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Journal of Immunology published online 10 June 2016
http://www.jimmunol.org/content/early/2016/06/10/jimmunol.1502656

Supplementary Material
http://www.jimmunol.org/content/suppl/2016/06/10/jimmunol.1502656
6.DCSupplemental

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Loss of the Protein Tyrosine Phosphatase PTPN22 Reduces Mannan-Induced Autoimmune Arthritis in SKG Mice

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The cytoplasmic phosphatase, protein tyrosine phosphatase nonreceptor type 22 (PTPN22), is a negative regulator of T cell signaling. Genome-wide association studies have shown that single-nucleotide polymorphisms in PTPN22 confer an increased risk of developing multiple autoimmune diseases in humans. The precise function of PTPN22 and how the variant protein contributes to autoimmunity is not well understood. To address this issue, we investigated the effect of PTPN22 deficiency on disease susceptibility in a mouse model of autoimmune arthritis. The SKG mouse expresses a hypomorphic mutant allele of ZAP70, which, upon exposure to fungal Ags, predisposes the mice to a CD4+ T cell-mediated autoimmune arthritis that closely resembles rheumatoid arthritis in humans. Surprisingly, SKG Ptpn22−/− mice developed less severe mannainduced arthritis compared with SKG mice. Diminution of disease was not due to significant alterations in thymocyte development or repertoire selection in SKG Ptpn22−/− mice, even though T cell-mediated signal transduction was improved. Instead, Ptpn22 deficiency appeared to bias CD4+ Th cell differentiation away from the Th17 lineage, which is pathogenic in this setting, to a more Th1/T regulatory–focused response. These data show that even small perturbations in TCR signal transduction pathways can have profound consequences on the differentiation of T cell lineages and thus for the development of autoimmune diseases. The Journal of Immunology, 2016, 197: 000–000.

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease that predominantly affects the synovial membranes of the primary joints. Although autoreactive CD4+ T cells and dysregulated B cell homeostasis are prime mediators of RA, the precise etiology remains unknown (1). Recent genome-wide association analysis has identified a single-nucleotide polymorphism, R620W, in the PTPN22 gene as a predominant risk factor for RA and other autoimmune diseases (reviewed in Ref. 2).

PTPN22 encodes a phosphatase known as lymphoid tyrosine phosphatase in humans or proline, glutamic acid, serine, threonine–enriched phosphatase in mice. The role of PTPN22 is best characterized in T cells, where it functions to inhibit TCR signaling by dephosphorylating key members of the T cell signaling pathway, including Src family kinases such as Lck, ITAM residues on TCR-ζ and CD3 chains, ZAP70, and Vav (3, 4). Ptpn22−/− mouse increased TCR signaling particularly in effector cells with an age-dependent increase of effector-memory CD4+ and CD8+ T cells (5, 6). On a C57BL/6 (B6) background, Ptpn22−/− mice do not show signs of autoimmunity (5, 6) unless the PTPN22 deletion is combined with another susceptibility mutation such as CD45-E163R (7). PTPN22 R619W knock-in mice, which are analogous to the human R620W variant and show a similar, albeit milder phenotype to that of the Ptpn22−/− mouse (8, 9), were found to develop multiple features of autoimmunity on a susceptible mixed B6/129 background (8). Collectively, these data indicate that there is synergy between susceptibility alleles that contribute to an autoimmune phenotype.

An association of PTPN22 dysfunction with autoimmunity fits well with the known roles of phosphatases in attenuating signaling pathways integral to immune cellular reactivity (10). However, the underlying molecular mechanism by which PTPN22 regulates the adaptive immune responses to contribute to maintenance or breaking of tolerance is not well understood. Although a number of PTPN22 targets have been identified in vitro, it is unclear which of these are most relevant to the development of autoimmunity. To address this issue, we used the ZAP70−/− (hereafter called SKG) mouse that has a defect in ZAP70 kinase activity and expression and develops CD4+ T cell–mediated autoimmune arthritis (11). In mice reared under specific pathogen-free conditions, arthritis can be elicited either through activation of innate immunity, by administration of fungal extracts, or by provoking homoeostatic proliferation of self-reactive T cells upon transfer to the T cell–deficient environment (12). The SKG mouse has a spontaneous point mutation in ZAP70, resulting in an amino acid change, W166C within the Src homology 2 domain. ZAP70−/− homozygous mice have a ~80% decrease in ZAP70 protein abundance compared with wild-type (WT) BALB/c mice and decreased signaling through the TCR complex (11). Substrate trap experiments...
show that ZAP70 is one of the substrates of PTP22, which dephosphorylates the activating tyrosine residue (Tyr695) on ZAP70 (4). We generated double-mutant SKG Ptpn22 deficient mice (SKG Ptpn22<sup>−/−</sup>) by backcrossing SKG mice with mice containing global deletion of Ptpn22 to ask what influence combining these two mutations would have on the development and progression of arthritic disease.

We show that PTPN22 deficiency enhanced TCR-mediated signaling in SKG Ptpn22<sup>−/−</sup> thymocytes and that the early stages of thymus positive selection were partially restored in SKG Ptpn22<sup>−/−</sup> mice. However, the selected peripheral TCR repertoire of SKG Ptpn22<sup>−/−</sup> mice was not substantially changed from that of SKG mice. Consistent with increased signaling observed in the thymocytes, there were improved in vitro proliferation and IL-2 production of CD4<sup>+</sup> T lymphocytes from SKG Ptpn22<sup>−/−</sup> mice compared with SKG mice. Surprisingly, SKG Ptpn22<sup>−/−</sup> mice developed less severe mannan-induced arthritis than SKG mice and showed decreased proportions of Th17 and slightly higher numbers of T regulatory cells (Tregs). Therefore, the absence of PTPN22 partially compensates for the defect in ZAP70 activity in the SKG mouse. The increase in TCR signaling capacity influenced the differentiation outcomes of SKG Ptpn22<sup>−/−</sup> Th cells with a corresponding decrease in the incidence of autoimmune disease.

Materials and Methods

**Mice**

Ptpn22<sup>−/−</sup> Exon1<sup>−/−</sup> × PC3-Cre (global Ptpn22<sup>−/−</sup>) (5) mice were backcrossed onto the ZAP70<sup>−/−</sup> mouse line (11) for more than eight generations to generate SKG Ptpn22<sup>−/−</sup>/Ptpn22<sup>−/−</sup> mice. When crossed onto the ZAP70<sup>−/−</sup> background, the CD4<sup>+</sup> T cell population was then sorted on a FACS Aria I (BD Biosciences) to obtain pure populations of CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>+</sup> naive T cells (>99% purity).

**Flow cytometry and Abs**

Cells were resuspended in FACS buffer (PBS containing 1% FCS and 0.05% sodium azide) and incubated with Abs for 30 min on ice, followed by washes in FACS buffer before acquisition on MACSQuant (Miltenyi Biotec) or LSR-II (BD Biosciences) flow cytometers and analyzed using FlowJo software v9.6 (Tree Star). Fluorochrome-conjugated mAbs against the following Abs were from eBioscience: CD3-bio (145-2C11), CD8-PE (53-6.7), CD4-FITC (48-18144), CD25allophycocyanin (PC61.5), CD44-allophycocyanin eFluor 780 (IM7), CD45RBPE (C363.16A), CD62L-PE (MEL-14), TRC-FTTC and -biotin (H57-597), IL-17 allophycocyanin (eBio17B7), Vbeta3-FTTC (K125), Vbeta5.2PerCp (MR9.4), Vbeta1.8/2.6 (K16), and Vbeta11-FTTC (CTVB11). The following Abs were purchased from BioLegend: CD4-Pacific Blue and -PerCp (RMA 4-5), CD6-PerCp (53-67), CD69-biotin (H12.2F13), and IFN-γ-FTTC (XMGI2.1). CFSE and Cell Trace Violet were purchased from Invitrogen. For intracellular staining, LN cells prestained with CD4 Ab were stimulated with 50 ng/ml phorbol 12,13-dibutyrate (PDBu; Sigma-Aldrich) and 0.5 mM ionomycin (Sigma-Aldrich) in the presence of 5 μg/ml brefeldin A (Sigma Aldrich) for 4 h, stained with Live/Dead (Molecular Probes, Invitrogen), fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), and stained with cytokines anti-IL-10, anti-IL-17, anti-TNF, and anti-IFN-γ. The data were collected using a MacsQuant (Miltenyi Biotec) flow cytometer and analyzed using FlowJo software v9.6 (Tree Star).

**Western blotting**

Cells were lysed in 1% Triton X-100, 0.5% n-dodecyl-β-maltoside, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 20 mM EDTA, 1 mM NaF, and 1 mM sodium orthovanadate containing protease inhibitors, and samples were separated by SDS-PAGE. Membranes were incubated in blocking reagent (LI-COR Biosciences) before immunoblotting with Abs to ZAP70 and β-actin. Proteins were detected with secondary Abs and visualized using an infrared imaging system (Odyssey; LI-COR Biosciences).

**Calcium flux**

Single thymocyte cell suspension, 1 × 10<sup>6</sup> cells/ml in prewarmed PBS, from BALB/c, SKG, and SKG Ptpn22<sup>−/−</sup> mice were labeled with varying concentration of CFDA-SE dye (Life Technologies) for 10 min, protected from light at 37°C. The individual populations were mixed in a 1:1:1 ratio at final cell concentration of 3 × 10<sup>5</sup> cells/ml and incubated with 2 μM solution of the ratiometric indo-1-AM dye (Life Technologies) for 40 min at 37°C in prewarmed PBS, before washing and staining with 10 μg/ml biotinylated anti-TRC (H57-597), anti-CD3 (145-2C11), and anti-CD4 (RMA 4.5) mAbs. Anti-CD8a-PerCP (53-6.7) and anti-CD4-allophycocyanin (RMA 4.4) Abs were used to identify the thymus populations. After the surface staining, cells were resuspended in prewarmed IMDM (Life Technologies) containing 1% FCS and supplemented with 2.5 mM CaCl<sub>2</sub> and 2.8 mM MgCl<sub>2</sub>. The baseline Ca<sup>2+</sup> levels were measured for 45 s before cross-linking the biotinylated Abs with 1 μg/ml streptavidin–PE conjugates, and samples were collected on a flow cytometer (LSR II; BD Biosciences). The increase in Ca<sup>2+</sup> was measured as a function of time.

**TCR repertoire analysis**

mRNA was purified from 10<sup>6</sup> naive CD4 T cells using a mRNA direct purification kit (ThermoFischer Scientific), and cDNA was synthesized using SMART<sup>®</sup>scribe reverse transcriptase (Clontech Laboratories) to produce bead-coupled mRNA libraries. The entire volume of mRNA beads was added to 4 μl first-strand buffer, 0.5 μl 20 mM DTT, 2 μl 10 mM dNTP mix, 2 μl SMART synthesis oligo (5′-GGCGAACGAGTTGTAT-CAACGCGAGTGACGGrGrG-3′), 0.5 μl RNase inhibitor, and 2 μl SMART<sup>®</sup>scribe reverse transcriptase (Clontech), and the reaction was incubated for 42°C for 1 h.

TCR-β V-region amplicons were generated from cDNA by PCR using indexed forward primers composed of the SMART synthesis oligo sequence fused to a P7 illumina tag, and a reverse primer within the TCR-β C region fused to a P5 illumina tag. Amplified products were purified by extraction from excised agarose gel bands and concentrated by ethanol precipitation prior to sequencing. Single-end 1 × 400 bp sequencing was performed on an Illumina MiSeq sequencer using custom read primers and indexing primers complementary to the amplification primer sequences. Sequence data were processed using the MiTCR utility (13) and a customized pipeline of python scripts to analyze and plot the MiTCR output.

**Cell cultures and assays**

Cells were cultured in IMDM (Sigma-Aldrich) supplemented with 5% heat-inactivated FBS (Serotec), 2 × 10<sup>-3</sup> M L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 × 10<sup>-5</sup> M 2-ME (Sigma-Aldrich). Sorted naive CD4<sup>+</sup> T cells were labeled with 2.5 μM Cell Trace Violet (Molecular Probes, Life Technologies) in prewarmed PBS at 37°C for 20 min in dark and washed, and 1 × 10<sup>5</sup> Cell Trace–labeled T cells were incubated with 2 × 10<sup>5</sup> irradiated BALB/c Rag2<sup>−/−</sup> spleen cells in presence of 0.04, 0.2, and 1 μg/ml anti-mouse CD3e Abs (eBioscience) in round-bottomed 96-well plate at 37°C. Cells were harvested at 72 h after stimulation and stained with anti-CD4 and anti-CD8, and proliferation was assessed and analyzed by flow cytometry. Supernatant from inactivated LN cultures were analyzed for IL-2 by ELISA with paired capture and detection Ab from eBioscience. Plates (NUNC Maxisorp) were washed with 0.05% Tween 20 in PBS and blocked with blocking buffer (supplied with kit).

Supernatant samples were analyzed in triplicates. After the addition of 100 μL 3.3%, 5.5% tetramethylbenzidine substrate solution (supplied with kit) and 100 μL 0.18 M H<sub>2</sub>SO<sub>4</sub> (Carl Roth), absorbance was read at 450 nm with a LabSystems Multiscan Ascent Spectrophotometer plate reader.

In vitro Th17 induction. 5 × 10<sup>5</sup> sorted naive CD4<sup>+</sup> T cells from LN were in cultured 1 ml media in a 48-well plate and stimulated with immobilized anti-CD3 (1.5 μg/ml) and anti-CD28 (3 μg/ml) in the presence of a mixture of the following cytokines: TGF-β (1 ng/ml), IL-1β (10 ng/ml; PeproTech), IL-23 (10 ng/ml; R&D Systems), IFN-γ (300 ng/ml; Enzo Life Science), IL-6 (2, 0.2, 0.02, 0.02 μg/ml; eBioscience), anti-IFN-γ (5 μg/ml; RA12; Biolegend), and anti-IL-12 and anti-IL-4 (from BD Pharmingen) for 4 d. At the end of the culture, the cells were harvested and restimulated with PDBu and ionomycin before intracellular staining and analysis by flow cytometry.
Peritoneal exudate cells from unimmunized mice were adhered to plastic for 4 h and washed before adding mannans. Twenty-four hours later, supernatants were harvested, and the remaining cells were lysed for analysis of protein content. IL-6 was assayed using an ELISA kit (eBioscience), and the IL-6 concentrations obtained from a standard curve were normalized to the protein values per well.

For the in vivo Treg suppression assay, FACS-sorted naïve BALB/c CD90.1+CD4+CD25+CD45RB+ cells (4 × 10⁶) were injected alone or together with FACS-sorted SKG or SKG Ptpn22⁻/⁻ CD45.2+CD4+CD25+ Tregs (2 × 10⁶) i.v. into BALB/c Rag2⁻/⁻ recipients in a 2:1 ratio, as indicated (n = 6 mice per group). After 12 d, LNs were assessed by flow cytometry for their proportions of CD90.1+Foxp3⁻ naïve cells and CD90.1 Foxp3⁺ Tregs.

Induction and scoring of arthritis
Six- to eight-week-old mice were injected once i.p. with 50–75 mg mannan reconstituted in sterile PBS. Mice were weighed and observed for clinical signs of arthritis weekly for 4 wk. Signs of arthritis were assessed by visual scoring where 0 = no joint swelling, 1 = mild to moderate swelling, 2 = substantial swelling of wrist or ankle, and 3 = severe swelling of wrist or ankle. Scores for all the wrists and ankles were combined for each mouse.

Results
Absence of Ptpn22 does not alter selection in SKG thymocytes
In SKG mice, decreased signaling results in defective positive and negative thymic selection (11). In contrast, Ptpn22 deficiency has little apparent influence on thymus selection in B6 mice (5, 6). However, in the context of Ptpn22 deficiency, there was a report of a slight increase in selection of CD4 cells, but no change in negative selection on a TCR transgenic background (6). Therefore, we asked whether loss of PTPN22 would improve selection of thymocytes on the SKG background.

SKG mice have a ~4-fold decrease in the abundance of ZAP70 protein (11). The absence of PTPN22 did not alter ZAP70 protein abundance with SKG Ptpn22⁻/⁻ thymocytes having a similar reduction compared with BALB/c mice (Fig. 1A). SKG thymocytes develop normally until the double-positive (DP) stage (11) in keeping with a limited role for ZAP70 in earlier stages of thymus differentiation (14). Consistent with this, analysis of thymocytes from SKG and SKG Ptpn22⁻/⁻ mice showed that loss of PTPN22 had little effect on the proportions (Fig. 1B) and absolute numbers (Table I) of double-negative and DP thymocytes between these two strains, although total thymocyte numbers for both were reduced relative to control thymocytes from BALB/c mice (11).

In SKG mice, impaired ZAP70 expression, and therefore function, leads to a failure of both positive and negative selection (11). There was a major reduction in the proportion of TCRbhigh DP thymocytes in SKG mice (11), and we saw an equivalent reduction in the SKG TCRbhigh DP subpopulation in the absence of PTPN22 (Fig. 1C). SKG DP thymocytes have lower CD5 expression than BALB/c DPs, whereas levels of CD5 expression on SKG Ptpn22⁻/⁻ and BALB/c DP thymocytes were similar (Fig. 1C). The higher expression of CD5 on DP thymocytes in the absence of PTPN22 was consistent with reports from Ptpn22⁻/⁻ mice on the C57BL/6 background (5, 6); however, the upregulation of CD5 at this stage did not seem to impact on subsequent selection events. Detailed analysis of the stages of positive selection can be identified by gating DP thymocytes on CD5 and TCR expression to reveal three stages of maturation, as follows: DP1 (CD5⁺ TCR⁻), DP2 (CD5⁺⁺ TCR⁺), and DP3 (CD5⁺⁺⁺ TCR⁺⁺) (Fig. 1D) (15). The abundance of both CD5 and TCR depends on the levels and function of ZAP70 (17), and consequently SKG mice have a lower proportion of DP2 and DP3 cells compared with BALB/c mice, whereas the proportions of DP2 and DP3 subsets were slightly elevated in SKG Ptpn22⁻/⁻ mice compared with SKG mice (Fig. 1E). Selection signals can also be monitored by CD69 upregulation. In BALB/c thymocytes, CD69 expression increased progressively as the cells transited from DP1 to DP3, consistent with these subpopulations becoming positively selected. Although both SKG and SKG Ptpn22⁻/⁻ thymocytes upregulated CD69 between DP1 and DP2 subsets, thereafter CD69 expression was unchanged (Fig. 1F). These data suggest that selection signals at this later stage in the SKG background strains of mice are not maintained to the same extent as they are in BALB/c mice. Indeed, in both SKG and SKG Ptpn22⁻/⁻ mice, there were significantly fewer mature CD4 SP and CD8 SP cells generated than in BALB/c mice (Table I), consistent with the impaired positive selection described previously for SKG mice (11). However, there was no significant change in the overall numbers of positively selected thymocytes between SKG and SKG Ptpn22⁻/⁻ mice.

Given that ZAP70 has been reported to be a target of PTPN22, we asked whether we could detect differences in signaling through the TCR between SKG and SKG Ptpn22⁻/⁻ DP thymocytes. To control the timing of the delivery of the TCR signal between the individual samples, thymocytes from BALB/c, SKG, and SKG Ptpn22⁻/⁻ mice were labeled with different amounts of CFSE so that they could be mixed together in a 1:1 ratio and yet distinguished by FACS. Cells were stimulated by incubating with biotinylated Abs that were cross-linked by addition of streptavidin. Cross-linking CD3 with CD4, but not TCR alone, induced a robust peak of intracellular Ca²⁺ flux in WT BALB/c DP thymocytes, whereas Ca²⁺ flux in SKG thymocytes was impaired, as has been reported previously (11) (Fig. 2A). The absence of PTPN22 partially restored the ability of CD3 plus CD4 cross-linking to induce a Ca²⁺ flux in the SKG DP cells, although it remained lower than WT BALB/c control (Fig. 2A).

In BALB/c mice, thymocytes expressing TCR Vß3, Vß5, Vß11, and Vß12 chains are reactive with endogenous viral superantigens encoded by Mtv-8 and Mtv-9, and are consequently deleted (18). A graded decrease in TCR signal strength by the expression of aberrant ZAP70 was shown to lead to failure of deletion of self-reactive autoimmune clones in the thymus and thus to alter the peripheral TCR repertoire (19). SKG mice were shown to have an increased frequency of thymocytes bearing these TCR Vß subfamilies, indicating a failure of negative selection (20). Given that lack of PTPN22 partially restored Ca²⁺ signaling in DP thymocytes, and the strength of Ca²⁺ signaling is directly linked with induction of negative selection at this stage (21), we sought to determine whether there was any effect of Ptpn22 deficiency on deletion of these particular TCR Vß families. As described previously (18), the frequency of mature CD4⁺ single-positive (SP) thymocytes and CD4⁺ peripheral T cells expressing TCR Vß3, Vß5, or Vß11 chains was increased in SKG as compared with BALB/c mice (Fig. 2B). Additionally, there was a compensatory decrease in the proportion of TCR Vß8.1/8.2⁺ cells in SKG mice (20). Loss of PTPN22 did not impact on this failure of negative selection on the SKG background, as there was no significant difference in the proportions of these specific TCR Vß-expressing cells in either the thymus or periphery of SKG Ptpn22⁻/⁻ compared with SKG mice (Fig. 2B). This result agrees with previous studies reporting that PTPN22 has minimal role in negative selection (6). Overall, these data indicate that PTPN22 deficiency does not impact substantially on either positive or negative selection in the thymus of SKG background mice. We observed a small, but significant increase in Vß11-expressing T cells in the periphery of SKG Ptpn22⁻/⁻ compared with SKG mice, which was not apparent in the thymus, and it may be that exposure to viral superantigens in the periphery causes greater expansion of this Vß subset in the absence of PTPN22.
Similar TCR repertoire but increased responsiveness of SKG Ptpn22<sup>−/−</sup> CD4<sup>+</sup> T cells

To address more specifically whether the peripheral repertoire was similar between SKG and SKG Ptpn22<sup>−/−</sup> mice, we sequenced their TCR-β CDR3 regions. Naive CD4<sup>+</sup> T cells were sorted from LN of naive BALB/c, SKG, and SKG Ptpn22<sup>−/−</sup> mice, and TCR-β CDR3 sequences were obtained by next generation sequencing. Linear regression analysis of the TCR V<sub>β</sub> repertoires between BALB/c and SKG or BALB/c and SKG Ptpn22<sup>−/−</sup> mice showed there were considerable differences in VJ usage, indicated by the poor r<sup>2</sup> values, 0.87 and 0.85, respectively, in comparison with that between SKG and SKG Ptpn22<sup>−/−</sup> T cells, which were highly

Table I. Comparison of thymocyte subpopulation numbers in BALB/c, SKG, and SKG Ptpn22<sup>−/−</sup> mice

| Genotype          | Total    | DN      | DP       | CD4 SP   | CD8 SP   |
|-------------------|----------|---------|----------|----------|----------|
| BALB/c (<i>n</i> = 9) | 125.2 ± 13.8 | 7.0 ± 2.7 | 98.1 ± 8.8 | 3.2 ± 1.0 | 1.9 ± 0.9 |
| SKG (<i>n</i> = 9)   | 113.3 ± 3.2  | 4.7 ± 1.5  | 102.5 ± 3.5 | 0.5 ± 0.1 | 0.06 ± 0.04 |
| SKG Ptpn22<sup>−/−</sup> (<i>n</i> = 12) | 112.1 ± 10.4 | 4.7 ± 3.0  | 100.8 ± 9.6 | 0.7 ± 0.3 | 0.1 ± 0.07 |

Thymocytes were obtained from 7-wk-old age-matched mice, and the absolute number within each subpopulation was calculated based on the expression of the markers CD4 and CD8 using the gating strategy shown in Fig. 1B.

DN, double negative.
alleles in gated mature CD4+CD5hi thymocytes (their response to TCR stimulation. Naive CD4+ T cells, gated as compensate for the reduced expression of ZAP70 and improve CD45RBhiCD25 gates. Arrows on the histogram indicate the time of addition of streptavidin–PE conjugates. The data are representative of three independent experiments. (B) Graphs show expression of TCR Vβ3, Vβ5, Vβ11, and Vβ8 alleles in gated mature CD4+CD5hi thymocytes (left) and peripheral CD4+ T cells (right) for the individual mouse strains, as indicated. The data are representative of three independent experiments and are shown as averages of n = 4 for each strain ± SD. Statistical significance was calculated by one-way ANOVA and post hoc Holm–Sidak test for multiple comparisons. **p < 0.0001.

Correlated, r² = 0.96 (Fig. 3A). More detailed analysis represented by heatmaps of V versus J segment usage showed that there were some subtle changes in VJ usage between the SKG and SKG Ptpn22−/− populations (Supplemental Fig. 1A). However, these differences were found to be due to a few VJ combinations that were represented at very low frequencies in SKG or BALB/c repertoires, and were slightly increased in SKG Ptpn22−/− mice (Supplemental Fig. 1B). It had been shown previously (22) that arthritogenic T cells in the SKG model were found among TCRs expressing Vβ6, Vβ8.1/8.2, and Vβ10 gene segments. We examined the representation of these specific Vβ alleles among the TCR sequence data and found these Vβs to be at least equally, if not slightly more represented in the absence of PTPN22 (Fig. 3B). Overall, the repertoires of SKG and SKG Ptpn22−/− naïve CD4 T cells were highly similar, and quite distinct from that of BALB/c mice, further confirming that loss of PTPN22 had little effect on the selection of the naïve SKG TCR repertoire.

We asked whether Ptpn22 deficiency in SKG T cells was able to compensate for the reduced expression of ZAP70 and improve their response to TCR stimulation. Naive CD4+ T cells, gated as CD45RBhiCD25−, were sorted from LNs of young (6-wk-old) BALB/c, SKG, and SKG Ptpn22−/− mice, and proliferation was measured following stimulation with anti-CD3 Ab and irradiated splenocytes from BALB/c Rag2−/− mice as APC. As previously reported, there was decreased proliferation of SKG CD4+ cells compared with that of WT BALB/c in response to all doses of anti-CD3 after 72 h of stimulation in vitro (Fig. 3C, 3D (11)). Although the proliferation of SKG Ptpn22−/− cells was reduced compared with BALB/c T cells, SKG Ptpn22−/− cells proliferated significantly more than SKG cells, particularly at lower concentration of anti-CD3, as indicated by the division index (Fig. 3C, 3D). In addition, we measured IL-2 cytokine production following stimulation. Although considerably less than produced by WT BALB/c T cells (compare different axes between SKG and BALB/c), SKG Ptpn22−/− cells produced more IL-2 than SKG T cells at all anti-CD3 concentrations (Fig. 3E).

Ptpn22 deficiency decreases the incidence and severity of arthritis in SKG mice

The frequency and clinical score of spontaneous arthritis in SKG mice are dependent on the cleanliness of the environment and microbial colonization. In our animal facilities, SKG mice up to the age of 6 mo did not develop any spontaneous arthritis or any systemic inflammation such as interstitial pneumonitis, as previously reported (23), and loss of PTPN22 did not make them any more susceptible to spontaneous disease up to ~8 mo of age.

It has been shown that autoimmune disease can be triggered by a single i.p. injection of fungal extracts, such as zymosan, a crude yeast wall extract (12), or mannan, a fungal wall polysaccharide (24). We were unable to elicit any signs of arthritis with the published doses of mannan (0.2, 2, and 20 mg) (25), possibly because, rather than being specific pathogen free (SPF), our mice are of specific opportunistic pathogen-free health status, which may impact on the generation of Th17 T cells (26, 27). Therefore, we used a higher dose of mannan of 50 mg/mouse to provoke disease. A single i.p. injection of 50 mg mannan triggered joint inflammation in both SKG and SKG Ptpn22−/− mice. Contrary to expectation, Ptpn22 deficiency delayed the onset and severity of arthritis in SKG mice. Moreover, the incidence of disease was decreased in SKG Ptpn22−/− mice with 3 of the 10 SKG Ptpn22−/− mice failing to develop any symptoms of arthritis, compared with only 1 of 10 SKG mice (Fig. 4A). Arthritic joints from mannan-treated SKG and SKG Ptpn22−/− mice with comparable arthritis scores showed similar levels of cellular infiltrates (data not shown); however, loss of PTPN22 decreased both the likelihood of getting disease and the overall severity of the resulting arthritis (Fig. 4B).

IL-6 is a key cytokine for the development of arthritogenic Th17 cells in SKG mice, and mannan stimulates IL-6 production by macrophages. We tested whether there was a difference in IL-6 production by SKG or SKG Ptpn22−/− peritoneal macrophages after exposure to a range of mannan concentrations, and found the response to be equivalent for both genotypes (Fig. 4C). It is unlikely, therefore, that differences in innate cell responses to
mannan were responsible for the resistance to arthritis in the SKG Ptpn22<sup>−/−</sup> background. Loss of PTPN22 does not affect Treg numbers or function on the SKG background. We and others (5, 28) have previously reported that Ptpn22<sup>−/−</sup> mice on the B6 background have an increase in Treg numbers. Furthermore, on a per cell basis, Ptpn22<sup>−/−</sup> Tregs were more potent than WT Tregs and suppressed colitis induced by Ptpn22<sup>−/−</sup> effector cells, which WT Tregs were unable to control (5). We examined whether increased Treg production or efficacy accounted for the reduced arthritis found in the SKG Ptpn22<sup>−/−</sup> mice. TCR signal strength is crucial in development of thymic Tregs (29), and the decrease in TCR signaling capacity in SKG mice results in a decrease in the proportion of thymic Tregs (19). Given that SKG Ptpn22<sup>−/−</sup> mice had slightly improved signaling in DP thymocytes (Fig. 2A), we asked whether they had any changes in proportions of thymic Tregs. Both the percentages and the absolute cell numbers of FoxP3<sup>+</sup> cells were similar in the thymuses of SKG Ptpn22<sup>−/−</sup> and SKG mice (Fig. 5A), indicating comparable efficiency of selection of Tregs between these strains. The overall numbers of thymic Tregs in SKG were significantly reduced compared with control BALB/c mice (Fig. 5A), as reported previously (19).
showed that the former had a small, but significant increase in the proportions of Tregs recovered from LN and spleen of mannan-treated SKG and SKG Ptpn22−/− mice (Fig. 5D). We noted a consistent, albeit not statistically significant, increase in the proportions of Tregs in the LN and spleen of SKG Ptpn22−/− compared with SKG mice. Furthermore, a significant increase in the proportions of Tregs in the mesenteric LN, a key site of inducible Treg induction, was seen in SKG Ptpn22−/− mice, suggesting that Ptpn22 deficiency might enhance the propensity of naive T cells to differentiate into this lineage (Fig. 5D).

We tested the ability of SKG and SKG Ptpn22−/− Tregs to suppress expansion of naive BALB/c T cells in vivo by cotransfer into empty Rag−−/− mice. In the absence of Tregs, the naive T cells expanded in the empty hosts, and the expansion was controlled to a similar extent by Tregs from either SKG or SKG Ptpn22−/− mice (Fig. 5E). Therefore, on the SKG background, PTPN22-deficient Tregs are not more suppressive than SKG Tregs, in contrast to our previous findings that Tregs from C57BL/6 mice with normal ZAP70 expression are more potent suppressor cells when PTPN22 is absent (5). Given this result, we conclude that, although there was a slight increase in Treg numbers when PTPN22 was absent, it is unclear that this increase in numbers alone would have sufficient impact to account for the resistance to arthritis in this model.

**Effector SKG Ptpn22−/− CD4 T cells have reduced propensity to polarize to the Th17 lineage**

On the B6 background, there is an expansion of Ptpn22−/− T effector/memory cells with age (5, 6). Perturbations in T cell homeostasis are linked to onset of autoimmunity and are seen with autoimmune-prone PTPN22R620W-expressing individuals (30), so we asked whether there was equivalent expansion of effector cells in SKG mice or whether the SKG phenotype might suppress PTPN22-mediated expansion and thus explain the reduced arthritis in SKG Ptpn22−/− mice. We analyzed the T cell phenotype of young (~6 wk of age) and older (~21 wk) at 6 wk and saw a small, but not statistically significant increase of effector cells (CD44hi CD62Llo) on the SKG background compared with SKG Ptpn22−/− mice. In older animals, SKG Ptpn22−/− mice showed a clear expansion of effector cells, which was significantly greater than that seen in SKG mice, which in turn were significantly expanded compared with BALB/c mice (Fig. 6A). Analysis of the cytokine-producing potential of CD4 T cells from these animals ex vivo showed that, compared with BALB/c cells, both SKG and SKG Ptpn22−/− CD4 T cells from LN had an increased, but similar capacity to produce IL-17, whereas the incidence of IFN-γ–producing cells was not significantly different between the strains (Fig. 6B). A higher incidence of IL-17–producing cells has been noted for SKG mice (25), although the percentage of spontaneous Th17+ cells is considerably lower in our animal facility than previously published, presumably reflecting different exposure to commensal microbiota.

Development of arthritis in SKG mice is dependent on IL-17–producing arthritogenic Th17 cells, which are promoted by IL-6 secreted predominantly by APCs (31). Deficiency of either IL-6 or IL-17 completely inhibits the arthritis, whereas lack of IFN-γ can exacerbate disease in SPF mice (31, 32). As SKG Ptpn22−/− mice developed less severe arthritis than SKG mice, we compared

Compared with BALB/c mice, numbers of T effector/memory cells and peripheral Tregs are increased in SKG mice, as the reduced generation of SP thymocytes results in a more lymphopenic peripheral environment (19). Comparison of Treg numbers in peripheral lymphoid organs of SKG Ptpn22−/− and SKG mice showed that the former had a small, but significant increase in the numbers of CD25+FoxP3+ Tregs in spleen of adult animals (Fig. 5B). This change was confined to the spleen, as LNs from these mice showed comparable numbers of Tregs irrespective of Ptpn22 status (Fig. 5C). We further assessed whether PTPN22 deficiency influenced Treg expansion upon mannan challenge. Compared with either untreated SKG or BALB/c control mice, increased proportions of Tregs were recovered from LNs and spleen in mannan-treated SKG and SKG Ptpn22−/− mice (Fig. 5D). We noted a consistent, albeit not statistically significant, increase in the proportions of Tregs in the LN and spleen of SKG Ptpn22−/− compared with SKG mice. Furthermore, a significant increase in the proportions of Tregs in the mesenteric LN, a key site of inducible Treg induction, was seen in SKG Ptpn22−/− mice, suggesting that Ptpn22 deficiency might enhance the propensity of naive T cells to differentiate into this lineage (Fig. 5D).

FIGURE 4. Milder mannan-induced arthritis in SKG Ptpn22−/− mice. (A) Incidence of arthritis and (B) joint scores from 11- to 12-wk-old female SKG and SKG Ptpn22−/− mice that received 50 mg mannan i.p. Data representative of two independent experiments. Error bars are mean ± SD of duplicate samples from two biological replicates, and represent one of two independent experiments.

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cytokine production in peripheral CD4+ T cells in these animals following injection of mannan and induction of arthritis. Peripheral and mesenteric LNs were isolated separately from mice 14 d after mannan injection and stained for cytokine production after a brief stimulation with PDBu and ionomycin. Production of key cytokines IL-17 and TNF-α (Fig. 7A, 7B) was significantly reduced in SKG Ptpn22−/− mice following arthritis induction compared with SKG mice. IL-17–producing cells from both mouse strains were CD4+, αβ-TCR+, and CCR6+, indicating they were conventional Th17 cells (data not shown). There was a tendency of SKG Ptpn22−/− mice to produce more IFN-γ–secreting cells (Fig. 7C); however, this was significant only in the

**FIGURE 5.** Treg numbers are similar in SKG and SKG Ptpn22−/− mice. (A) Representative FACS plots and total cell numbers of Tregs present in the thymus of 7-wk-old mice show no difference between SKG and SKG Ptpn22−/− mice. (B) Older mice, >21 wk old, on the SKG background show expansion of Tregs in the spleen compared with BALB/c mice, and there was a statistically significant increase in absolute numbers of Tregs between SKG and SKG Ptpn22−/− mice. (C) In contrast, LN Treg proportions and numbers are equivalent in SKG and SKG Ptpn22−/− mice, although raised in comparison with BALB/c mice. Each symbol in (A-C) represents an individual of six to nine mice total, and bars represent SD. Statistical significance was calculated using a Kruskal–Wallis test with Dunn’s posttest, adjusted for multiple comparisons. (D) Proportions of Tregs were expanded to a similar extent in SKG, and SKG Ptpn22−/− mice 3 wk following mannan treatment, with only MLN, showed a slight but significant increase of Tregs in the absence of PTPN22. Each symbol represents an individual of six mice total per genotype (+SD) from two independent experiments. (E) In vivo Treg suppression assay. WT sorted BALB/c naive CD90.1+CD4+ cells (4 × 10⁵) were injected alone or together with SKG or SKG Ptpn22−/− CD45.2+CD4+CD25+ Tregs (2 × 10⁵) into BALB/c Rag2−/− recipients (n = 6 mice per group). After 12 d, recovery of CD90.1+Foxp3+ T cells was determined. Each symbol represents individual mouse. Bars represent SD. Statistical significance was calculated using one-way ANOVA and post hoc Tukey’s test for multiple comparisons. *p < 0.05, **p < 0.005, ***p < 0.0005.
mesenteric LNs. To ascertain whether the reduced tendency of cells from SKG Ptpn22<sup>−/−</sup> to differentiate to the IL-17 lineage was cell intrinsic or a consequence of the cytokine milieu, we differentiated purified naive CD4<sup>+</sup> T cells from SKG and SKG Ptpn22<sup>−/−</sup> mice in vitro in the presence of increasing concentrations of IL-6 together with TGF-β. Significantly fewer IL-17–producing cells were generated from SKG Ptpn22<sup>−/−</sup>CD4 T cells than from SKG mice (Fig. 7D, 7E), suggesting that loss of PTPN22 produces an inherent bias against polarization to the IL-17 lineage that may contribute to the reduction in susceptibility to arthritis observed in this strain of mice.

**Discussion**

In this study, we examined the influence of the phosphatase PTPN22 on the development of autoimmune disease in combination with an established model of autoimmune arthritis, the SKG mouse. The SKG mouse develops arthritis as a consequence of having a reduction in functional ZAP70 protein, and given that ZAP70 is both a direct target of PTPN22 and a target of the Src-family kinase, Lck, whose activity is regulated by PTPN22, we anticipated that the combined mutations might exacerbate development of disease. On the contrary, we found that SKG mice lacking PTPN22 had both lower incidence and decreased severity of arthritis following induction of disease by administration of mannan.

Susceptibility to autoimmune diseases involves complex interactions between genetics and environmental factors. The major genetic contribution to diseases such as arthritis is the MHC (33), but recent genome-wide association studies have identified a number of other contributing genes, the most predominant of which is PTPN22 (10). PTPN22 is present in all hematopoietic cells (34), and the major disease-associated polymorphism, PTPN22-C1858T, has been shown to affect myeloid and B cell, as well as T cell, function (2). The SKG arthritic mouse is a good model for the human RA, as joint inflammation and damage mirror that found in patients (24). Similar to humans, arthritic disease in SKG mice is triggered by environmental factors. Sakaguchi and colleagues (31) showed that subclinical fungal infections drove the inflammatory signals that instigated spontaneous arthritis, an effect that was recapitulated by direct administration of fungal products, such as zymosan or mann, to SPF-bred SKG mice. These fungal products induce IL-6 production, which in turn drives differentiation of autoreactive T cells to the Th17 lineage. In the context of a more self-reactive TCR repertoire, which differentiates in the presence of the mutant ZAP70 alleles (11), SKG Th17 CD4 T cells migrate to the joints causing the characteristic tissue damage.

The PTPN22<sup>C1858T</sup> mutation in human cells of the myeloid lineage has been shown to interfere with upregulation of type 1 IFNs after TLR4 stimulation by LPS, and mouse Ptpn22<sup>−/−</sup> bone
marrow–derived macrophages showed a similar defect (35, 36). This reduction in type I IFNs may impede their tissue-protective effects in the presence of inflammatory cytokines and thus contribute to autoimmune disease susceptibility in humans. In contrast, production of proinflammatory cytokines, TNF-α and IL-6, was shown to be equivalent to WT cells following TLR4 stimulation for either mouse *Ptpn22*+/− or human *PTPN22*<sup>C<sup>1858T</sup></sup> myeloid cells (36). Given the central role of macrophage-derived IL-6 in driving autoimmune disease in SKG mice (25), we confirmed that SKG *Ptpn22*+/− mice were activated with plate-bound anti-CD3 and anti-CD28 in the presence of TGF-β, IL-1β, IL-23, and varying amounts of IL-6 for 3 d, and then restimulated with PDBu/ionomycin for 4 h in presence of brefeldin A. (D) Quantitation of IL-17+ cells after culture with varying doses of IL-6, and (E) FACS dot plots are shown for IL-17 and IFN-γ expression with 20 ng/ml IL-6. Statistical significance calculated with two-way ANOVA; *p < 0.05, **p < 0.005, ***p < 0.0005.

Peripheral tolerance is maintained in vivo by keeping an appropriate balance between self-reactive T cells and Tregs (39), and *Ptpn22* deficiency has been shown to be protective in NOD and experimental autoimmune encephalomyelitis mice model of disease due to increased Treg numbers (28, 40). Surprisingly, however, with the exception of spleen, we did not see a consistent expansion of Tregs in lymphoid tissue of SKG *Ptpn22*+/− older mice beyond that seen in SKG mice, despite the former having almost twice as many CD44+ effector T cells, nor did we find any increased potency in suppressive ability between Tregs from SKG and SKG *Ptpn22*+/− mice, which are further expanded after injection of zymosan. In contrast to the SKG *SLAP<sup>−/−</sup>* mutant mice, where there was ∼300-fold increase in Treg numbers, the increase of Tregs in SKG *Ptpn22*+/− mice was only ∼20% above that found in SKG mice and not observed consistently in all lymphoid organs.

Although the SKG mutation is a robust predisposition allele for development of arthritis following innate stimulation, it is...
clear that in this model the tipping point between generation of pathogenic T cells and maintenance of tolerance is finely balanced. To date any modulation of precisely SKG levels of ZAP70 function has tended to reduce susceptibility to arthritis induction. As described above, combining SKG with SLAP knockout diminished arthritis by improving Treg numbers (41). In another model in which ZAP70 function is compromised, the knock-in mouse ZAP70YYAA in which Tyr residues 315 and 319 are substituted with Ala, ZAP70 function was also considerably impaired compared with WT ZAP70 (20). ZAP70YYAA generated rheumatoid factor following zymosan injection, but the mice were very resistant to the development of arthritis. Ca2+ flux in DP thymocytes and responses of peripheral ZAP70YYAA T cells were intermediate between SKG and WT, similar to the levels we reported in this work with SKG Ptpn22−/− mutant T cells. Yet ZAP70YYAA thymocytes showed improved deletion of Mtv-reactive TCR Vβ sequences for selection events in the thymus, and yet significantly improved the activity of mutant ZAP70skg/skg, this had little conduction of RA, and similar mechanisms may contribute to human which, when combined with infection by pathogens, leads to in-...
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