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Unique tailoring of Th17 at the gingival oral mucosal barrier

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Abstract (150 words)

Our recent work highlights unique requirements for the induction of Th17 cell immunity at the oral and gingival mucosal barrier. Unlike other barrier sites, such as the skin and gastrointestinal tract, we found that Th17 cells can develop at the gingiva independently of commensal microbiota colonization. Instead, we identified that damage, which occurs physiologically due to mastication, promotes induction of Th17 cells and therefore tones homeostatic immunity at the gingiva.
**Discovery!**

The T-helper subset 17 and its signature cytokine IL-17 (Amatya et al., 2017) are considered vital mediators of immunity at barrier surfaces such as the gastrointestinal tract and skin (Belkaid and Harrison, 2017). Indeed, Th17 cells are enriched in these environments where they participate in immune surveillance and maintenance of barrier integrity (Stockinger and Omenetti, 2017) (Veldhoen, 2017) . As such, residence of Th17 cells at barrier sites has been shown to ensure protective immunity against both bacterial and fungal challenge (Stockinger and Omenetti, 2017; Veldhoen, 2017). However, dys-regulated Th17 cell responses have, over the last decade, been continuously associated with auto-inflammatory pathologies (Gaffen et al., 2014; Stockinger and Omenetti, 2017) (Burkett et al., 2015). With Th17 cells being key contributors to effective barrier homeostasis, as well as auto-inflammatory pathology, the factors that control their development, function and plasticity have been well explored. However, much of this work has focused on Th17 cells resident in the gastrointestinal tract and skin and by contrast the factors controlling Th17 cell biology at oral mucosal barriers remain less well explored. This is surprising given the critical role of Th17 cells in oral immunity. Indeed, defects in Th17 cells and/or their signature cytokines results in significant susceptibility to oral fungal infections (Conti et al., 2009) (Lionakis et al., 2014) (Abusleme and Moutsopoulos, 2016). Moreover, amplified/uncontrolled Th17 responses are documented in the oral chronic inflammatory disease periodontitis (Dutzan et al., 2016) (Moutsopoulos et al., 2014; Moutsopoulos et al., 2017; Zenobia and Hajishengallis, 2015). Thus, it is clear that appropriate Th17 cell function is vital to maintain immune homeostasis at the oral barrier, yet little is known regarding their developmental requirements at this site. Here we will discuss our recent work, (Dutzan et al., 2017) in which we elucidated the factors controlling the induction and
regulation of Th17 cells in the gingiva, outlining that novel, tissue-specific cues train immune function at this oral barrier.

To first place our findings at the oral barrier in the context of the larger field of mucosal immunity, we contrast Th17 development in the oral mucosa to findings from the gastrointestinal tract and skin. In these sites, Th17 cell development has been exquisitely tied to colonization by commensal bacteria (Ivanov et al., 2009) (Hooper et al., 2012), and as such Th17 cells are lacking in Germ Free animals. In the gastrointestinal tract, specific bacterial species have been identified that support acquisition of the Th17 cell fate, most well characterized being segmented filamentous bacteria (SFB) (Ivanov et al., 2009). Moreover, gastrointestinal Th17 cells have been shown to be commensal specific (Yang et al., 2014), further reinforcing the vital role of microbes in ensuring Th17 cell residence at this site. Importantly, in both the gastrointestinal tract and skin, the downstream mediators that drive Th17 development in response to commensal colonization have also been defined. It is well established that the cytokines TGFβ, IL-1β, IL-23 and IL-6 play crucial roles in Th17 cell development (Zuniga et al., 2013)(Stockinger and Omenetti, 2017). Exploring the roles of these cytokines in generation of skin and GI tract resident Th17 cells has demonstrated that IL-1β-signaling is a common mediator of tissue Th17 cell specification (Naik et al., 2012; Shaw et al., 2012). In both barrier environments IL-1β is produced in response to commensal colonization (Naik et al., 2012; Shaw et al., 2012). Yet production of this cytokine can only be stimulated by certain bacterial species, further highlighting the commensal-specific nature of this response. Importantly, in the absence of either commensal colonization or IL-1-signals, gut and skin resident Th17 cells fail to develop. Our understanding of Th17 cell specification in the GI tract does not end here, the antigen presenting cells that drive development of Th17 cells in this environment have also been defined. CD103^+CD11b^+ IFR4-dependent dendritic cells (DCs) have been shown to be important drivers of Th17 cell development (Persson et al., 2013). Alongside this,
CCR2-dependent cells, likely monocyte-derived DCs, have also been reported to be required for Th17 cell induction upon colonization by SFB (Panea et al., 2015).

With this detailed mechanistic insight in mind, we set out to establish the factors shaping Th17 cell immunity at the gingiva. Our first surprise was that at in health, the gingiva of young mice was home to very few Th17 cells, contrasting other barrier sites. However, when mice were aged to 24-weeks of age, this resident population of Th17 cells was significantly increased in the gingiva, but not at other barriers (Figure 1A). An increase in Th17 cells with age was also evident in healthy human gingiva. IL-17 producing cells in gingiva were increased in individuals aged 40-50 years compared to adults younger than 25, even in the absence of oral inflammation. Importantly, and perhaps most surprisingly, this increase in Th17 cells with age was not dependent upon commensal bacteria. Not only was the oral microbiome comparable in young compared to older mice, but the Th17 cell population increased with age in germ free mice, meaning both germ free and conventionally housed animals had a similar sized gingiva resident population of Th17 cells (Figure 1B). Thus we outlined that in the gingiva Th17 cell development occurs via commensal-independent mechanisms; starkly contrasting other barrier sites.

These data demonstrated that Th17 cell specification at the gingiva occurred via mechanisms distinct to those controlling this process at other barrier sites. Highlighting this further was the demonstration that gingival Th17 cell generation was IL-6-dependent and not dependent upon IL-1β-signals, which are critical in other barrier microenvironments (Figure 1C). In elucidating the upstream driver of IL-6 production in the gingival environment, we considered the local, tissue-specific stimuli encountered at this barrier. We reasoned that during mastication the gingiva experiences a considerable amount of tissue damage. We hypothesized that this physiological mechanical damage could be training immune function in the gingiva. It is well recognized that tissue injury is a trigger of immune responses, initiated in
response to orchestrated signals from damaged or stressed cells (Kono and Rock, 2008). Thus, the concept that such signals, which would arise continuously in the oral environment, could contribute to educating T cell responses in the gingiva formed an attractive proposition.

To address whether local gingival damage could be a stimulus promoting Th17 cell development, we examined Th17 cell generation following alterations in the levels of gingival damage. First, we directly damaged the gingiva of young mice, which resulted in a significant expansion of barrier resident Th17 cells (Figure 1D). This damage-induced expansion of Th17 cells was dependent on IL-6 and the presence of cognate antigen. Secondly, we placed weanling mice on either a softened or hardened diet and examined gingival Th17 cells at 24 weeks of age. Compared to mice aged on normal diet, those aged on the softened diet had a reduced population of Th17 cells, whereas those aged on a hard diet exhibited increased proportions of Th17 cells (Figure 1E). Combined these data clearly demonstrate that physiological mechanical damage promotes Th17 cell development in the gingiva.

Further exploring this novel pathway of Th17 specification in the gingiva we outlined that in response to gingival damage, epithelial cells produced IL-6, which was subsequently vital for Th17 cell generation at this site. A core component of cellular response to damage and/or stress is the production of pro-inflammatory mediators, including IL-6. Our data demonstrated that epithelial cells function as local, gingival resident sentinels that produce IL-6 in response to mechanical damage and therefore coordinated Th17 specification at this site.

These data suggested that gingiva mechanical damage was the major driver promoting accumulation of Th17 cells. As the gingiva is an environment experiencing constant physiological mechanical damage from mastication, it was important to understand the contribution of damage-induced Th17 cells to local immunity. We demonstrated that damage stimulated induction of IL17-dependent defenses,
revealing its role as an ongoing signal that tones protective immunity at this site. Thus, we identify physiological mechanical damage as a key educator of immune function in the gingiva. These data do not preclude a role for the local oral microbiome in shaping gingival immunity, but highlight a novel stimulus capable of training immune function at this unique site. A damage-induced pathway of Th17 cell generation could well be active at other sites during pathological settings when levels of damage are elevated and approach those experienced in the gingiva. That tissue damage is often associated with expansions of Th17 cells adds value to this proposition. Indeed, we demonstrated that repeated damage to the skin could promote increases in local Th17 cells, revealing the activity of this pathway even at a site where, during steady-state, Th17 cells are dominantly educated by commensals. However, the levels of damage that occur physiologically at the oral barrier are substantial and continuous compared to other sites, with constant occlusal forces, epithelial abrasion and mechanical stresses arising with every chew of food. That this local immune stimulus could be a dominant mechanism educating immune functions at this site is therefore not surprising.

Yet, as with all immune signals, we show that exaggeration of damage over time contributes to local immunopathology and periodontal bone loss. In this regard, mice develop periodontal bone loss with age, the severity of which was altered by changing the hardness of the diet consumed and therefore the level of damage experienced. In line with a role for mechanical damage in periodontal bone loss, germ free mice were shown, both in our study and in that of others, to develop bone loss with age (Hajishengallis et al., 2011) (Baer and Newton, 1960) (Taubman et al., 1981). However, bone loss in germ free animals was less exaggerated than in control mice, indicating that the cooperative functions of the microbiome and damage exaggerate local inflammatory pathology. Thus, not only do our data outline a novel pathway of Th17 generation in the gingiva but uncover a mechanism that could contribute to dys-regulated Th17 responses in settings of pathology.
Our findings elucidate unique immunological triggers operating in the oral barrier yet raise further questions about the training of homeostatic immunity at this site. Is damage a unique signal for the induction of Th17 immunity or does it trigger additional mechanisms of immune responsiveness promoting barrier fitness? Indeed, continued damage would likely promote a plethora of immune functions. Are there unique functional capabilities exhibited by gingival-resident, damage-induced Th17 cells compared to Th17 cell at other barriers? As a product of their novel developmental program, gingival Th17 cells may well exhibit unique functions specifically supporting defense and/or integrity of the gingival barrier. How stable are these damage-induced Th17 cell populations? Functional plasticity and reduced stability are well described phenotypes of Th17 cells (Hirota et al., 2013) (Muranski and Restifo, 2013), given the pathogenic potential of these cells within the gingival environment it will be important to establish the long-term stability of this cellular fates. Importantly, given the amplification of Th17 cells in oral inflammatory disease, what are the critical signals participating in the dysregulation of Th17 immunity at the oral barrier? Elucidation of unique requirements for the induction of immunity at the oral barrier and delineation of functionality of tissue resident populations at this interface is critical to support our understanding of immunity and inflammation at this unique mucosal site.

**Figure Legends**

**Figure 1: The ongoing damage of mastication promotes residence of Th17 cells in the gingiva.** Figure outlines the key ways in which the novel pathway of Th17 cell development in the gingiva was elucidated. (A) Gingival Th17 cells increase by 24-weeks of age. The expansion at this time point is unique to the gingiva. (B) In 24-week old mice, the population of gingival Th17 is equal in germ free (GF) and conventionally housed (SPF) mice. (C) Gingival Th17 cells
development is dependent on the cytokine IL-6. (D, E) Altering levels of gingival damage impacts the development of gingival Th17. (D) Acute gingival damage in young mice results in an expansion of gingival Th17 cells. (E) Ageing mice on diet with different degrees of hardness influences the subsequent generation of Th17 cells with softer diets a smaller population of Th17 cells and harder diets giving rise to more Th17 cells.

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