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INSIGHTS

Finding NEMO in the thymus

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Rosain et al. (https://doi.org/10.1084/jem.20231152) describe the association between anti-type I interferon autoantibodies and severe viral infections in patients with incontinentia pigmenti and heterozygous loss-of-function *NEMO* variants, suggesting a role for canonical NF-κB signaling in immune tolerance. The mechanisms behind this selective autoimmunity remain unclear.

The thymus is a lymphoid organ where immature T lymphocytes, named thymocytes, are trained to discriminate self from non-self, preventing autoimmunity while generating a diverse T lymphocyte repertoire (Kreins et al., 2021b). Thymocyte instruction follows an intricate spatiotemporal sequence, guided mainly by thymic epithelial cells (TEC), which are specialized antigen-presenting cells (APC). TEC in the cortex of the thymus (cortical TEC, or cTEC) regulate the positive selection of thymocytes expressing T cell receptors (TCR) able to interact with human leukocyte antigen peptide (pHLA) complexes after successful VDJ gene rearrangement, a process mediated by recombination-activating genes (RAG). In the medulla, developing thymocytes undergo further scrutiny with the elimination of thymocytes that have a high affinity for self-pHLA. This negative selection process, mediated by medullary TEC (mTEC) and other APC, is accomplished by promiscuous expression of tissue-restricted antigens (TRA) under the control of transcriptional regulators, such as primarily the autoimmune regulator (AIRE). Rather than being eliminated, thymocytes with an intermediate affinity for self-peptides can additionally be redirected toward T regulatory lymphocyte (Treg) development. Those thymocytes that escape clonal deletion and diversion, egress into the periphery as self-tolerant, naïve T lymphocytes. These steps are critical for the induction of immune tolerance and the prevention of autoimmunity.

The thymus involutes with age, and loss of immune tolerance is a feature of immune senescence. This is associated with dramatic changes in the lympho-stromal compartments, including adipogenesis following an epithelial-to-mesenchymal transition (EMT) of TEC (Kousa et al., 2024). Using single-cell omics, age-associated changes in the TEC subsets have recently been described more comprehensively in mice and, to a lesser extent, in humans (Kousa et al., 2024; Yayon et al., 2023, *Preprint*). How these changes contribute to progressive aberrations in thymic function remains poorly understood, but the incidence of cancer and autoimmune diseases significantly increases over time (Kooshesh et al., 2023). Most autoimmune disorders, whether systemic or not, are typically associated with autoantibodies. The COVID-19 pandemic revealed that with age the higher prevalence of autoantibodies neutralizing type I interferons (AAN-I-IFN), found in ∼5% of the elderly population, lead to more severe disease due to SARS-CoV-2 and other viruses (Bastard et al., 2024). Previously, these autoantibodies were known to be pathognomic, but considered clinically silent, in a rare inborn error of immunity (IEI) called autoimmune polyglandular syndrome type 1 (APS-1). APS-1 is caused by biallelic

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disease variants in *AIRE*, enabling autoreactive T lymphocytes to evade mTEC-orchestrated negative selection and leading to autoimmunity against multiple organs (Kreins et al., 2021b). AAN-I-IFN have since been associated with severe viral diseases in APS-1 and in a number of other rare IEI, in the context of more or less broad autoimmune manifestations (Table 1) (Bastard et al., 2024). Specifically, they have been reported in patients with IEI of the non-canonical NF-κB signaling pathway (Le Voyer et al., 2023, 2024) (see below) and in Treg-deficient patients with X-linked FOXP3 deficiency, as well as patients with partial RAG deficiencies (Bastard et al., 2024). Overall, the discovery of AAN-I-IFN in aging populations and in these IEI raises the hypothesis that their presence is related to a breakdown of central thymic tolerance secondary to senescence of thymic stromal

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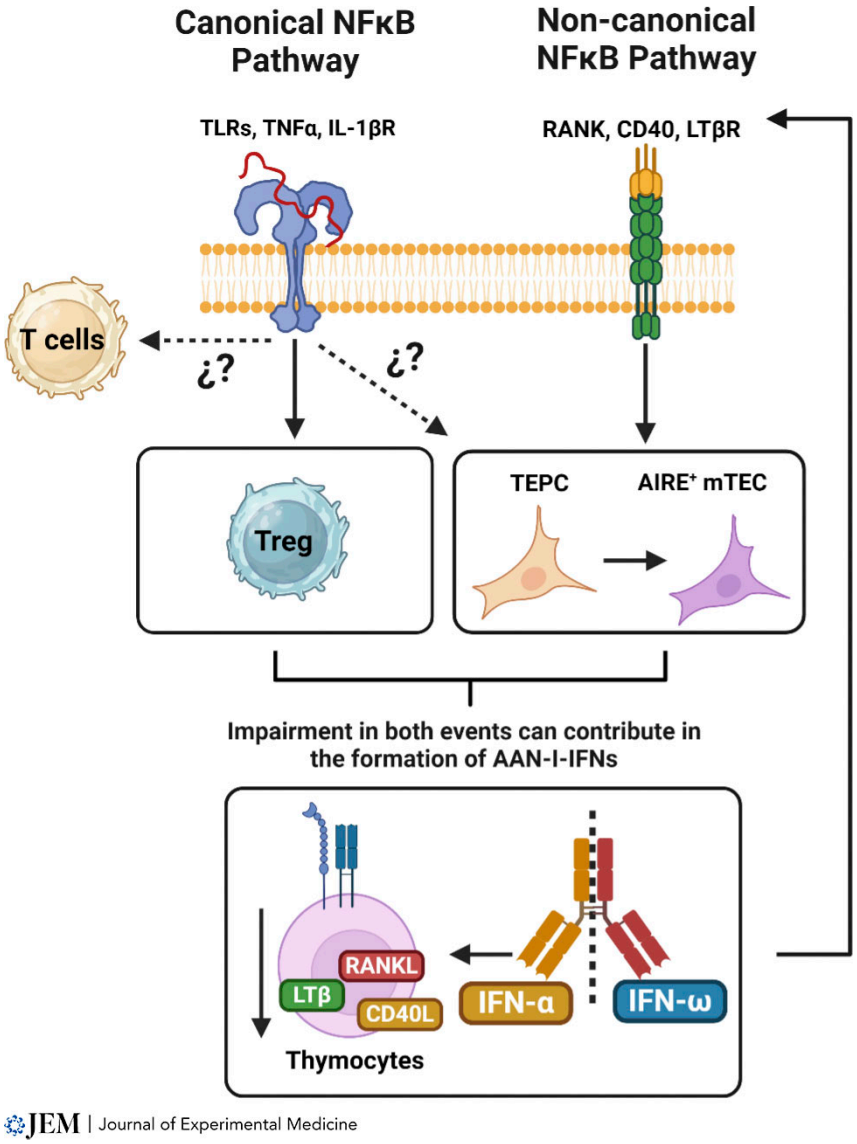

AAN-I-IFN production is linked to AIRE⁺ mTEC dysfunction. The differentiation of TEPC into AIRE⁺ mTEC relies on the non-canonical NF-κB pathway. Defects in this pathway have been associated with AAN-I-IFN. Rosain et al. identified AAN-I-IFN in IP patients. The canonical NF-κB pathway is crucial for Treg production, but a TSC defect in IP has not been ruled out. Additionally, the AAN-I-IFN could further disrupt AAN-I-IFN thymocyte development by altering intrathymic interferon signaling, reducing RANKL, LTβ, and CD40L production and leading to a secondary AIRE⁺ mTEC differentiation defect with thymic dystrophy.

cells (TSC), including TEC, or in a developing T lymphocyte–intrinsic manner (Bastard et al., 2024).

Rosain et al. (2024) now report the presence of AAN-I-IFN in female patients with incontinentia pigmenti (IP), an X-linked dominant genodermatosis. It is most frequently caused by a loss-of-function deletion of exons 4–10 of the inhibitor of nuclear factor κ B kinase regulatory subunit γ (*IKBKG* or *NEMO*) gene. NEMO is part of a regulatory kinase complex (IKK) that controls rapid NF-κB activation and nuclear translocation of NF-κB dimers in its canonical signaling pathway mediated by transcription factors NFKB1 (p105/p50) and RelA (p65) or c-Rel (Maubach et al., 2017) (see figure). IP is characterized by typical skin, eye, and central nervous system anomalies, with variable clinical penetrance. Whilst immunodeficiency and autoimmunity were not known features, Rosain et al. found AAN-I-IFN in about one-third of a large cohort of 131 IP patients, predisposing them to severe viral infections. These autoantibodies appeared between the ages of 5 and 15, with the proportion of patients positive for AAN-I-IFN remaining stable afterward. Broader autoimmunity was absent. Without access to thymus tissue from IP patients, they attempted to investigate whether the presence of these autoantibodies could be linked to thymic abnormalities by assessing thymus size and structure in magnetic resonance imaging and by evaluating thymic output. They observed small thymi with straight edges in young IP patients, consistent with premature thymic involution, yet the thymic output of naïve T lymphocytes was normal in the overall cohort. IP patients had slightly decreased Treg counts, but Treg TCR repertoire and immunosuppressive function were not studied. They further investigated a murine model for IP; however female IP mice do not recapitulate the natural history of IP in humans, as they die within 10 days from birth and do not produce AAN-I-IFN. Notwithstanding these limitations, they describe hypotrophic thymi in postnatal IP mice with altered corticomedullary tissue organization, including ghost areas depleted of TEC.

AAN-I-IFN have recently also been detected in patients with inborn errors of the non-canonical NF-κB pathway (Le Voyer et al., 2023, 2024), which triggers slower but more sustained NF-κB signaling mediated by transcription factors NFKB2 (p100/p52) and RelB upon the activation of the NF-κB–inducing kinase (NIK) by certain ligands of the tumor necrosis factor (TNF) superfamily, including RANKL. In mice, RANKL-mediated activation is known to induce the differentiation of thymic epithelial progenitor cells (TEPC) into AIRE⁺ mTEC (Wells et al., 2020) (see figure). Consistently, a decrease in AIRE⁺ mTEC has been reported in humans and mice with impaired non-canonical NF-κB signaling (Le Voyer et al., 2023). In the murine IP model, AIRE expression was preserved. How loss-of-function of the canonical NF-κB signaling pathway underlies thymus dysfunction and production of AAN-I-IFN in IP thus remains to be elucidated. Given that IFN-I signaling pathways play a pivotal role in the production of RANKL by developing thymocytes (Hikosaka et al., 2008), their impairment by AAN-I-IFN could hypothetically indirectly disrupt the non-canonical NF-κB signaling cascade through a reduction in RANKL production (see figure).

Table 1. **IEI associated with AAN-I-IFN**

IEI	Affected gene (inheritance)	Primary cellular defect	Prevalence AAN-I-IFN	AAN against other cytokines
APS-1	*AIRE* (AR)	AIRE$^+$ mTEC	97%	IL-17A, IL-17F, IL-22, IFN-λ1
RAG deficiency	*RAG1* or *RAG2* (AR)	T-lymphocytes	50%	TNF-α, IL-1Ra, IL-6, IL-12p70, IL-17A, IL-17B, IL-17D, CCL1, IFN-γ, IFN-λ 1, IFN-λ2
IPEX	*FOXP3* (X-linked recessive)	Treg	83%	IL-17A
NF-κB2 deficiency[a]	*NFKB2* (AD)	AIRE$^+$ mTEC	59%	
NIK deficiency	*NIK* (AR)	AIRE$^+$ mTEC	100%	
RELB deficiency	*RELB* (AR)	AIRE$^+$ mTEC	87%	
IP	*NEMO* (X-linked dominant)	?	36%	

[a]$p52^{LOF}/I\kappa B\delta^{GOF}$ deficiency. AR autosomal recessive. AD autosomal dominant.

Despite the growing body of single-cell transcriptomic data from thymus tissue, the study of *NEMO* activity is complicated by its adjacent pseudogene. Whilst NEMO is ubiquitously expressed, thymus single-cell RNA sequencing data analysis, most likely erroneously, indicates low *NEMO* expression levels in all lymphoid and stromal cell subsets. Additionally, human TEC are difficult to culture and manipulate ex vivo (Kreins et al., 2021a). Recent progress in TSC culture facilitated the identification of a subset with stem cell properties, named polykeratin (polyKRT) cells or corticomedullary TEC (Yayon et al., 2023, *Preprint*; Ragazzini et al., 2023). These cells also share characteristics with mesenchymal stem cells, suggesting they may be in EMT. Whilst preliminary evidence indicates that polyKRT cells have the potential to differentiate into multiple TEC subtypes, including cTEC and mTEC (Ragazzini et al., 2023), it remains unknown whether this differentiation process is also regulated by the canonical NF-κB signaling pathway or by other ligands of the non-canonical NF-κB pathway beyond RANKL (see figure).

A promising research strategy may be the generation of a transgenic mouse model using the $Rosa26^{Cre\text{-}ERT2}$ Cre/lox recombination system for spatiotemporal knockout (KO) of NEMO in vivo upon tamoxifen administration to study the impact of NEMO deficiency in thymus-seeding progenitors, thymocytes, and TSC, including polyKRT cells and other TEC. This model has already been applied to KO the other two subunits of the IKK complex (Fischer et al., 2021), with the targeted KO of IKKβ in T lineage–specific cells disrupting the development of Tregs and memory T lymphocytes (Schmidt-Supprian et al., 2003). Single-cell omics could then be applied to profile the different cellular components of the thymus, including various stages of thymocyte differentiation, and also to study whether NEMO KO and disruption of the canonical NF-κB signaling pathway lead to altered expression of TRA associated with type I interferons and other peptides. This could be complemented by the introduction of *NEMO* variants by CRISPR-Cas9 gene-editing in murine TEC lines used in expandable organoids (Lim et al., 2024). As different types of NEMO dysfunction have been associated with different diseases (Maubach et al., 2017), such as ectodermal dysplasia, anhidrotic, with immunodeficiency, and various cancers in addition to IP, the latter approach could be particularly useful for the detailed in vitro study of NEMO's possibly pleiotropic functions in TEC. This is relevant for the design of drug candidates targeting specific NEMO domains or specific NEMO protein modifications or interactions, while avoiding broader cytotoxicity. IP patient–derived induced pluripotent stem cells could also be used for disease modeling and future treatment development (Kreins et al., 2021a). In the meantime, the study by Rosain et al. highlights the need to screen IP patients for the presence of AAN-I-IFN. If available, the use of antiviral drugs, possibly combined with plasma exchange to remove the autoantibodies, should be considered to prevent severe disease in AAN-I-IFN-positive patients with a viral infection.

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