as an increase in the ratio of Foxp3+ T cells among CD4+ T cells (Fig. 6A). There was no significant difference between NOD skg/skg and NOD +/+ mice in the ratio and number of Foxp3+ cells (data not shown). The NOD skg/skg mice spontaneously developed a higher number of Th17 cells than did NOD mice (∼10% vs <1% of CD4+ T cells), as seen in skg/skg mice on the BALB/c background (Fig. 6A). The IFN-γ-secreting cells, which are required for diabetes development in NOD mice (34), were also increased in NOD skg/skg mice compared with NOD mice. Notably, no NOD skg/skg mice became diabetic during 10 mo of observation, whereas 37.5% of NOD skg/+ and 57.1% of NOD +/+ mice developed the disease (Fig. 6B). In terms of histology, NOD skg/skg mice exhibited no inflammation in Langerhans islets in contrast with severe insulitis in NOD +/+ or NOD skg/+ mice (Fig. 6C).
a separate experiment, ∼30% NOD\(^{skg/skg}\) mice that had survived up to 12 mo of age developed mild but macroscopically evident joint swelling even in the SPF environment (Fig. 6D). No joint swelling was observed in 20 NOD\(^{skg/+}\) or NOD\(^{+/+}\) male mice that had survived 12 mo without developing diabetes (Fig. 6D and data not shown). Histologically, arthritis in NOD\(^{skg/skg}\) mice was more fibrotic and less inflammatory compared with SKG mice at 12 mo of age (Fig. 6E).

Furthermore, transfers of splenic cells from NOD\(^{+/+}\) mice to NOD.SCID mice elicited the development of diabetes in all recipient NOD.SCID mice by 16 wk after transfer despite no overt diabetes in the donor NOD\(^{+/+}\) mice at the time of transfer (Fig. 6F). In contrast, no recipients of NOD\(^{skg/skg}\) splenocytes suffered from the disease during 16 wk of observation. The recipients of NOD\(^{skg/skg}\) splenocytes, which contained an equivalent number of Foxp3\(^+\) cells as NOD\(^{+/+}\) splenocytes, exhibited an intermediate disease incidence. Histologically, the recipients of NOD\(^{skg/skg}\) spleen cells were protected from insulitis, which was present in almost all islets of the diabetic and even normoglycemic recipients of NOD\(^{+/+}\) or NOD\(^{skg/+}\) spleen cells (data not shown). One recipient of NOD\(^{skg/skg}\) spleen cells developed histologically evident arthritis (data not shown). When NOD\(^{skg/skg}\) spleen cells were transferred after deleting CD25\(^+\) T cells, the recipient mice became debilitated and developed severe colitis within a month after transfer, making it difficult to assess whether diabetes or arthritis was elicited by Treg depletion (data not shown).

Thus, the skg mutation appears to exert a similar effect on the Th cell differentiation on either NOD or BALB/c background. However, the mutation inhibits the development of diabetes in a mutation dose-dependent fashion and instead induces arthritis on the NOD background, although the incidence of arthritis was not as high as on the BALB/c background.

Altered thymic selection of TCR transgenic T cells specific for self or non–self-Ag

To examine the effect of the skg mutation on thymic T cell selection at the clonal level, we introduced the mutation into OVA peptide-specific RAG-2–deficient (RAG\(^{-/-}\)) DO TCR transgenic mice, which express no endogenous TCR \(\alpha\)- and \(\beta\)-chains and develop few Foxp3\(^+\) Tregs (24). We similarly introduced the mutation into RAG\(^{-/-}\) T26 transgenic mice that express a TCR transgene derived from a gastritogenic T cell clone, which was established from BALB/c NTx mice with autoimmune gastritis (25). RAG\(^{-/-}\) T26 transgenic mice on the BALB/c background spontaneously developed autoimmune gastritis (25). These two TCR transgenic strains on the BALB/c background allowed us to trace the differentiation of T cell clones specific for self or non–self-Ag (Fig. 7A, 7B).

In contrast with the development of mature CD4SP thymocytes in RAG\(^{-/-}\) DO or T26 mice with ZAP-70\(^{+/+}\) genotype, mice with ZAP-70\(^{skg/skg}\) genotype produced very few CD4SP thymocytes. By staining with KJ1.26 mAb specific for the DO transgenic TCR, or anti-V\(^b\)14 detecting T26 transgenic TCR, we found...
that transgenic TCR-expressing cells constituted ~20% and ~5% of spleen cells in DO and T26 RAG−/−ZAP-70+/+ mice, respectively. In contrast, very few KJ1.26+ or Vb14+CD4+ T cells were detected in the spleens of DO or T26 RAG−/−ZAP-70+/+ skg/+/+ mice, respectively. In DO or T26 RAG−/− mice with ZAP-70+/+, the change was intermediate between ZAP-70+/+ and ZAP-70+/+ skg/+/+ mice. The levels of the transgenic TCR expression on CD4+CD8+ thymocytes was the lowest in mice with ZAP-70+/+ skg/+/+ genotype and intermediate in mice with ZAP-70+/+ skg/+/+ genotype. The levels on mature CD4SP thymocytes were equivalent among the three strains. Notably, T26 RAG−/− mice of ZAP-70+/+ or ZAP-70+/+ skg/+/+ genotype developed histologically evident gastritis, whereas those with ZAP-70+/+ skg/+/+ genotype did not (Fig. 7C).

Furthermore, CD4SP thymocytes developed in ZAP-70+/+ skg/+/+ DO-RAG−/−transgenic mice at an equivalent level as in ZAP-70+/+ DO-RAG−/− mice (Supplemental Fig. 7). The expression levels of transgenic TCR by CD4+CD8+ thymocytes were comparable between the two strains, contrasting with much reduced expression in ZAP-70+/+ skg/+/+ DO-RAG−/− CD4+CD8+ thymocytes as shown in Fig. 7A (Supplemental Fig. 7). These findings, together with the result that CD4SP thymocytes failed to develop in DO-RAG−/−ZAP-70+/+ skg/+/+ mice (Fig. 7A), indicate that there is no functionally significant dominant-negative effect, if any, exerted by the mutated ZAP-70 proteins on normal ones, unlike other ZAP-70 mutations (35).

Taken together, gastric Ag-specific T26 and OVA-specific DO T cells with skg ZAP-70 mutation exhibited reduced expression of transgenic TCRs at the CD4+CD8+ thymocyte stage and therefore fail to undergo positive selection into CD4SP thymocytes. The extent of this defective positive selection depends on the dose of the mutation.

Discussion

The W163C missense mutation of the ZAP-70 gene in the region encoding the carboxyl-terminal Src homology 2 domain in SKG mice leads to a conformational change of the mutant ZAP-70 protein, thereby reducing its binding to ITAMs of TCRζ and CD3 chains (6, 35–37). The mutation also facilitates the degradation of the mutated ZAP-70 protein (Supplemental Fig. 3). The degree of the reduced ZAP-70 protein and the impairment of its signaling function were dependent on the dose of the mutation, with increased severity in skg homozygotes versus heterozygotes. Other than the skg mutation, it has been shown that other mutations of murine ZAP-70 gene affect thymic T cell selection. Some mutations in the catalytic domain resulted in total T cell deficiency, whereas other mutations of the same domain produced a mild T lymphopenia with or without autoimmune manifestations (8, 9, 12–14). For example, BALB/c mice with the mutations of Y315 and Y319 to alanine in interdomain B exhibited reduced signaling apparently corresponding to the degree somewhere between skg/skg and skg/+ mice (judging from the degree of TCRβ skewing and impaired development of Foxp3+ T cells) and developed rheumatoid factor without histologically evident arthritis (14). These findings collectively indicate that the signaling intensity through ZAP-70 changes quantitatively depending on the site of a mutation in the ZAP-70 gene and homo- or heterozygosity of the mutation. This change accordingly affects, at various degrees, the function of conventional T cells and Tregs as well as the sensitivity of developing T cells to thymic T cell
selection by endogenous self-peptide/MHC. The resulting T cell abnormalities range from the total T cell deficiency due to the absence of positive selection to the development of T cell-mediated autoimmune disease owing to altered positive and negative selection of aberrant self-reactive T cells and presumable dysfunction of natural Tregs (Fig. 8). Additionally, the degree of T cell signaling abnormality via ZAP-70 alters the dependency of autoimmune development on the environment.

The comparisons among Treg-depleted or nondepleted skg/skg, skg/+ and +/+ BALB/c or NOD mice indicate that incremental allelic doses of the skg ZAP-70 mutation renders the host susceptible to some autoimmune diseases and, interestingly, resistant to others. For example, whereas ZAP-70–intact T cells that solely expressed a transgenic TCR α-and β-chain specific for a gastric self-Ag were positively selected in +/+ BALB/c mice and were able to induce autoimmune gastritis, ZAP-70–mutated transgenic T cells with the same Ag specificity were not positively selected and therefore failed to induce the disease (Fig. 8). The result, however, does not solely mean that all gastritogenic T cells are not positively selected in skg/skg mice, because transfer of a large number of Treg-depleted skg/skg peripheral T cells was able to produce autoimmune gastritis in a T lymphopenic environment (Fig. 4B). It rather suggests that dominant gastritogenic clones may fail in positive selection in skg/skg mice. Likewise, normal BALB/c mice are not devoid of all arthritogenic T cells, as indicated by our previous finding that Treg depletion and homeostatic proliferation could elicit autoimmune arthritis, albeit at a low incidence, in BALB/c mice (26). In addition to altered positive selection, the mice with the skg mutation produced self-reactive T cells that were otherwise negatively selected in ZAP-70 intact mice, as illustrated by the resistance of skg+ T cells to the deletion by endogenous superantigen. Furthermore, compared

FIGURE 7. Selection of T cells specific for an OVA peptide or a gastric self-Ag in RAG-2–deficient TCR transgenic mice with skg mutation. A, CD4+ and CD8+ thymocyte subsets, KJ1.26-expressing CD4+ splenic T cells, and thymocyte subsets in 7-wk-old DO.RAG2-/- mice with +/+ skg/+ or skg/skg genotype. Indicated numbers are percentages for each quadrant or the polygonal region. B, Similar analyses with 7-wk-old T26 TCR transgenic mice. CD4+ T cells expressing transgenic TCR were detected as Vβ14+ cells. C, Representative histology of intact gastric mucosa in 4-mo-old T26 RAG-2-/-ZAP-70skg/skg mice (left) and gastritis in T26 RAG-2-/-ZAP-70skg/+ mice (right). H&E staining (original magnification ×100). Scale bars, 200 μm. Histological scores of gastritis in each strain are also shown. Shaded area in A and B indicates negative control for staining. A representative of three to five independent experiments are shown in A and B.

FIGURE 8. “Selection shift” and Treg anomaly can determine the phenotype of autoimmunity. A, Graded attenuation of ZAP-70 signaling among +/+ skg/+ skg/skg, skg/skg, and −/− mice changes the spectrum of affected organs, the severity of disease, the dependency on environmental stimuli for disease development. B, A model of autoimmune induction by skg ZAP-70 mutation. In this model, the skg mutation results in “selection shift” of TCR repertoire toward higher self-reactivity; consequently, a putative arthritogenic clone (red circle), normally deleted, will be positively selected. By contrast, weakly self-reactive clones (green, blue, and yellow), which are physiologically produced in ZAP-70 intact mice, are no longer positively selected after the “selection shift” in skg/skg mice, and autoimmunities associated with these clones fail to occur. In skg/+ mice, these clones are partially selected and the autoimmunities may develop mildly and/or at low incidences.
with normal mice, TCR-transgenic skg\(^+\) mice harbored a larger number of T cells that expressed endogenous TCR \(\alpha\)-chains paired with the transgenic \(\beta\)-chain; such endogenous \(\alpha\)-chain–expressing T cells showed high proliferative activity, hence high self-reactivity, when subjected to homeostatic proliferation in a T cell-deficient environment (Supplemental Figs. 4, 5). Thus, assuming that the positive selection of a developing T cell (or evasion of “death by neglect”) requires TCR signal intensity above a certain threshold, only those skg mutant T cells with high enough TCR affinities for self-peptide/MHC ligands would be positively selected and consequently induce high enough TCR-proximal signaling intensities to compensate the attenuated signaling through the aberrant ZAP-70. Conversely, those T cells that are negatively selected in normal mice because of their high affinity for thymic self-peptide/MHC ligands are not deleted if they bear skg mutation. These alterations in thymic T cell selection result in the skewing of the whole T cell repertoire toward higher self-reactivity. This leads to the production of highly self-reactive T cells (more specifically, T cells presumably reactive with systemically expressed self-Ag; see below), including autoimmune ones, and also hampers positive selection of weakly self-reactive T cells (or T cells reactive with tissue-localized self-Ag) that are normally produced in ZAP-70 intact mice (e.g., gastriegenic T cells and anti-OVA T cells in BALB/c mice and diabetogenic T cells in NOD mice; Fig. 8). Importantly, the degree of this “selection shift” in whole T cell repertoire depends on the homo- or heterozygosities of skg mutation. That is, not only the severity but also the phenotype of autoimmune disease (i.e., which self-Ag is predominantly targeted) may differ depending on the dose of a particular mutation affecting T cell signaling.

How, then, are SKG mice prone to predominantly develop autoimmune arthritis irrespective of presumable production of self-reactive T cells bearing various other specificities? We have shown that some arthritogenic T cells in SKG mice may recognize self-Ags ubiquitously expressed in various tissues. For example, SKG mice spontaneously develop not only arthritis but also interstitial pneumonitis (6), and T cell clones prepared from arthritic joints of SKG mice mediated both arthritis and interstitial pneumonitis (6), and T cell clones prepared from arthritic joints of SKG mice mediated both arthritis and interstitial pneumonitis (6), and T cell clones prepared from arthritic joints of SKG mice mediated both arthritis and interstitial pneumonitis (6), and T cell clones prepared from arthritic joints of SKG mice mediated both arthritis and interstitial pneumonitis (6), and T cell clones prepared from arthritic joints of SKG mice mediated both arthritis and interstitial pneumonitis (6), and T cell clones prepared from arthritic joints of SKG mice mediated both arthritis and interstitial pneumonitis (6), and T cell clones prepared from arthritic joints of SKG mice mediated both arthritis and interstitial pneumonitis (6), and T cell clones prepared from arthritic joints of SKG mice mediated both arthritis and interstitial pneumonitis (6), and T cell clones prepared from arthritic joints of SKG mice mediated both arthritis and interstitial pneumonitis (6). By sequestration of self-Ag in the joint, the latter to be activated by self-Ag because of their high-affinity TCRs to overcome signal attenuation. Even if the signal attenuation is inadequate to shift the overall TCR signaling to the critical range where overt autoimmune diseases spontaneously occur, it changes genetic predisposition of the host to various autoimmune diseases and the dependency of disease development on environmental stimuli. These findings are conducive to our understanding of the genetic basis of a variety of autoimmune diseases and may help designing preventive and therapeutic measures.

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