Genetic evidence for the role of plasmacytoid dendritic cells in systemic lupus erythematosus

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Systemic lupus erythematosus (SLE) is an autoimmune disorder characterized by the production of antibodies to self-nucleic acids, immune complex deposition, and tissue inflammation such as glomerulonephritis. Innate recognition of self-DNA and -RNA and the ensuing production of cytokines such as type I interferons (IFNs) contribute to SLE development. Plasmacytoid dendritic cells (pDCs) have been proposed as a source of pathogenic IFN in SLE; however, their net contribution to the disease remains unclear. We addressed this question by reducing gene dosage of the pDC-specific transcription factor E2-2 (Tcf4), which causes a specific impairment of pDC function in otherwise normal animals. We report that global or DC-specific Tcf4 haplodeficiency ameliorated SLE-like disease caused by the overexpression of the endosomal RNA sensor Tlr7. Furthermore, Tcf4 haplodeficiency in the B6.Sle1.Sle3 multigenic model of SLE nearly abolished key disease manifestations including anti-DNA antibody production and glomerulonephritis. Tcf4–haplodeficient SLE-prone animals showed a reduction of the spontaneous germinal center reaction and its associated gene expression signature. These results provide genetic evidence that pDCs are critically involved in SLE pathogenesis and autoantibody production, confirming their potential utility as therapeutic targets in the disease.
IFN-α receptor (IFNAR) ameliorates experimental SLE in the Tlr7-overexpressing model (Buechler et al., 2013) as well as in NZB/NZW-derived strains (Santiago-Raber et al., 2003; Agrawal et al., 2009). Conversely, IFN overexpression strongly exacerbates experimental SLE (Liu and Davidson, 2013). Hence, the blockade of IFN signaling (for example, using antibodies to IFN or IFNAR) represents a potential therapeutic approach to SLE (Bronson et al., 2012).

Plasmacytoid DCs (pDCs) are a distinct lineage of DCs specialized in IFN production in response to viral nucleic acids sensed through TLR7 and TLR9 (Gilliet et al., 2008; Reizis et al., 2011). In addition to virus-derived DNA and RNA, pDCs can be activated by self-nucleic acids complexed with antibodies (Bäve et al., 2003) or DNA/RNA-binding proteins such as HMGB1 (Tian et al., 2007). In particular, DNA complexes released from activated neutrophils induce pDCs to secrete IFN, which fuels the “vicious circle” of myeloid cell activation in SLE patients (Garcia-Romo et al., 2011; Lande et al., 2011). TLR-activated pDCs become resistant to glucocorticoids, underlying the limited efficacy of these drugs in SLE (Guiducci et al., 2010a). Therefore, pDCs have been proposed as a key source of aberrant IFN production and a major driver of SLE progression (Rönnblom and Alm, 2001). In experimental SLE, minor signs of pDC activation have been described in the Tlr7 transgenic model (Buechler et al., 2013), and antibody-mediated pDC ablation prevented trauma-induced skin inflammation in the (NZBxNZW)F1 model (Guiducci et al., 2010b). However, the precise role and significance of pDC function in SLE remains moot, largely because models for specific, long-term pDC ablation have not been available.

We have previously identified the transcription factor E2-2 (official symbol Tcf4) as a specific regulator of pDC development in mice and in humans (Cisse et al., 2008). Tcf4 is expressed in pDCs but not in classical DCs (cDCs), and its deletion abolishes the development of pDCs but not of cDCs or other immune cell types. Importantly, even monoallelic loss of Tcf4 causes specific impairment of pDC function in mice and human patients. For example, Tcf4<sup>−/−</sup> mice fail to produce IFN in response to the TLR9 ligand CpG but show normal IFN induction by the TLR3/RIG-I ligand poly-I:C and normal T cell–dependent antibody responses (Cisse et al., 2008). Thus, Tcf4 haploinsufficiency represents a specific tool for constitutive functional blockade of pDCs. In this study, we applied this tool to determine the role of pDCs in two distinct genetic models of SLE.

RESULTS AND DISCUSSION

Tcf4 haploinsufficiency ameliorates SLE caused by Tlr7 overexpression

To validate Tcf4 haploinsufficiency as a pDC-specific tool, we first used a model of SLE induced by the administration of saturated hydrocarbon tetramethylpentadecane (pristane). This model is characterized by autoreactivity to small ribonucleoproteins (anti-Smith antigen, anti-Sm), which is dependent on TLR7-induced IFN production by inflammatory monocytes rather than by pDCs (Lee et al., 2008). We found that the expression of IFN-inducible genes and anti-Sm antibody production were similar in pristane-treated WT and Tcf4<sup>−/−</sup> animals (unpublished data). Thus, Tcf4 haploinsufficiency does not generally affect autoantibody production that is not dependent on pDCs.

Next, we used a monogenic SLE model based on multiple transgenic copies of the Tlr7 locus. These Tlr7.Tg animals develop an SLE-like disease characterized by anti-RNA antibody production, expansion of monocyte-like myeloid cells in the blood, massive immune activation, and glomerulonephritis (Deane et al., 2007). We have used a line of Tlr7.Tg animals with moderate Tlr7 mRNA overexpression (4–8-fold) and the transgene integrated on the Y-chromosome. On the hybrid B6129F1 background, nearly all Tlr7.Tg males became

![Figure 1. Tcf4 haploinsufficiency ameliorates SLE-like disease in Tlr7 transgenic mice. Tlr7.Tg males, haploinsufficient for Tcf4 (Tg/het) or their Tcf4-sufficient littermates (Tg), were analyzed. (A) Kaplan–Meier survival plot (n = 7–10). Significance was determined by log-rank test. (B) Splenic size (left) and splenic weights were determined for individual 50–60-wk-old transgenic animals and WT controls (right). (C) Peripheral blood cells from the indicated mice were analyzed by flow cytometry (left); thresholds of positive staining and percentages of cells within the resulting quadrants are indicated. The frequencies of the CD11c<sup>+</sup> MHC cl. II<sup>+</sup> population (top left quadrant) in individual 30–60-wk-old animals were determined (right). Data were pooled from 3 independent experiments. (D) Anti-RNA IgG levels in the sera of 30–40-wk-old animals were determined by ELISA. Data were pooled from 3 independent experiments. (E) Kidney cryosections of 50–60-wk-old animals were stained for IgG (red) and DNA (blue) and analyzed by fluorescence microscopy (bars, 100 µm). Arrows show kidney glomeruli. Shown is a representative of 5 animals in each group from 2 independent experiments. Horizontal bars indicate mean. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.](image)
Tcf4 haploinsufficiency in DCs ameliorates Tlr7-induced SLE

Although Tcf4 deficiency primarily affects pDCs, Tcf4 is also expressed at low levels in B cells, as well as in nonimmune tissues including the brain. To confirm that the effect of Tcf4 haploinsufficiency in Tlr7.Tg mice originates in pDCs, we combined a single conditional (floxed) allele of Tcf4 with the Itgax-Cre deleter strain. This strain (also known as CD11c-Cre) mediates Cre recombination specifically in the DC lineage in tissues including cDCs and pDCs (Caton et al., 2007). However, Tcf4 is not expressed in cDCs; therefore, any consequences of Itgax-Cre-mediated deletion of Tcf4 would reflect its function in cells within the DC lineage, which correspond to pDCs.

Because Tlr7.Tg animals on pure B6 background survive for >1yr, differential survival could not be assessed in this experiment. Nevertheless, similar to the germline Tcf4 haploinsufficiency, DC-specific Tcf4 haploinsufficiency significantly reduced splenomegaly (Fig. 2 A) and completely abolished myeloid cell expansion (Fig. 2 B). It also significantly reduced the fraction of activated T cells, although the latter was still increased relative to WT (Fig. 2 C). Control Tlr7. Tg animals at 30–40 wk showed increased levels of total serum IgG and IgM (Fig. 2 D), the presence of anti-RNA IgG (Fig. 2 E), and the associated autoreactivity to cytoplasmic antigens (Fig. 2 F). In contrast, Tlr7.Tg CKO animals had normal Ig levels and no significant anti-RNA autoreactivity (Fig. 2, D–F). This was accompanied by reduced IgG deposition in the kidneys of CKO mice (Fig. 2 G). Thus, Tcf4 haploinsufficiency in the DC lineage reduces autoreactivity and immune activation in Tlr7.Tg animals, similar to the germline Tcf4 haploinsufficiency. These data confirm that the effect is intrinsic to Tcf4-expressing cells within the DC lineage, which correspond to pDCs.
Tcf4 haplodeficiency ameliorates SLE in B6.Sle1.Sle3 animals

Although Tlr7.Tg animals develop robust SLE-like disease, this monogenic model contrasts with the complex polygenic nature of human SLE. Furthermore, Tlr7-driven SLE does not recapitulate several important features of the human disease, including the production of antibodies against dsDNA (anti-dsDNA) and prominent glomerulonephritis. We therefore turned to a polygenic model that harbors large genomic intervals from the NZW strain (Sle1, Sle2, and Sle3) on B6 background (Morel et al., 2000). Notably, the homozygosity for only two intervals, Sle1 and Sle3, is sufficient to cause full-blown SLE with nearly complete penetrance (Morel et al., 2000). We therefore analyzed animals on B6 background that were homozygous for Sle1 and Sle3, heterozygous for Sle2, and either haplosufficient or haplodeficient for Tcf4.

The spleens of B6.Sle1.Sle3 Tcf4+/- animals at 30 wk were significantly smaller than in Tcf4+/- littermates, although still enlarged relative to WT (Fig. 3 A). Peripheral blood leukocytes of B6.Sle1.Sle3 mice showed no myeloid cell expansion or other major changes except for a slight decrease of B cell fraction (unpublished data). In contrast, the observed increase in the fraction of activated T cells was abolished in Tcf4+/- animals (Fig. 4 B). Peripheral blood leukocytes in B6.Sle1.Sle3 animals showed increased expression of Sca-1, an IFN-inducible gene whose induction on B and T cells is associated with persistent IFN signaling (Lee et al., 2008). In Tcf4+/- animals, the levels of Sca-1 on B and T cells were significantly reduced to normal or near-normal levels, respectively (Fig. 3 C). Similar changes in T cell activation and Sca-1 expression were observed in the spleens of experimental animals (unpublished data).

B6.Sle1.Sle3 mice showed increased levels of total serum IgG (particularly IgG1) and IgM, which were reduced to normal levels by Tcf4 haplodeficiency (Fig. 3 D). The majority (9 out of 11) of control B6.Sle1.Sle3 mice showed high titers of anti-dsDNA IgG and 5 out of 9 showed anti-RNA IgG (Fig. 3 E). Both the frequency and titers of these autoantibodies were significantly reduced in Tcf4+/- animals (Fig. 3 E), accompanied by the loss of anti-nuclear IgG reactivity (Fig. 3 F) and reduced IgG deposition in the kidneys (Fig. 3 G). We conclude that Tcf4 haplodeficiency greatly reduced autoimmune production and immune activation in the B6.Sle1.Sle3 model of SLE.

Tcf4 haplodeficiency ameliorates kidney inflammation in SLE models

Because glomerulonephritis is a key inflammatory manifestation and morbidity cause in SLE, we analyzed the effect of Tcf4 haplodeficiency on kidney pathology in both experimental models. Control Th7.Tg animals at 50 wk manifested a relatively mild kidney inflammation with minimal glomerular deposits (Fig. 4 A and not depicted). Nevertheless, Th7.Tg KO animals showed significant reductions in mean glomerular size (Fig. 4 B), extent of endocapillary proliferation and leukocyte infiltration, and median cumulative score of the disease (Fig. 4 C). Control B6.Sle1.Sle3 animals at 30 wk manifested prominent glomerulonephritis with interstitial inflammation, leukocyte infiltration, and glomerular deposits. In contrast, Tcf4+/- B6.Sle1.Sle3 littermates
Tcf4 haplodeficiency reduces the germinal center (GC) signature of SLE

To explore the molecular correlates of SLE amelioration by Tcf4 haplodeficiency, we performed microarray expression analysis of total splenocytes from WT, B6.Sle1.Sle3 (Sle), and B6.Sle1.Sle3 Tcf4+/− (Sle/het) animals. Hierarchical clustering showed that Sle and Sle/het samples were highly similar yet distinct from the WT (Fig. 5 A). Indeed, principal component analysis (PCA) identified two major clusters of genes that were coordinately up- or down-regulated in both Sle samples compared with WT (Fig. 5 B and Dataset S1). Genes that were up-regulated in both Sle and Sle/het splenocytes (Fig. 5 B, cluster 1b) included proliferation-associated genes and granulocyte-specific genes, as well as genes associated with immune activation such as high mobility group proteins and defensins (Dataset S1). Consistent with previous observations in the B6.Sle1.Sle2.Sle3 mice (Sriram et al., 2012), IFN-inducible genes were not significantly up-regulated in either Sle sample. Notably, PCA identified a distinct cluster of 49 genes that were up-regulated in Sle but not in Sle/het splenocytes (Fig. 5 B, cluster 2a). This cluster included several genes that are highly specific for GC B cells, including the Aida gene encoding activation-induced cytidine deaminase (AID) (Dataset S1). Also present were several genes expressed in plasma cells (e.g., Xbp1) and in follicular helper T cells (Il21 and Pdcd1). The reduction of Aida overexpression in splenocytes from Tcf4-haplodeficient animals was confirmed by qRT-PCR for both B6.Sle1.Sle3 and Tlr7.Tg models (Fig. 5 C). Furthermore, immunofluorescent staining revealed massive GC reaction in the spleens of B6.Sle1.Sle3 mice that was virtually abolished in Tcf4-haplodeficient littermates (Fig. 5 D). We conclude that Tcf4 haplodeficiency does not generally affect the SLE-associated gene expression profile, but specifically reduces the GC reaction and the associated expression signature.

A recent work used germline deletion of the transcription factor Irf8 or proton-coupled oligopeptide transporter Slc15a4 to test the role of pDCs in NZB and B6. Fas<sup>mod</sup> models of SLE, respectively (Baccala et al., 2013). However, Irf8 deletion affects the development and/or function of multiple immune cell types (Wang and Morse, 2009), whereas Slc15a4 regulates cytokine responses to Tlr9 and NOD-like receptor ligands in both pDCs and cDCs (Sasawatari et al., 2011). Here, we used haplodeficiency for Tcf4, a specific regulator of pDC development, to target pDC function in experimental SLE. In the case of the Tlr7.Tg model, we confirmed that the effect was intrinsic to the DC lineage, in which only pDCs express Tcf4. Although a minor subset of noncanonical CD8+ cDCs is depleted by full Tcf4 deficiency (Bar-On et al., 2010), it is unaffected by reduced gene dosage of Tcf4 (our unpublished data). Furthermore, Tcf4 haplodeficiency did not affect autoreactivity in the pDC-independent model of pristane-induced SLE. Therefore, our approach specifically interrogates the net contribution of the pDC lineage to experimental SLE.

We found that Tcf4 haplodeficiency significantly ameliorated Sle manifestations in both Tlr7.Tg transgenic model...
and B6.Sle1.Sle3 multigenic model. Unlike human SLE patients, these and other mouse SLE models do not manifest a prominent IFN signature (Perry et al., 2011); hence, we could not detect a consistent reduction of IFN-inducible genes. It is therefore likely that the role of pDCs in experimental SLE and possibly in human SLE patients goes beyond IFN production. Indeed, a recent study in human systemic sclerosis revealed an important role for chemokine production by pDCs (van Bon et al., 2014). In that respect, it is notable that Tif4 haplodeficiency profoundly decreased autoantibody production, GC reaction, and the ensuing glomerulonephritis in both SLE models. These data suggest a prominent IFN signature (Perry et al., 2011); hence, we consider that pDCs might contribute to SLE predisposition in part through their activity in the pDC lineage. In addition, our results highlight the beneficial effect of functional pDC impairment on SLE pathogenesis, providing the rationale for pDC-targeted therapeutic approaches. Indeed, pDC ablation or functional impairment would be less immunosuppressive than a global blockade of IFN signaling, and would target multiple pDC functions such as secretion of chemokines. Collectively, our data suggest the targeting of the pDC lineage (e.g., by depleting antibodies) as a viable therapeutic strategy to ameliorate SLE.

**MATERIALS AND METHODS**

**Animals.** All experiments were performed according to the investigator’s protocol approved by the Institutional Animal Care and Use Committee of Columbia University. Tif4+/− animals (Zhuang et al., 1996) were on pure 129SveTac background (>N12); all other animals were on pure C57BL/6 (B6) background (>N12). Pristane was administered i.p. into Tif4+/− animals or Tif4+/− littermates as previously described (Lee et al., 2008). Th7.Tg males (Y−linked transgenic line 7.6; Deane et al., 2007) were crossed with Tif4+/− females to generate B6129F1 Tif4+/− Th7.Tg males or Tif4+/− Th7.Tg littermates. Age-matched B6129F1 males bred in the same colony were used as WT controls. For conditional targeting of Tcf4, Th7.Tg males carrying the Igαx-Cre transgene (Caton et al., 2007) were crossed with Tif4h+/−.
females (Bergqvist et al., 2000) to generate TgfβRS+/− Th7.Tg mice with or without the Cre transgene. Age-matched B6 mice bred in the same colony were used as WT controls.

For the cross with B6.Sle1.Sle3 strain, a new null allele of Tgfβ4 was derived by recombining the Tgfβ4−/− allele in the female germ line using the Tek-Cre transgenic strain (The Jackson Laboratory). After crossing out the Cre transgene, Tgfβ4−/− animals were crossed to BcN.LmoJ animals homozygous for Sle1, Sle2, and Sle3 loci (Morel et al., 2000; The Jackson Laboratory). The resulting Tgfβ4−/− animals heterozygous for Sle1, Sle2, and Sle3 were backcrossed to BcN.LmoJ to achieve homozygosity for Sle1 and Sle3. The integrity of Sle loci was confirmed by PCR genotyping for microsatellite markers spanning each locus. No differences in any SLE manifestations have been observed between male and female B6.Sle1.Sle3 animals; thus, both male and female mice were included in the analyses along with age-matched B6 WT controls.

Flow cytometry. Suspensions of peripheral blood leukocytes or splenocytes were subjected to red blood cell lysis, washed, and stained with directly conjugated fluorescent antibodies to the indicated surface markers (eBioscience). The samples were acquired on the LSR II flow cytometer (BD) and analyzed using FlowJo software (Tree Star).

Autoantibody measurement. Immunoglobulin levels in the serum were determined by ELISA using alkaline phosphatase–conjugated antibodies to IgM, IgG, and IgG isotypes (SouthernBiotech). Anti–RNA and anti–dsDNA IgG concentrations were determined by ELISA as previously described (Blanco et al., 1991) using yeast RNA or calf thymus DNA as antigens.

Histopathology. 2-µm sections of formalin-fixed kidneys were stained with H&E and evaluated by a pathologist (V. D’Agati) who was blinded to sample identity. Mesangial and endocapillary proliferation, leukocyte infiltration, glomerular deposits, and apoptosis were scored separately on a scale from 0 (none) to 4 (highest) and added to yield a cumulative score. The percentage of cortical parenchyma with interstitial inflammation was also determined. To measure immune complex deposition, kidneys were fixed in 4% paraformaldehyde, dehydrated in 30% sucrose, and frozen in OCT (TissueTek). Frozen sections (5 µm) were stained with DAPI and PE-labeled anti–mouse IgG and DAPI. Images were captured on a confocal fluorescent microscope (LSM 710 NLO) and processed by Zen software (Carl Zeiss).

Gene expression analysis. Total splenocytes from individual WT B6 (n = 2), B6.Sle1.Sle3 (n = 3), or B6.Sle1.Sle3.Tgfβ4−/− (n = 3) animals at 30 wk were used for microarray analysis. Total RNA was isolated, reverse transcribed, amplified, labeled, and hybridized to Mouse Genome 1.0 ST arrays (Affymetrix). The results were gcRMA-normalized by the manufacturer’s software and analyzed using the NIA Array software (Sharov et al., 2005). The qRT-PCR analysis of total splenocytes from individual mice was performed as previously described (Cisse et al., 2008; Ghosh et al., 2010).

Statistics. Unless noted otherwise, significance was estimated by nonparametric Mann–Whitney test.

Accession nos. Microarray data have been deposited in the Gene Expression Omnibus (GEO) database under the accession no. GSE57324.

Online supplemental material. Dataset S1 shows genome-wide expression analysis of splenocytes from B6.Sle1.Sle3 mice with or without Tgfβ4 haplodeficiency. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20132522/DC1.

We thank Dr. Nicole Heise for reagents and advice.

This research was supported by the National Institutes of Health grant AI072571 and the Lupus Research Institute (B. Reizis), the S.L.E. Lupus Research Foundation (D. Gangul), and the Cancer Research Institute (V. Sirirak). The authors declare no competing financial interests.

Submitted: 5 December 2013
Accepted: 10 July 2014

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