

Is a human in vitro enthesitis model relevant to SpA-associated enthesitis? Response to: 'Beware of wolves in sheep's clothing: immune cell plasticity and instability in health and disease' by Alunno *et al*

We thank Alunno *et al*¹ for their comments on our paper reporting on the isolation and preliminary characterisation of normal human enthesis CD4+ and CD8+ conventional T-cells.² Our work showed that up to 2% of CD4+ T cells were Th17 in nature as demonstrated by intracellular flow cytometry. We were unable to identify FOXP3+ Tregs at the enthesis, but these were readily demonstrable in peripheral blood from the same patients. Alunno and colleagues¹ point towards the transdifferentiation between Th17s and Tregs that may occur in vivo and which can be modulated by pivotal spondyloarthritis (SpA) associated cytokines including TNF. Our results were generated in an artificial 'in vitro enthesitis' model, and we agree with the comments by Alunno *et al*¹ in regard to the role of TNF and other factors and that Th17/Treg plasticity will ultimately need to be addressed in tissue from patients with active SpA. While access to spinal enthesal tissue has been reported at one timepoint, we think that it would be technically very difficult to do this due to tissue inaccessibility from such sites for a second post-therapy biopsy.³ It would be possible to model the impact of TNF on our in vitro system using anti-CD3/CD28 stimulation and by either including or blocking TNF in the system. We also agree that the stroma, including mesenchymal stem cells, at the enthesis may play a very important role in regulating T-cell responses which are well defined for native stroma from bone but this has not been studied specifically at the enthesis thus far.⁴

Alunno and colleagues¹ also point out that double negative (DN) CD3+ T cells could be responsible for IL-17A production and that this adds another layer of complexity to the analysis. DN CD3+ T cells include TCR $\gamma\delta$ T-cells that have been previously reported to make IL-17A protein in enthesal tissue.⁵ The other significant population of DN T-cells are the TCR $\alpha\beta$ CD3+ CD4- C8- T-cells that have been repeatedly linked to autoimmunity and inflammation but remain somewhat enigmatic. This DN T-cell population, as the authors pointed out, has been incriminated in psoriasis, which may be relevant to SpA. Given the technical difficulty in rapid tissue digestion and rapid sample preparation for functional studies, our work thus far has focused on the more abundant conventional T-cells but we agree that the role of TCR $\alpha\beta$ CD3+ CD4 T-cells, both at the healthy enthesis and from diseased tissue, awaits elucidation including proinflammatory cytokine induction and production.

Finally, Alunno *et al*¹ point to cells ex-Th17-cells that no longer produce IL-17 but produce IFN- γ , as emergent Th1-like lymphocytes that have recently been reported in psoriatic arthritis (PsA) and that this could be important for disease pathology.⁶ This is an important point and raises the wider issue of the link between such populations in the synovium versus the enthesis and their putative microanatomical associations. It also raises questions pertaining to potential links between such resident populations in health. Further insight into these interactions will be possible after labelling specific T-cell populations followed by single-cell RNA sequencing, prestimulation and

poststimulation with relevant cytokines. To conclude, we agree that the demonstration of a functional adaptive immune system, although with very preliminary characterisation, is key to understanding the enthesitis process in humans with respect to the local biomechanical environment.

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