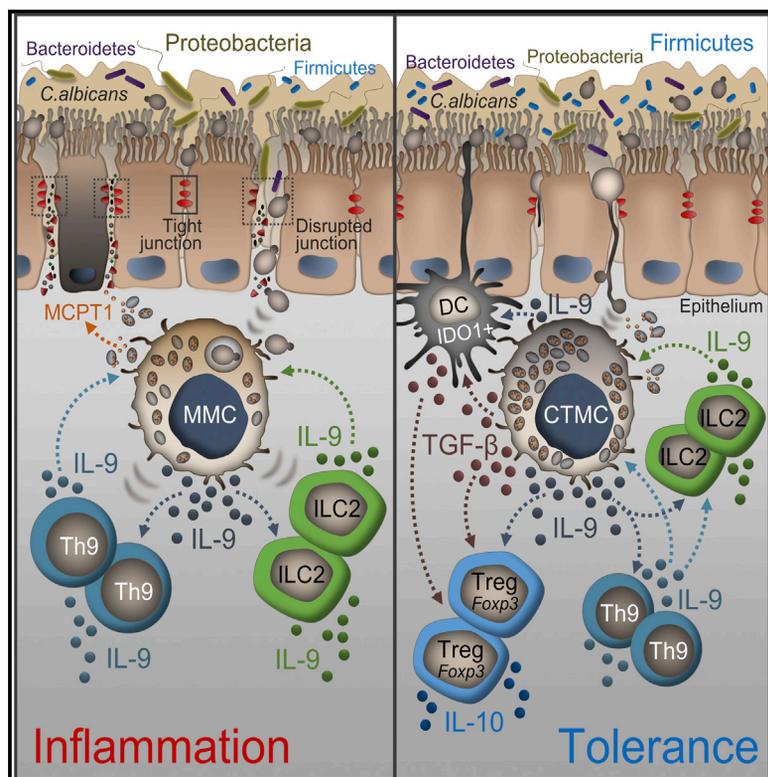


IL-9 and Mast Cells Are Key Players of *Candida albicans* Commensalism and Pathogenesis in the Gut

Graphical Abstract



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In Brief

Deciphering the mechanisms by which *Candida albicans* promotes either pathology or protective tolerance in the gut could be clinically relevant. Renga et al. show a key role for IL-9 and mast cells in promoting either inflammatory dysbiosis and pathology or tolerance in leaky gut models and human celiac disease.

Highlights

- IL-9/IL-9R signaling affects MC function in mucosal candidiasis
- IL-9 and mucosal MCs contribute to barrier function loss in leaky gut models
- IL-9 and stromal MCs induce local protective tolerance in infection via IDO1
- IL-9 and mucosal MCs expand and IDO1 decreases in human celiac disease



IL-9 and Mast Cells Are Key Players of *Candida albicans* Commensalism and Pathogenesis in the Gut

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SUMMARY

Candida albicans is implicated in intestinal diseases. Identifying host signatures that discriminate between the pathogenic versus commensal nature of this human commensal is clinically relevant. In the present study, we identify IL-9 and mast cells (MCs) as key players of *Candida* commensalism and pathogenicity. By inducing TGF- β in stromal MCs, IL-9 pivotally contributes to mucosal immune tolerance via the indoleamine 2,3-dioxygenase enzyme. However, *Candida*-driven IL-9 and mucosal MCs also contribute to barrier function loss, dissemination, and inflammation in experimental leaky gut models and are upregulated in patients with celiac disease. Inflammatory dysbiosis occurs with IL-9 and MC deficiency, indicating that the activity of IL-9 and MCs may go beyond host immunity to include regulation of the microbiota. Thus, the output of the IL-9/MC axis is highly contextual during *Candida* colonization and reveals how host immunity and the microbiota finely tune *Candida* behavior in the gut.

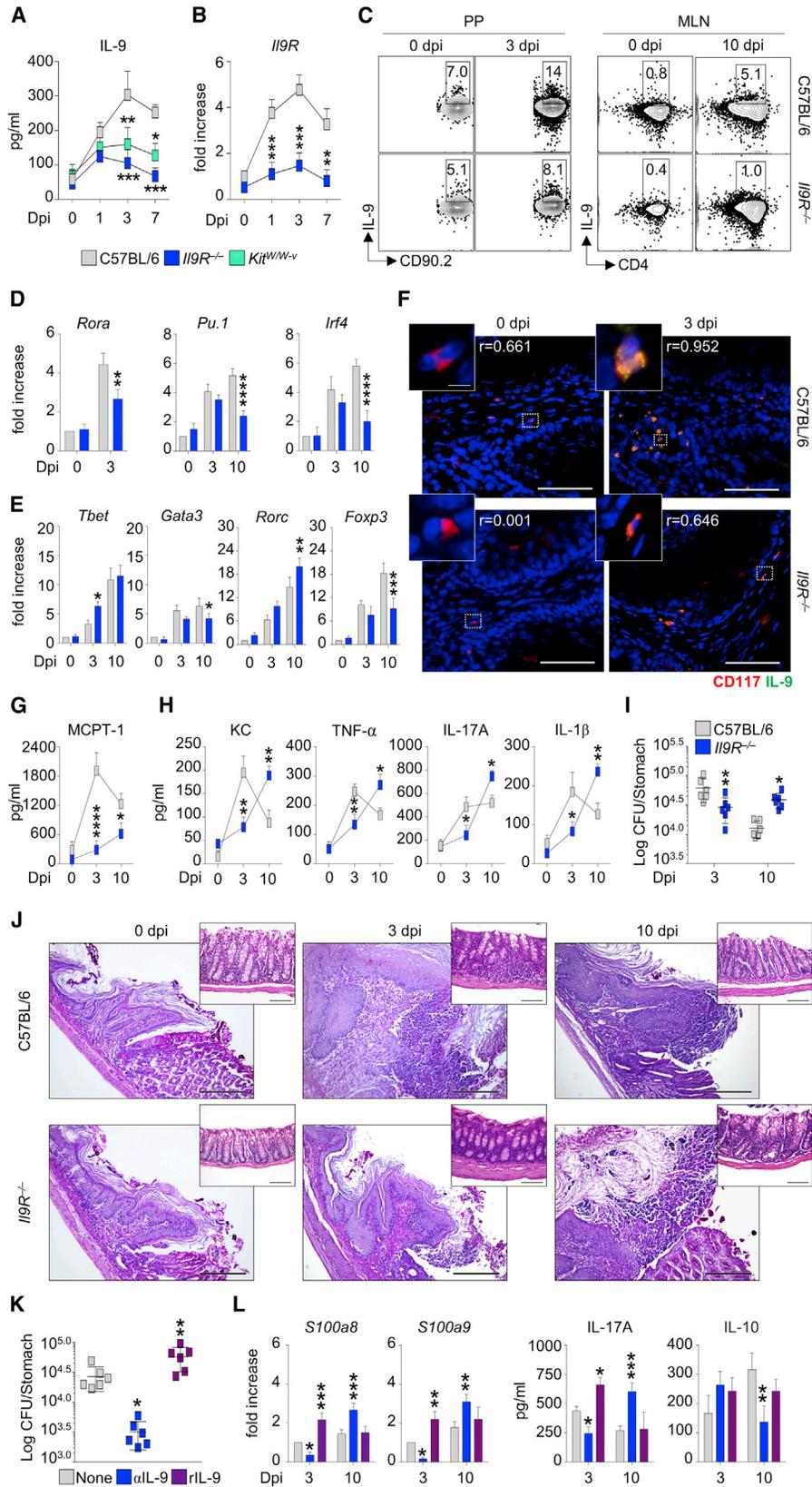
INTRODUCTION

Candida albicans is well adapted for growth in the gastrointestinal tract, where the colonization levels reflect an interplay between host immunity, the microbiota, and the fungus (Noble et al., 2017; Romani, 2011; Underhill and Pearlman, 2015). Much like antibiotics, intestinal inflammation may perturb the resident bacterial community, creating conditions that favor

both high-level *Candida* colonization and inflammation. However, despite being implicated in gut immunopathology (Gerard et al., 2015), including celiac disease (CD) (McDermott et al., 2003; Nieuwenhuizen et al., 2003) and sensitization to food antigens (Yamaguchi et al., 2006), *C. albicans* colonization protects against local (Bonifazi et al., 2009; Montagnoli et al., 2002) and distant (Noverr and Huffnagle, 2004) immune pathologies in mice. In humans, the clinical significance of fungi in gastric diseases is still controversial, and the need for antifungal therapy has not reached a consensus (Sasaki, 2012). Thus, identifying host signatures that discriminate between the pathogenic versus protective role of the fungus becomes important.

Recent studies have suggested the possible participation of mast cells (MCs) in *Candida*/host interaction at mucosal surfaces (Lopes et al., 2015; Schlapbach et al., 2014). *In vitro*, human MCs mount a specific temporal pattern of responses toward *C. albicans* that includes an initial phase characterized by secretion of granular proteins, neutrophil recruitment, and reduced fungal viability, followed by a late stage of release of mediators with known anti-inflammatory activity (Lopes et al., 2015). Because of their strategic location at vascularized mucosal surfaces combined with a unique versatility (Frossi et al., 2017), MCs are well positioned to respond to allergens and pathogens and modulate mucosal immune responses (Abraham and St John, 2010; Reber et al., 2015), thus contributing to a wide variety of human infections and diseases (Frossi et al., 2017). The phenotypic and functional characteristics of MCs can be tuned by many genetic and environmental factors, including changes in the cytokine milieu associated with inflammatory or immune responses (Frossi et al., 2017; Galli et al., 2005). However, despite their potential phenotypic “plasticity,” two types of MCs have been described in mice based on their protease content and location: connective tissue-type MCs (CTMCs), derived from fetal liver progenitors and primarily located in





(legend on next page)

stromal tissue, and mucosal MCs (MMC9s), of bone marrow origin and residing in the gut and lung (Gurish and Austen, 2012; Reber et al., 2015).

Among MMCs, interleukin-9 (IL-9)-producing mucosal MCs (MMC9s) are the principal producers of IL-9 (~2.0 pg/mL per cell) (Chen et al., 2015), the key cytokine that autocrinally drives mastocytosis (Renauld et al., 1990). MMC9s are scarce in the small intestines of immunologically naive mice and expand considerably after repeated ingested antigen exposure (Chen et al., 2015). In addition to MMC9s, innate lymphoid cells (ILCs) 2 and T helper (Th) 9 cells may also serve as alternative cellular sources of IL-9, thus amplifying intestinal mastocytosis involved not only in food allergy and systemic anaphylaxis (Chen et al., 2015; Shik et al., 2017) but also in intestinal inflammation (Boeckxstaens, 2015; Gerlach et al., 2014; Nalleweg et al., 2015) and in the onset and progression of CD (Frossi et al., 2017). However, several lines of evidence show that MCs and IL-9 may also suppress chronic inflammatory responses and promote immune tolerance (de Vries and Noelle, 2010; Lu et al., 2006; Metz et al., 2007). Thus, by integrating multiple signals and mechanisms, MCs promote either inflammatory immunity or immune tolerance.

It has recently been reported that the MC-ILC2-Th9 pathway exacerbates aspergillosis and promotes lung inflammation in cystic fibrosis (CF) (Moretti et al., 2017). In candidiasis, Th9 responses were defective in patients with chronic mucocutaneous candidiasis (Becker et al., 2016) and abundant in *Candida*-driven skin inflammation (Schlapbach et al., 2014). Thus, a role for the IL-9/MC axis in mucosal candidiasis is plausible but has never been directly demonstrated. In the present study, we reveal that IL-9 and MCs are key players of *Candida* pathogenicity at mucosal surfaces. IL-9 and mucosal MCs contributed to barrier function loss, dissemination, and inflammation in experimental models of impaired intestinal barrier function and were upregulated in patients with CD. However, by inducing the indoleamine 2,3-dioxygenase enzyme in response to the fungus, IL-9 pivotally contributed to local immune tolerance. Indeed, inflammatory dysbiosis occurred in the relative absence of IL-9 and MCs, a finding indicating that the activity of IL-9 and MCs may go beyond host immunity to include regulation of the microbiota.

RESULTS

The IL-9/IL-9R Signaling Pathway Is Involved in Innate and Th Immunity to *C. albicans*

We resorted to primary and secondary gastrointestinal infection in C57BL/6 and *I19R*^{-/-} mice to measure IL-9 production and *I19R* expression in infection. Increased production of IL-9 and expression of *I19R* were observed in the stomach of C57BL/6 but not *I19R*^{-/-} mice (Figures 1A and 1B), a finding suggesting that IL-9 production is IL-9R-dependent. Both innate and adaptive immune responses contributed to IL-9 production, as indicated by the high levels observed early and late in infection and defective production in *Rag2*^{-/-}*I19R*^{-/-} mice (Figure S1C). IL-9 production was not defective in IL-4- or IL-17RA-deficient mice (Figure S1C), which indicates that Th2 and Th17 cells do not contribute to IL-9 production, as suggested previously (Kaplan, 2013). Increased production of IL-9 was also observed in the ileum (from 20 ± 3 to 300 ± 13 pg/mL, naive versus 3-day infected mice) and colon (from 76 ± 6 to 540 ± 33 pg/mL, naive versus 3-day infected mice).

ILC2s, MMC9s, and Th9s are the most potent producers of IL-9 at mucosal surfaces (Kaplan, 2013; Wilhelm et al., 2011). We found that both ILC2s and Th9s (gating strategy shown in Figures S1A and S1B) produced IL-9 in infection, as indicated by flow cytometry (Figure 1C), and that expression of the *Rora* transcription factor of ILC2s and the *Pu.1* and *Irf4* transcription factors of Th9s (Figure 1D) were lower in *I19R*^{-/-} compared with C57BL/6 mice. Consistent with the significant reduction of IL-9 levels in MC-deficient C57BL/6-*Kit*^{W/W^{-v}} mice (hereafter called *Kit*^{W/W^{-v}}) (Figure 1A), IL-9⁺CD117⁺ MCs in the stomach (Figure 1F) and production of MC protease 1 (MCPT-1) (Figure 1G) were also lower in *I19R*^{-/-} compared with C57BL/6 mice. *Gata3* and *Foxp3*, but not *Tbet* or *Rorc*, were poorly upregulated in *I19R*^{-/-} mice during infection (Figure 1E). Together with the lower production of the neutrophil chemoattractant KC and inflammatory tumor necrosis factor alpha (TNF-α), IL-17A, and IL-1β cytokines observed in *I19R*^{-/-} compared with C57BL/6 mice early in infection (Figure 1H), this immune profile would predict an effect of defective IL-9/IL-9R signaling on infection. This appeared to be the case because the loss of IL-9R signaling led to reduced

Figure 1. The IL-9/IL-9R Signaling Pathway Orchestrates Innate and Th Immunity to *C. albicans*

- (A) IL-9 levels (ELISA) in stomach homogenates of C57BL/6, *I19R*^{-/-}, and MC-deficient C57BL6-*Kit*^{W/W^{-v}} mice infected i.g. with *C. albicans*.
 (B) *I19R* expression (RT-PCR) in the stomach.
 (C) Intracellular IL-9 staining by flow cytometry in CD90.2⁺ ILC2-gated cells from Peyer's patches (PPs) and CD3⁺CD4⁺ T cells from mesenteric lymph node (MLNs). The numbers in the quadrants indicate the percentage of CD90.2⁺IL-9⁺ and CD4⁺IL-9⁺ cells.
 (D) *Rora*- and Th9-specific transcript expression (RT-PCR) on PPs and purified CD4⁺ T cells from MLNs.
 (E) Th cell-specific transcripts (RT-PCR) on CD4⁺ T cells from MLNs.
 (F) Immunofluorescence staining of CD117⁺ IL-9⁺ MC cells in the stomach (the scale bars represent 100 and 50 μm). Numbers refer to the co-localization coefficient to quantify the overlap degree of CD117 and IL-9.
 (G) MCPT-1 levels (ELISA) in serum.
 (H) Cytokine production (ELISA) in stomach homogenates.
 (I) Fungal growth (log₁₀ CFUs).
 (J) Histopathology (periodic acid-Schiff [PAS]; the scale bar represents 200 or 100 μm) of the stomach and colon (H&E; insets).
 (K) Fungal growth (log₁₀ CFUs) in the stomach of infected C57BL/6 mice treated i.p. for a week with 10 mg/kg mAb neutralizing IL-9 or 10 μg/kg rIL-9. Control (None) mice received an isotype control antibody.
 (L) *S100a8* and *S100a9* expression (RT-PCR) and IL-17A and IL-10 concentration (ELISA) in the stomach.
 Data represent pooled results (n = 6, mean ± SD) or representative images from three experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, knockout versus C57BL/6 mice and treated versus untreated mice. One- or two-way ANOVA and Bonferroni and Tukey's post test. dpi, days post infection.

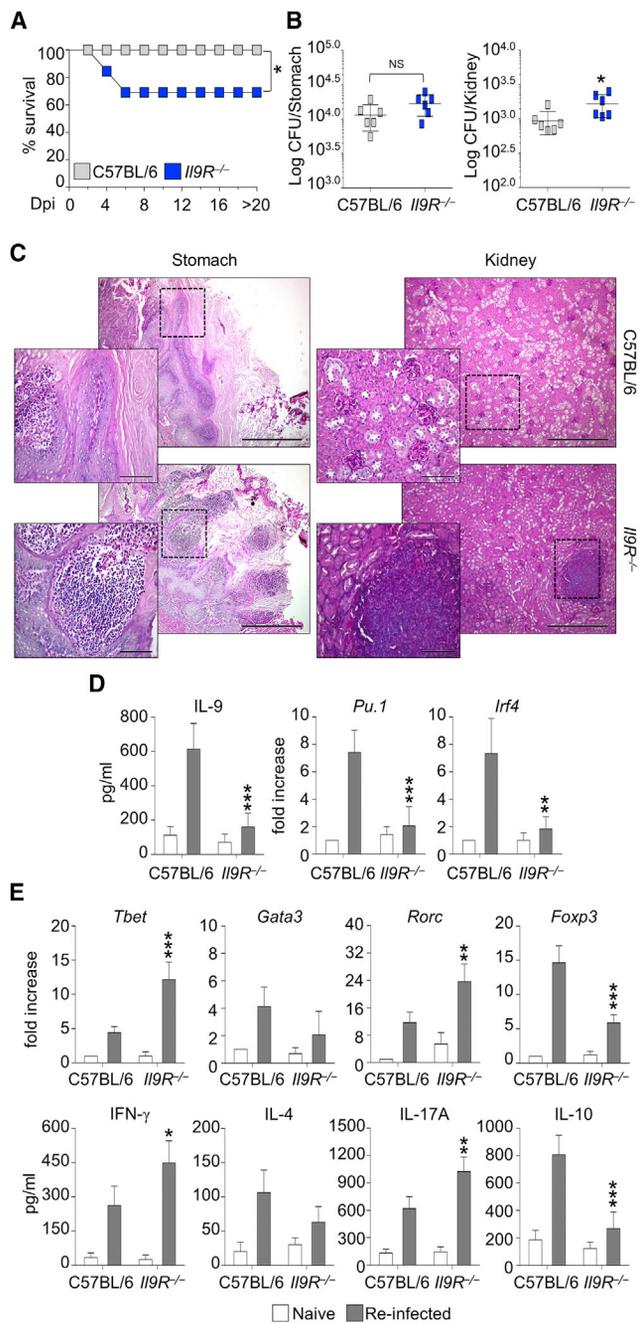


Figure 2. The IL-9/IL-9R Signaling Pathway Provides Resistance to Re-infection

(A) Survival (percent) of C57BL/6 and *I19R*^{-/-} mice subjected to secondary i.g. infection with *C. albicans*.
 (B) Fungal growth (log₁₀ CFUs).
 (C) Histopathology (PAS; the scale bar represents 500 μm or 100 μm) in the stomach and kidney.
 (D) IL-9 levels (ELISA) in stomach homogenates and expression of *Pu.1* and *Irf4* (RT-PCR) in CD4⁺ T cells from MLNs.
 (E) Expression of Th cell-specific transcripts (RT-PCR) on CD4⁺ T cells from MLNs and cytokine production (ELISA) in stomach homogenates.
 Assays were done 3 days after re-infection. Data represent pooled results (n = 6, mean ± SD) or representative images from three experiments. *p < 0.05,

inflammatory cell recruitment in the stomach (Figure 1J) and colon (inset in Figure 1J) and, paradoxically, reduced local fungal growth, particularly early in the course of infection (Figure 1I). Similar results were obtained in *Rag2*^{-/-}*I19R*^{-/-} mice (Figure S1), suggesting that defective IL-9/IL-9R signaling in innate cells contributes to the increased resistance. Importantly, IL-9 ablation by means of neutralizing antibody and exogenous IL-9 administration increased or decreased, respectively, resistance to infection, as indicated by local fungal growth (Figure 1K) and the pattern of inflammatory cytokine production (Figure 1L), suggesting a causal role of IL-9 in impairing early antifungal resistance. Together, these results suggest that IL-9/IL-9R signaling is dispensable for resistance, if not contributing to pathogenic inflammation, during initial *C. albicans* infection.

Surprisingly, the effects of IL-9 ablation or administration were different late in infection, at a time when resistance to infection was instead promoted by IL-9 and impaired upon IL-9 inhibition. When testing the hypothesis of a possible disparate activity of IL-9 on innate and adaptive immunity to the fungus, we assessed *I19R*^{-/-} mice for resistance to re-infection and parameters of adaptive Th immunity. *I19R*^{-/-} mice were less resistant than C57BL/6 mice to re-infection, as indicated by decreased survival (Figure 2A), the inability to control fungal dissemination despite a degree of control of fungal growth locally (Figure 2B), and increased inflammatory pathology both in the stomach and the kidneys (Figure 2C). IL-9 production and Th9 cells were expectedly lower in *I19R*^{-/-} than in C57BL/6 mice (Figure 2D). In contrast, Th1/Th17 cell activation was higher but regulatory T cell (Treg) activation was lower and Th2 apparently was unaffected in *I19R*^{-/-} compared with C57BL/6 mice (Figure 2E), a finding pointing to dysfunctional Th responses under conditions of IL-9R deficiency, which could account for the failure to mount memory responses to the fungus (Montagnoli et al., 2002). Together, these results suggest a temporally distinct activity of IL-9 in infection being required for optimal Th/Treg-mediated protection but dispensable for innate antifungal defense.

The IL-9/IL-9R Signaling Pathway Regulates MC Activity in Infection

The above results prompted us to investigate the mechanisms behind the opposite activity of IL-9 in infection and re-infection. Given the ability of IL-9 to inhibit the oxidative burst of effector phagocytes (Pilette et al., 2002), IL-9 could adversely affect the activity of effector phagocytes. Although IL-9 slightly decreased the oxidative burst and candidacidal activity *in vitro* (Figure S2), the antifungal activity was not impaired in *I19R*^{-/-} mice (Figure S2). Thus, the reduced inflammatory response of *I19R*^{-/-} mice was not secondary to a reduced fungal growth. To unravel mechanisms uncoupling the inflammatory response from the fungal growth, we looked for MC expansion and activity in infection, considering that MCs promote neutrophil recruitment (Malaviya et al., 1996), regulate acquired immune responses (Galli et al., 2005), and are directly responsible for increasing epithelial paracellular

p < 0.01, *p < 0.001. NS, not significant. *I19R*^{-/-} versus C57BL/6 mice. Statistical analyses of the survival curves were performed using the log rank (Mantel-Cox) test. Unpaired t test or two-way ANOVA, Bonferroni post test. Naive, uninfected mice.

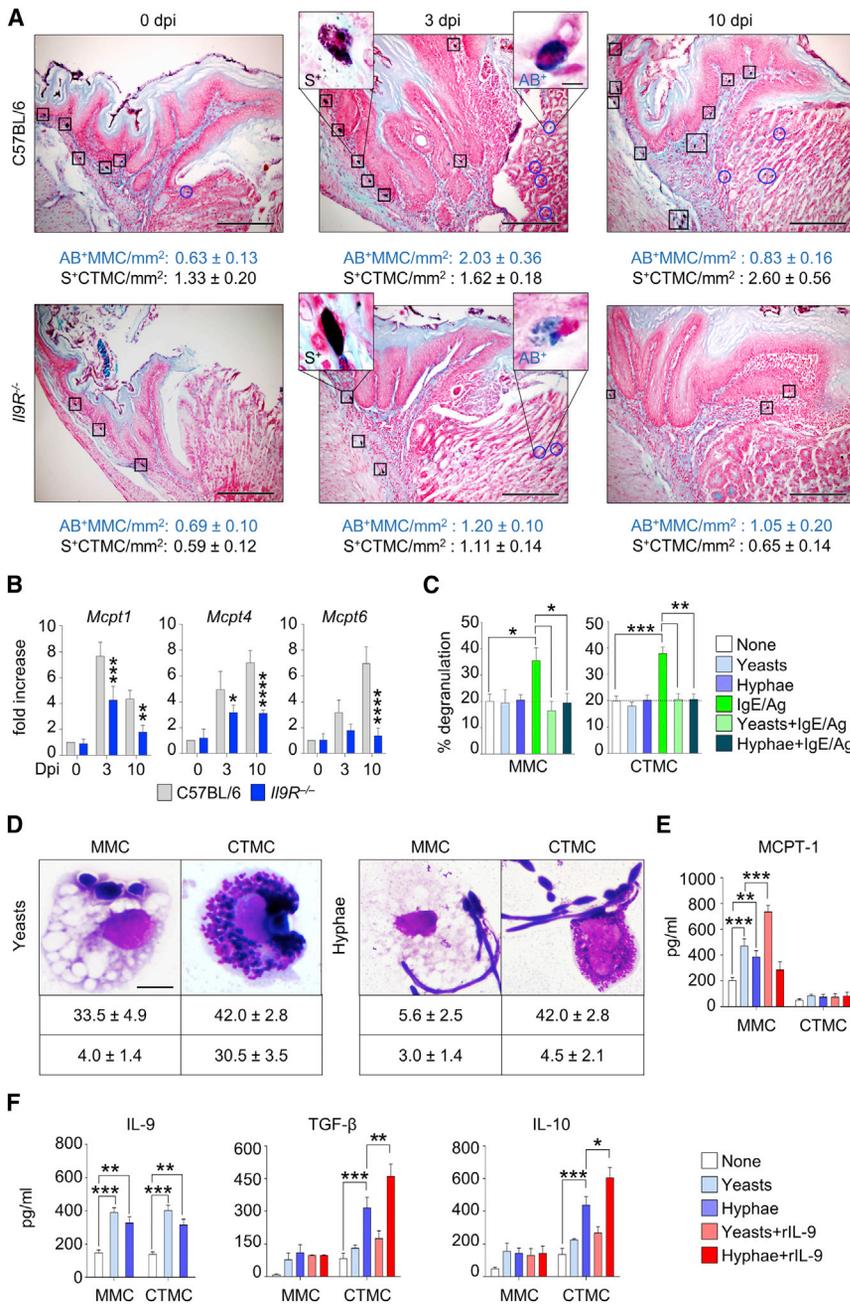


Figure 3. The IL-9/IL-9R Signaling Pathway Regulates MC Activity in Infection

(A) Alcian blue/safranin staining (the scale bar represents 200 μm or 50 μm) with relative MC number per square millimeter in stomach sections of C57BL/6 and *I19R*^{-/-} mice infected i.g. with *C. albicans*.

(B) MC protease expression (RT-PCR) in the stomach.

(C) Degranulation percentage in BM-derived MC in the presence of IL-3 with (connective tissue MC [CTMC]) or without (mucosal MC [MMC]) stem cell factor and stimulated with *C. albicans* yeasts, hyphae, or IgE/Ag.

(D) MC morphometry (May-Grünwald-Giemsa staining) and percentage of phagocytosis (top line) and killing (bottom line) of MMCs and CTMCs pulsed for 2 hr with viable *C. albicans* yeasts or hyphae.

(E and F) MCPT-1 (E) and cytokine (F) production (ELISA) in culture supernatants of MMCs and CTMCs pulsed with the fungus for 5 hr with or without 10 ng/mL rIL-9.

Data represent pooled results (n = 6, mean ± SD) or representative images from three experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, *I19R*^{-/-} versus C57BL/6 mice and treated versus untreated cells. One- or two-way ANOVA, Tukey's and Bonferroni post test.

ase gene expression, showing peak expression of the MMC-related chymase *Mcpt1* at 3 days (Figure 3B) and expression of the CTMC-related chymase *Mcpt4* and the tryptase *Mcpt6* continuously present throughout the infection (Figure 3B).

To functionally characterize the MC subtypes, we cultivated bone marrow (BM)-derived MCs in the presence of IL-3, alone or with stem cell factor, to obtain MCs considered to be the tissue culture equivalent of MMCs or CTMCs, respectively (Godfraind et al., 1998). We stimulated MC subtypes with *Candida* and/or immunoglobulin E (IgE)/Ag (antigen) and looked for degranulation, phagocytosis, and killing of the fungus

permeability during intestinal infection via MCPT-1 (McDermott et al., 2003). MCs expanded early in the forestomach and glandular stomach of C57BL/6 mice and less in *I19R*^{-/-} mice to decline thereafter (from 1.83 ± 0.26 MCs/mm² in uninfected mice to 3.50 ± 0.70 and 2.40 ± 0.40 at 3 and 10 days post infection (dpi), respectively), as seen by toluidine blue staining (Figure S3A). Of interest, safranin-negative/Alcian blue-positive MCs were localized in the mucosa early in infection, whereas Alcian-blue negative/safranin-positive MCs were continuously present in the connective tissue, indicating that both MMCs and CTMCs, respectively, expanded in infection (Figure 3A). This finding is consistent with the pattern of specific MC prote-

and cytokine production. Consistent with the ability of the fungus to degranulate human MCs at a high MOI only (Lopes et al., 2015), degranulation was not induced, if not inhibited, in either MC subtype in response to *Candida* yeasts or hyphae (Figure 3C). MC subtypes, despite being able to phagocytose unopsonized yeasts but not hyphae, exhibited different candidicidal activity, being MMCs unable to kill the ingested fungi (Figure 3D), as suggested previously (Trevisan et al., 2014). Actually, the massive MCPT-1 release would suggest MMC necrosis, an effect potentiated by IL-9 (Figure 3E). Killing of the ingested yeasts (Figure 3D) and no release of MCPT-1 (Figure 3E) were instead observed with CTMCs. Either MC subtype

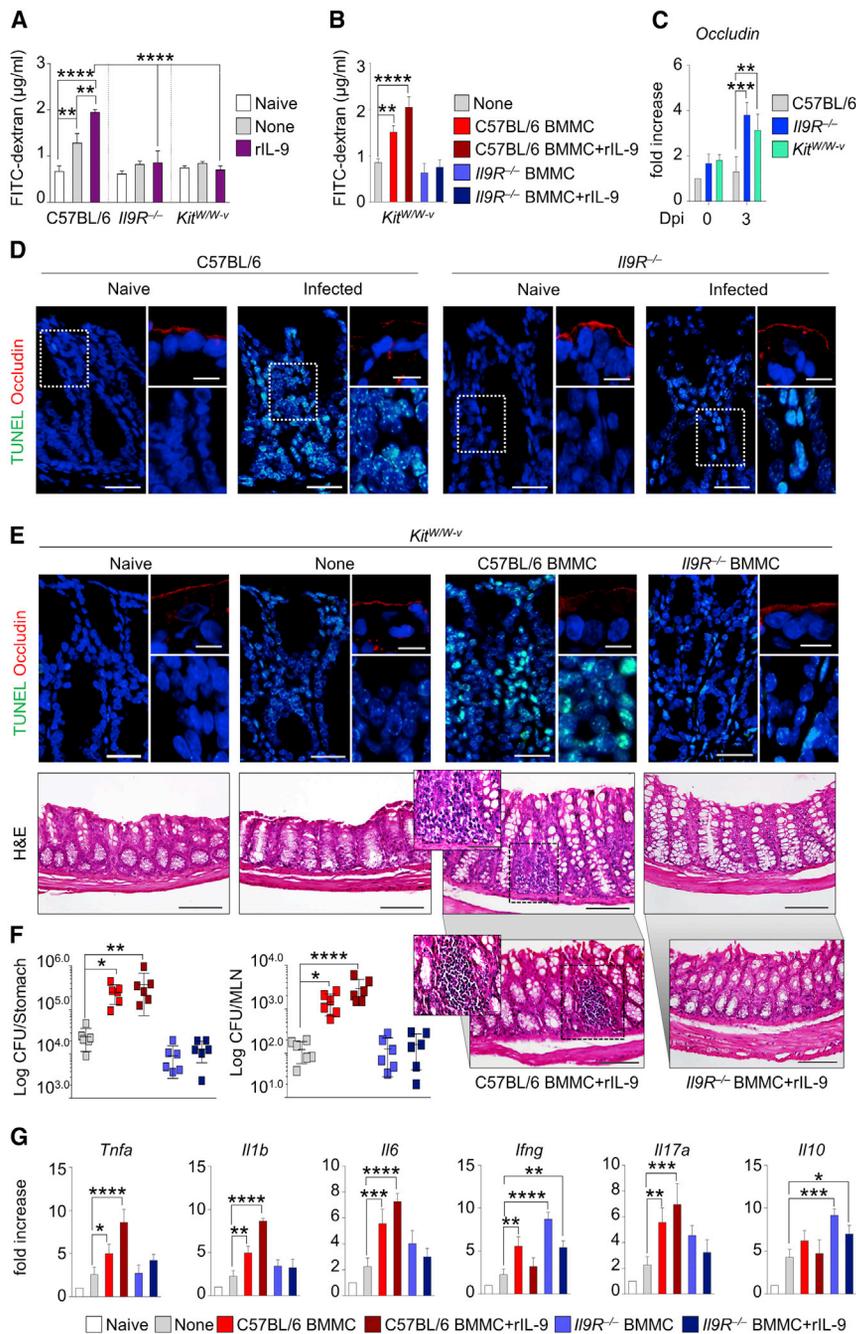


Figure 4. MCs Regulate Intestinal Permeability and Adaptive Immunity in *C. albicans* Infection

(A) FITC-dextran levels in the serum of C57BL/6, *I19R^{-/-}*, and *Kit^{W/W-v}* mice infected i.g. with *C. albicans* and treated for 3 days with 10 μg/kg rIL-9.

(B) FITC-dextran levels in *Kit^{W/W-v}* mice engrafted intravenously with C57BL/6 or *I19R^{-/-}* BM-cultured MCs (BMMCs), infected, and treated as in (A). Mice were sacrificed 3 dpi.

(C) *Occludin* expression (RT-PCR) in the colon.

(D) Expression of occludin by immunofluorescence and TUNEL staining in the gut of C57BL/6 and *I19R^{-/-}* infected mice.

(E) Occludin expression, TUNEL staining, and colon histopathology of *Kit^{W/W-v}* mice engrafted intravenously with C57BL/6 or *I19R^{-/-}* BMMCs, infected i.g., and treated with rIL-9 for 3 days. (H&E; the scale bars represent 100 μm or 50 μm).

(F) Fungal growth (\log_{10} CFUs).

(G) Cytokine expression (RT-PCR) in the colon of *Kit^{W/W-v}* mice.

Data represent pooled results (n = 6, mean ± SD) or representative images from three experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, treated versus untreated mice and knockout versus C57BL/6 mice. One- or two-way ANOVA, Tukey's or Bonferroni post test.

IL-9 and MCs Affect Intestinal Epithelial Permeability and Adaptive Immunity

The effect of IL-9 on MCPT1 production would predict an effect on epithelial cell permeability. We used fluorescein isothiocyanate (FITC)-dextran to detect leakage in the early stages of permeability induction. Leakage was observed at 3 dpi in C57BL/6 mice and was dependent on both IL-9 and MCs, as evidenced by the reduced leakage in *I19R^{-/-}* or *Kit^{W/W-v}* mice (Figure 4A). However, leakage was promoted upon IL-9 administration alone in C57BL/6 mice or together with engrafted wild-type but not *I19R^{-/-}* BM-cultured MCs (BMMCs) in *Kit^{W/W-v}* mice (Figure 4B), a finding indicating that IL-9 and MC work together in promoting epithelial leakage.

produced IL-9 in response to *Candida* (Figure 3F), and IL-9 affected cytokine production (Figure 3F) but not the antifungal effector activity of MCs (data not shown). Indeed, MCs discriminated between the fungal morphotypes in terms of TGF-β and IL-10 production; CTMCs more than MMCs were able to release both cytokines in response to hyphae, a release potentiated by IL-9 (Figure 3F). No differences were observed in *Il1b* and *Tnfa* gene expression in either MC subtype (data not shown). These findings indicate that *C. albicans* exploits MC functional versatility at mucosal surfaces to contribute to local damage and inflammation or protection.

Indeed, consistent with the upregulated expression of the sealing protein occludin in the colon of *I19R^{-/-}* mice (Gerlach et al., 2015), the occludin gene (Figure 4C) and protein expression (Figures 4D and 4E) were downregulated in C57BL/6 mice and upregulated in *I19R^{-/-}* mice as well as in *Kit^{W/W-v}* mice either untreated or engrafted with wild-type BMMCs. Paralleling the pattern of epithelial permeability, epithelial damage was also observed, as indicated by terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) assay staining (Figures 4D and 4E). In terms of infection and pathology, compared with C57BL/6 mice, inflammatory cell recruitment (Figure 4E), local

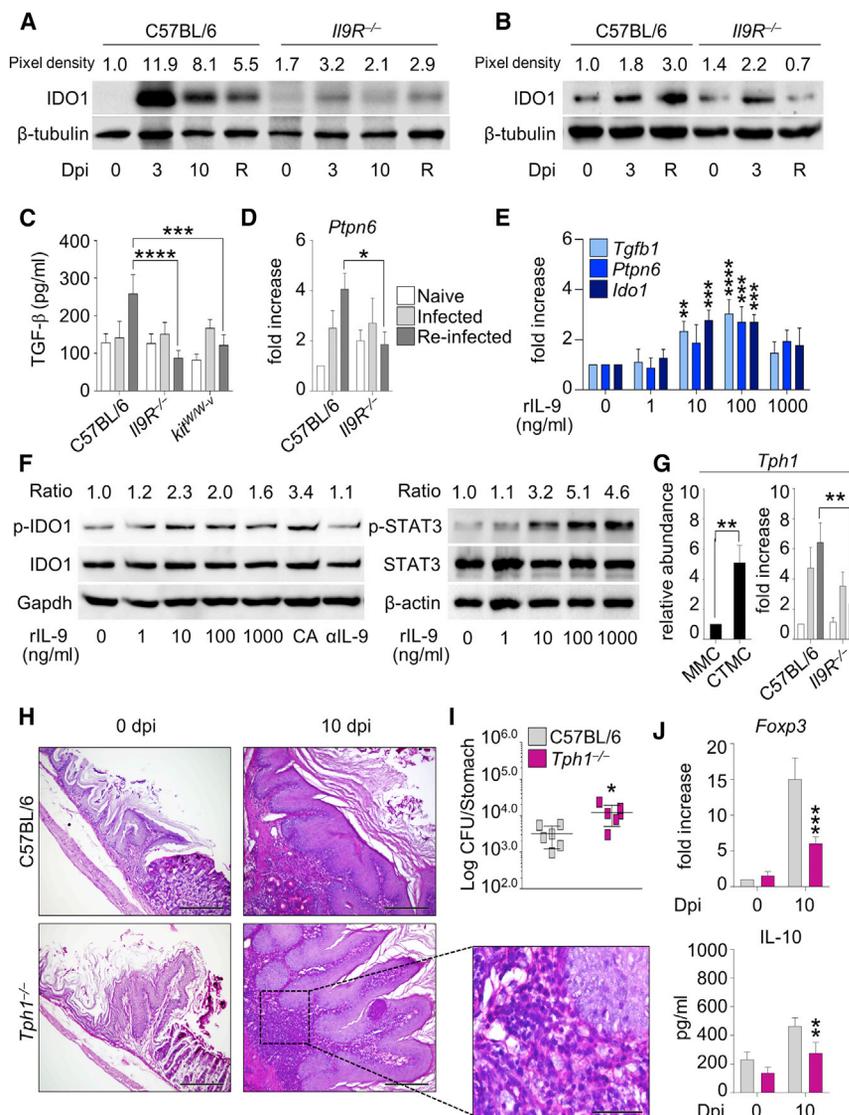


Figure 5. IL-9 Regulates IDO1 Activity

(A and B) Expression of IDO1 by immunoblotting in the spleen (A) and colon (B) of C57BL/6 and *I19R*^{-/-} mice subjected to primary and secondary *C. albicans* infection. Mice were sacrificed 3 days after secondary infection.

(C) TGF-β production (ELISA) in stomach homogenates of infected C57BL/6, *I19R*^{-/-}, and *Kit*^{W/W-v} mice.

(D) Expression of the *Ptpn6* gene (RT-PCR) in the stomach.

(E and F) Expression of the *Tgfb1*, *Ptpn6*, and *Idol1* genes (RT-PCR) (E) and production/phosphorylation of IDO1/STAT3 (F) in purified splenic dendritic cells pretreated for 1 hr with different doses of rIL-9 or αIL-9 before pulsing with the fungus for an additional 5 hr.

(G) *Tph1* expression (RT-PCR) in unstimulated MMCs and CTMCs and in the stomach of C57BL/6 and *I19R*^{-/-} mice.

(H) Stomach histopathology (PAS; the scale bar represents 200 μm or 50 μm) of C57BL/6 and *Tph1*^{-/-} mice i.g. infected with *C. albicans*.

(I) Fungal growth (log₁₀ CFUs).

(J) *Foxp3* expression (RT-PCR) and IL-10 production (ELISA) in the stomach.

Data represent pooled results (n = 6, mean ± SD) or representative images from three experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, knockout versus C57BL/6 mice and rIL-9-treated versus untreated (0) cells. Unpaired t test or two-way ANOVA, Tukey's or Bonferroni post test. R, 3 days after re-infection.

fungal growth and dissemination (Figure 4F), and inflammatory cytokine gene expression (Figure 4G) were reduced in *Kit*^{W/W-v} mice early in infection and promoted upon administration of wild-type but not *I19R*^{-/-} BMDCs with and without IL-9. Similar to *I19R*^{-/-} mice, *Kit*^{W/W-v} mice failed to control both fungal growth (Figure S3B) and inflammatory pathology (Figure S3C) upon re-infection and to activate memory-protective Th1/Treg cell responses (Figure S3D). These results highlight the potent immunomodulatory role of MCs that both augment (Shelburne et al., 2009) and regulate the quality (Mekori et al., 2016) of adaptive immunity. Together, these findings point to IL-9/MC-dependent negative activity on intestinal permeability that may account for inflammation, *Candida* dissemination, and dysregulated Th immunity.

IL-9 Regulates IDO1 Activity

TGF-β is known to provide long-term immune tolerance by regulating the activity of indoleamine 2,3-dioxygenase (IDO1) (Pallotta et al., 2011), known to be crucially involved in Treg-

mice as opposed to C57BL/6 mice (Figures 5A and 5B) and paralleled the decreased production of TGF-β observed in *I19R*^{-/-} and *Kit*^{W/W-v} mice (Figure 5C). The decreased expression of *Ptpn6* (coding for SHP-1) (Figure 5D) causes a functional deficiency of TGF-β that is required for long-term tolerance (Pallotta et al., 2011). This finding suggests that IL-9 may regulate IDO1 activity via MC-dependent TGF-β production. This appeared to be the case because IL-9 activated *Tgfb1*, *Ptpn6*, and *Idol1* gene expression (Figure 5E) and STAT3/IDO1 phosphorylation on splenic dendritic cells at optimal concentrations ranging from 10 to 100 ng/mL (Figure 5F). Thus, IL-9 appears to act as a potent regulator of IDO1 activity via transcriptional and post-transcriptional mechanisms. Of great interest, IL-9 also mediated IDO1 phosphorylation induced by the fungus, as indicated by defective phosphorylation observed in the presence of IL-9-neutralizing antibody (Figure 5F). Together, our results offer a plausible explanation for the dual role for IL-9 in *C. albicans* colonization. IL-9 negatively affected innate antifungal resistance by

expanding inflammatory MMCs, eventually leading to barrier function loss; however, it was required for the activation of an optimal antifungal Th/Treg response by promoting IDO1 activity with the contribution of CTMCs producing TGF- β . However, MCs are also known to mediate immune suppression via Tph-1 (tryptophan hydroxylase-1), a synthase that catalyzes the conversion of tryptophan to serotonin (Nowak et al., 2012). When assessing the possible contribution of Tph-1, we found that CTMCs, but not MMCs, express *Tph1* (Figure 5G) and that *Tph1* expression was lower in *IIR^{-/-}* compared with C57BL/6 mice (Figure 5G). Consistent with the tolerogenic potential of Tph-1, inflammation (Figure 5H) and fungal growth (Figure 5I) were increased whereas tolerance decreased (Figure 5J) in *Tph1^{-/-}* mice. Thus, CTMCs may contribute to ensure tolerance at the host/*Candida* interface through multiple mechanisms.

IL-9 and MMCs Are Upregulated in Experimental Leaky Gut Models and CD Patients

To accommodate the above findings in a clinically relevant setting, we evaluated the presence of IL-9 and MC in experimental models of impaired barrier function, such as in homozygous *F508del-Ctfr* C57BL/6 mice (*Ctfr^{F508del/F508del}*, hereafter referred to as *Ctfr^{F508del}* mice) or mice with gluten sensitivity (Papista et al., 2012) infected with *C. albicans* as well in duodenal biopsies from CD patients, scored according to the Marsh classification modified by Oberhuber et al. (1999) (AGA Institute, 2006). *Ctfr^{F508del}* mice and mice with gluten sensitivity, although highly susceptible to *C. albicans* gastrointestinal infection in terms of fungal growth and inflammatory pathology (Figure S4), showed increased production of IL-9 (Figures 6A and 6E), increased *Pu.1* and *Irf4* expression (Figures 6B and 6F), and increased MMC activity (Figures 6C and 6G). Consistent with the remarkable fungal dissemination to visceral organs (Figure S4), occludin expression was decreased in these mice (Figures 6D and 6H), a finding suggesting impaired intestinal permeability, to which the fungus greatly contributed. Prompted by these findings, we looked for IL-9 and MCs in CD patients in whom both *Candida* (Corouge et al., 2015; Nieuwenhuizen et al., 2003) and MCs (Frossi et al., 2017) contribute to pathology. IL-9 positivity was increased in duodenal biopsies from CD patients and correlated positively with infiltration of CD3⁺ T cells, CD117⁺, tryptase⁺ MCs, and disease severity and negatively with IDO1 expression (Figure 6I; Figure S5). Thus, IL-9/MMC expansion is a distinctive feature of disease severity in murine models of leaky gut, such as in the CF gut, gluten sensitivity, and human CD.

DISCUSSION

This study shows a plausible mechanism by which *C. albicans* could promote either immune pathology or protective tolerance in the gut. It is well known that MCs and IL-9 are associated with intestinal diseases, from food allergy (Chen et al., 2015; Forbes et al., 2008) to inflammation (Boeckxstaens, 2015; Gerlach et al., 2014; Nalleweg et al., 2015; Weigmann and Neurath, 2017). However, that *C. albicans* was able to exploit the versatility of the IL-9/MC axis for balancing commensalism versus pathogenesis is an interesting finding. Consistent with the ability

of MCs to respond to pathogens via Toll-like receptors (Abraham and St John, 2010), phagocytosis of the fungus by MCs was dependent on fungal morphotype and MC subtype, with hyphae promoting anti-inflammatory cytokine production by CTMCs and yeasts promoting MMC death and damage. IL-9 pivotally contributed to the discriminative responses of MCs. Produced by both MC types, in addition to ILC2s and Th9 cells, IL-9 stimulated secretion of proteases and cytokines, including its own. A positive amplification loop between ILC2s producing IL-9 and MMC9s likely maximizes IL-9 production and promotes epithelial damage and inflammation. Thus, although exerting divergent antifungal effector activity, the different MCs, finely tuned by IL-9, appear to discriminate between fungal morphotypes and, in so doing, promote basal immune tolerance or epithelial damage.

Although the small intestine is usually considered the primary site of uptake of food antigens (Walker et al., 1972), uptake of food antigens can also occur in the stomach (Hatz et al., 1990). Therefore, *C. albicans* colonization of the stomach, where mastocytosis occurs, could be associated with barrier function loss, a finding suggesting a possible pathogenic role of the fungus in the development of gut diseases. In addition to known risk factors favoring fungal colonization in the stomach (Scott and Jenkins, 1982), epithelial damage promoted through MMC activation could be the key event promoting transition from fungal commensalism to pathogenesis, as already suggested (Moyes et al., 2016). Given the inflammatory activity of *C. albicans*-specific Th9 cells in the skin (Schlapbach et al., 2014), a pathogenic role for Th9 cells is also plausible. However, we found that Th9 cells are dispensable for immune pathology and are not detected under conditions of failure to activate Treg cells, a finding in line with the functional plasticity of Th9 cells capable of exerting protective and pathogenic roles in different clinical settings (Elyaman et al., 2009; Schmitt et al., 2014). It is intriguing that a sex dimorphism at IL-9 and IL-9R loci (Schuurhof et al., 2010) increased the risk of *Aspergillus* allergy in females more than in males (Moretti et al., 2017). Should this risk extend to *Candida* allergy, this would be in line with the increased resistance to mucosal candidiasis (including vaginal infection; data not shown) observed in *IIR^{-/-}* mice and predict an increased risk of candidiasis in carriers of this polymorphism.

The above results confirm the patho-inflammatory role of the IL-9/MC pathway in CF (Moretti et al., 2017) and suggest that the IL-9/MC axis could be an attractive druggable pathway to prevent the clinically unwanted consequences of fungal colonization. However, the occurrence of *Candida* pneumonia in patients receiving imatinib mesylate treatment suggests that MC-targeted therapy might predispose patients to opportunistic and life-threatening fungal infections (Speletas et al., 2008). This study provides a plausible explanation for MC-mediated protection. Consistent with their immunoregulatory role in adaptive immune responses (Morita et al., 2016), tissue remodeling, homeostasis, and peripheral tolerance (Galli et al., 2008; Lu et al., 2006), CTMCs greatly contribute to the activation of intestinal immune tolerance via Tph1 and IDO1. That MCs transactivate IDO1 in dendritic cells (DCs) has already been described (Rodrigues et al., 2016). Here we found that IL-9 may not only directly affect IDO1 gene expression via STAT3

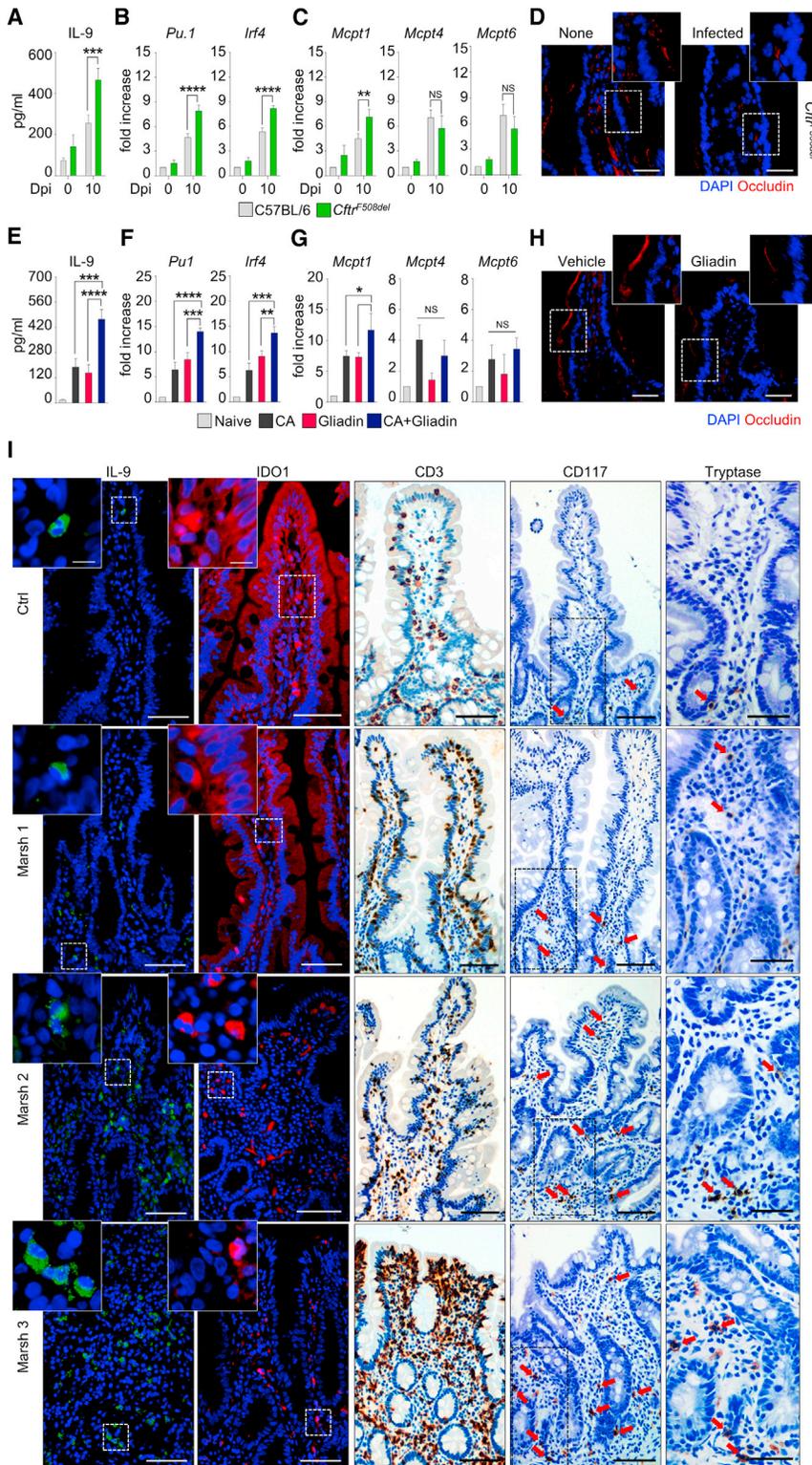


Figure 6. IL-9/MMCs Are Expanded in Murine Models of Leaky Gut and in Human CD (A–C) IL-9 production (A) (ELISA), Th9 cell-specific transcripts (B), and MC proteases (C) (RT-PCR) in the stomach of C57BL/6 and *Cfr^{F508del}* mice infected i.g. with *C. albicans*.

(D) Occludin expression by immunofluorescence in the gut of *Cfr^{F508del}* mice (the scale bar represents 100 μ m).

(E–G) IL-9 production (E) (ELISA), expression of Th9-cell specific transcripts (F), and MC proteases (G) (RT-PCR) in the small intestine of gluten-sensitive BALB/c mice infected i.g. with *C. albicans*.

(H) Expression of occludin in the gut by immunofluorescence.

(I) IL-9 and IDO1 expression by immunofluorescence and detection of CD3⁺, CD117⁺, and trypsinase⁺ cells by immunohistochemical staining in duodenal biopsies from patients with Marsh 1, Marsh 2, and Marsh 3 scores and controls (Ctrl). The scale bars represent 200 μ m or 100 μ m.

Data represent pooled results (n = 6, mean \pm SD) or representative images from three experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. *Cfr^{F508del}* versus C57BL/6; gliadin and infected versus gliadin or infected mice. Unpaired t test or two-way ANOVA, Bonferroni post test.

(Demoulin et al., 1999) in MCs but also promote IDO1 long-term tolerance via TGF- β (Pallotta et al., 2011). Together with IL-4, TGF- β is able to promote Th9 cell development *in vitro* (Goswami et al., 2012), a finding highlighting the potential role of TGF- β in reinforcing Th9 cell activity *in vivo*. Interestingly, TGF- β production by MCs in response to IL-9 promotes lung fibrosis in aspergillosis (Moretti et al., 2017), a finding suggesting that modules of effector immunity may have different functional outcomes contingent upon the context.

Tryptophan abundance under conditions of defective host IDO1 expression is known to expand *L. reuteri*, which provides colonization resistance to *Candida* via the AhR/ILC3/IL-22 axis (Zelante et al., 2013). All of these microbial signatures were present in *Il9R*^{-/-} mice and were responsible for the decreased inflammatory pathology and increased antifungal resistance. Indeed, Firmicutes were more abundant in *Il9R*^{-/-} and *Kit*^{W/W-v} mice, with Lactobacillaceae represented more than Clostridiaceae in *Il9R*^{-/-} mice and Clostridiaceae more than Lactobacillaceae in *Kit*^{W/W-v} mice (Figure S6A). Of interest, *L. johnsonii* was abundant in both C57BL/6 and *Il9R*^{-/-} mice, but *L. reuteri* was expanded in *Il9R*^{-/-} mice either uninfected or in infection (Figure S6B), and, consistently, *AhR* expression, IL-22 production (Figure S6C), and *Lcn2* expression (Figure S6D) were all increased in these mice. Microbiota transplantation between C57BL/6 and *Il9R*^{-/-} mice revealed that antifungal resistance was increased and inflammation decreased in C57BL/6 mice receiving fecal content from *Il9R*^{-/-} mice, whereas the opposite occurred in *Il9R*^{-/-} mice transplanted with C57BL/6 feces (Figures S6E–S6G). This points to the contribution of local microbiota to antifungal resistance and mucosal homeostasis under conditions of IL-9 and, likely, MC deficiency, a finding also confirmed by the increased inflammatory pathology observed upon antibiotic treatment (Figures S6H and S6I).

Thus, the microbiota provides antifungal resistance under conditions of IL-9 deficiency much like in IDO1 deficiency. However, one most interesting and unexplained finding is the failure of the microbiota to provide antifungal resistance in re-infection, which suggests that dysregulated adaptive immunity affects the function of the microbiota. It is also likely that the activity of adaptive immunity encompasses an effect on MC plasticity (Daëron, 2016), including the ability of MCs to act as intestinal sensors of local microbiota. Indeed, we found a unique pattern of intestinal dysbiosis in MC-deficient mice in which Clostridiaceae and Enterobacteriaceae expanded early and late, respectively, during *Candida* colonization, suggesting a specific temporal pattern of MC-dependent regulation of local microbial composition. How nutrient availability, promotion of inflammation, or lack of tolerance contribute to microbial sensing and regulation by MCs at mucosal surfaces remains to be determined. Dysbiosis is a known risk factor for CD (Girbovan et al., 2017) and intestinal inflammation in CF (Garg and Ooi, 2017), conditions under which persistent symptoms are associated with a higher relative abundance of Proteobacteria and a lower abundance of Bacteroidetes and Firmicutes. This study provide evidence that the relatively high levels of IL-9 and MMCs observed in biopsies from patients with CD may promote inflammation-driven intestinal dysbiosis to which tryptophan deficiency may contribute. Thus, the IL-9/MC axis,

by integrating signals derived from perturbed host/microbiota homeostasis, may act as a signature that discriminates between the pathogenic versus protective role of the fungus in the gut.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6, *Rag2*^{-/-} and BALB/c mice were purchased from Charles River Laboratories (Calco, Italy). Homozygous MC-deficient C57BL/6-*Kit*^{W/W-v}, *Tph1*^{-/-}, *Il4*^{-/-}, and *Il17Ra*^{-/-} mice were bred under specific pathogen-free conditions in the animal facility at the University of Perugia. *Il9R*^{-/-} and *Rag2*^{-/-}*Il9R*^{-/-} mice were kindly provided by Prof. Jean-Christophe Renauld (Ludwig Institute for Cancer Research, Brussels). CF mice homozygous for the Phe508del-*Cfr* allele were described previously (Romani et al., 2017). Murine experiments were performed according to Italian Approved Animal Welfare Authorization 360/2015-PR and Legislative Decree 26/2014 regarding the animal license obtained by the Italian Ministry of Health lasting for 5 years (2015–2020). 5- to 8-week-old male and female mice were used in all experiments.

Infection and *In Vivo* Treatment

Candida albicans SC5314 cells were propagated on Sabouraud glucose agar at 37°C (Sigma-Aldrich, Milan, Italy). Gastrointestinal infection was performed by inoculating mice intragastrically (i.g.) with 10×10^8 *Candida albicans* cells in 200 μ L saline using an 18G 4-cm-long plastic catheter. Re-infection was performed by i.g. inoculation of 10×10^8 *Candida albicans* cells 15 days after the primary infection. Mice were sacrificed 3 days after re-infection. Fungal growth was expressed as colony-forming units (log₁₀ CFU) per organ, obtained by serially diluting homogenates on Sabouraud agar plates incubated at 35°C for 24 hr. Murine monoclonal anti-IL-9 antibody (clone MM9C1, Bio X Cell) or control isotype IgG (clone C1.18.4, Bio X Cell) were administered intraperitoneally (i.p.) at a dose of 10 mg/kg for a week starting on the day of infection (from 344.33 \pm 68.07 pg/mL to undetectable levels in the stomach, untreated *versus* treated mice at 3 dpi). Recombinant (r)IL-9 (R&D Systems) was administered i.p. at a dose of 10 μ g/kg.

Patients and Control Subjects

Biopsy specimens were obtained from the distal duodenum during upper gastrointestinal endoscopy from 8 adult patients undergoing diagnostic protocols at the University Hospital of Perugia. No individual patient identification was involved, and no study-driven clinical intervention was performed; therefore, no ethical approval was necessary. The CD diagnosis was established according to standard criteria, including human leukocyte antigen (HLA) genotyping, anti-TG2 serum titer measurement, and histologic analysis. Intestinal biopsy specimens were evaluated for villous architecture, crypt height, and intraepithelial lymphocytes and scored according to the Marsh classification modified by Oberhuber et al. (1999). Control samples with normal intestinal histology were obtained from 3 subjects without CD undergoing biopsies for screening procedures for abdominal symptoms and excluded from CD diagnosis.

Statistical Analysis

GraphPad Prism software 6.01 (GraphPad) was used for analysis. Data are expressed as means \pm SD. Horizontal bars indicate the means. Statistical significance was calculated by one- or two-way ANOVA (Tukey's or Bonferroni's *post hoc* test) for multiple comparisons and by two-tailed Student's *t* test for single comparisons. Statistical analysis of the survival curves was performed using log rank (Mantel-Cox) test. The distribution of levels tested by Kolmogorov-Smirnov normality test turned out to be non-significant. Fluorescence intensity was measured using ImageJ software. Cell fluorescence intensity was measured using ImageJ software. The co-localization program Fiji with the Just Another Co-localization Plugin (JACoP) was used to quantify the degree of overlap by calculating the co-localization coefficients (Pearson's correlation coefficient). The variance was similar in the groups being compared.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.04.034>.

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AUTHOR CONTRIBUTIONS

G.R. and V.O. designed the experiments and performed most of the *in vitro* and *in vivo* experiments. M. Borghi performed immunofluorescence experiments. T.Z., G.P., and M.D.Z. performed experiments with mastocytes. V.R.V. performed experiments in gluten-sensitive mice. V.R., R.D.S., M. Baldoni, and A.S. followed patients and provided clinical samples. J.-C.R. provided the knockout mice. A.B., S.M., C.C., E.G., L.M., C.P., and L.R. designed the experiments, analyzed the data, and wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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