SMOLDERING INFLAMMATION IN CARDIO-IMMUNE-METABOLIC DISEASES

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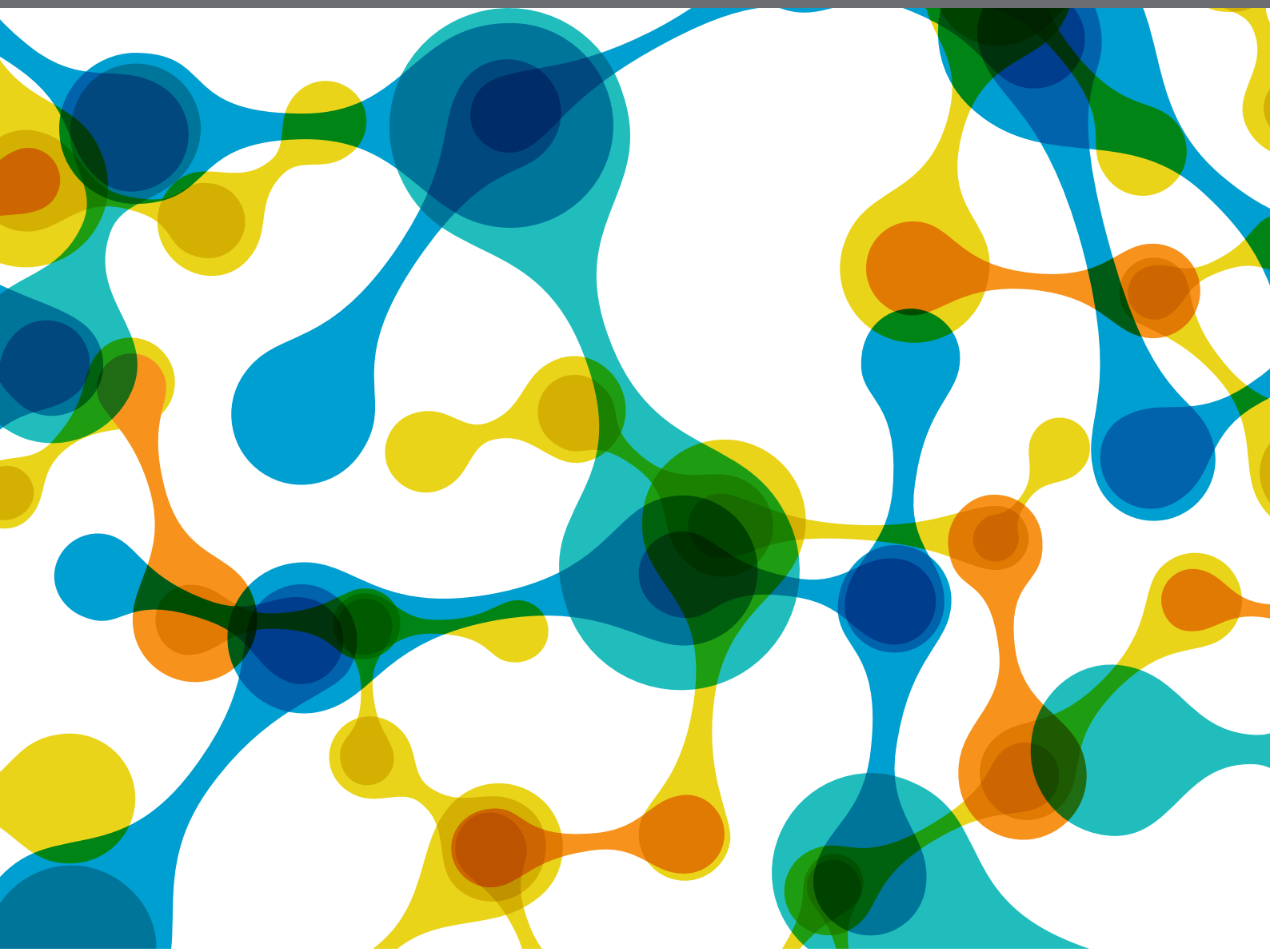

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SMOLDERING INFLAMMATION IN CARDIO-IMMUNE-METABOLIC DISEASES

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EDITORIAL
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Editorial: Smoldering Inflammation in Cardio-Immune-Metabolic Disorders

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Keywords: allergic disorders, diabetes, obesity, low grade inflammation, sinovitis, IL-5, mast cells, Alzheimer's disease

Editorial on the Research Topic

Smoldering Inflammation in Cardio-Immune-Metabolic Disorders

"If many remedies are prescribed for an illness, you may be certain that the illness has no cure."
Anton Chekhov - The Cherry Orchard -

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Smoldering or low-grade inflammation plays a pivotal role in both physiological and pathological conditions (Calder et al., 2017; Zelechowska et al., 2018; Ronnback and Hansson, 2019). Aging is accompanied by a physiological decline in immune competence, termed immunosenescence, characterized by inflammaging (Antonelli et al., 2006; Franceschi et al., 2017; Varricchi et al., 2020b). Besides, low-grade inflammation is a prodrome of a variety of cardiometabolic disorders (Wen et al., 2012, van Greevenbroek et al., 2016), including obesity (Avalos et al., 2018; Trim et al., 2018), diabetes (Zatterale et al.), and cardiovascular diseases (Hoogeveen et al., 2018; Xu et al., 2019). Immune cells, strategically localized also in white adipose tissue (Horckmans et al., 2018; Zelechowska et al., 2018; Merrick et al., 2019; Plotkin et al., 2019), are an important source of pro-inflammatory cytokines in pathophysiological conditions (Varricchi et al., 2019a,b, 2020c; Marone et al., 2020). There is increasing awareness that specific biomarkers of smoldering inflammation are predictive of cardiovascular risks (Weber et al., 2004; Wolber et al., 2007; Varricchi et al., 2020a). Chronic low-grade inflammation also participates in the initiation and progression of several disorders of the immune system such as rheumatoid arthritis (Rivellese et al.; Siouti and Andreakos, 2019), psoriatic arthritis (Gisondi et al.; Girolomoni et al., 2017), and allergic diseases (Weiss, 2005; Pelaia et al., 2015; Canonica et al., 2016; Ferrando et al., 2017).

This Research Topic's driving force was to collect new acquisitions on the role of smoldering inflammation in diverse clinical pathological settings, examining them through the lens of temporal and spatial changes in immune cells and their products, such as cytokines.

Pucino et al. highlighted the interplay between metabolism, immunity and inflammation in patients with rheumatoid arthritis. The authors provided evidence that metabolic alterations of tissue microenvironment plays a pivotal role in the pathophysiology of rheumatoid arthritis. On the same ground, Moschetta et al. illustrated the role of inflammatory sinovitis in the development of hemophilic arthropathy. They discussed the role of imbalance of pro- and anti-inflammatory cytokines in inducing hemophilic arthropathy. The authors suggested that modulation of synovial inflammation could represent a novel therapeutic approach to prevent hemophilic arthropathy. Calcaterra et al. discussed the "two hits" hypothesis of synovitis in hemophilic arthropathy.

Rivellese et al. evaluated the possible contribution of synovial mast cells and their mediators to histological features of synovitis in severe and/or early rheumatoid arthritis. The authors demonstrated that disease-modifying anti-rheumatic drugs (DMARDs) reduced synovial inflammation and mast cell infiltration only in half of the patients examined. The presence of mast cells after 6 months of treatment with DMARDs was associated with a higher disease activity. They concluded that synovial mast cell are associated with disease severity. Gisondi et al. discussed the pathophysiological relationship between psoriasis, a chronic, systemic immune-mediated disease and cardiometabolic comorbidities and the therapeutic strategies to modulate low-grade inflammation in these patients.

It is well-established that several cytokines (e.g., IL-4, IL-5, and IL-13) (Varricchi and Canonica, 2016; Peters and Wenzel, 2020; Marone et al.) and alarmins (e.g., TSLP, IL-33, IL-25; Afferni et al., 2018; Varricchi et al., 2018; Marone et al., 2019; Porsbjerg et al., 2020) play a pivotal role in different phenotypes of asthma. Pelaia et al. extensively reviewed the central role of IL- 5 in the pathogenesis of severe eosinophilic asthma. The latter condition can be responsive to inhaled and/or systemic glucocorticoids that reduce eosinophilia (Hong et al., 2020).

However, severe eosinophilic asthma may be resistant to glucocorticoids and require therapies with specific monoclonal antibodies (mAbs), targeting IL-5/IL-5Rα (Varricchi and Canonica, 2016). Experimental models and clinical studies have demonstrated that IL-13 is an important cytokine in chronic airway inflammation. IL-13 is produced by human basophils (Gibbs et al., 1996; Ochensberger et al., 1996; Redrup et al., 1998; Patella et al., 2000; Genovese et al., 2003; Galeotti et al., 2019) and mast cells (Fushimi et al., 1998; Lorentz et al., 2000), the primary effector cells of allergic disorders (Marone et al., 2014; Varricchi et al., 2019c; Miyake et al., 2020). Marone et al. analyzed the biochemical and immunological effects of IL-13 in the context of experimental models of asthma and in asthmatic subjects. Despite promising results in several *in vitro* and *in vivo* models of allergic inflammation, the efficacy of mAbs anti-IL-13 in patients with asthma has been surprisingly negative (Hanania et al., 2016; Russell et al., 2018).

Obesity is one of the major health burdens of the twenty-first century as it contributes to insulin resistance and type 2 diabetes (Calay and Hotamisligil, 2013). Chronic, low-grade inflammation in adipose tissue is a crucial risk factor for the development of obesity and type 2 diabetes. Obesity is characterized by activation of the innate and adaptive immune system which may explain the increase susceptibility to develop metabolic disorders such as diabetes mellitus (Saltiel and Olefsky, 2017). Zatterale et al. carefully examined the molecular pathways linking obesity-induced inflammation and insulin resistance. The authors elegantly discussed the complex role of innate and adaptive immunity in obesity. Finally, they provided evidence that low-grade inflammation might represent a novel therapeutic target for metabolic diseases. Osteopontin produced by several immune cells, endothelial cells, and fibroblasts, is involved in cardiovascular diseases (Abdelaziz Mohamed et al., 2019; Vianello et al., 2020). Moschetta et al. reported that osteopontin is linked to pathological dysregulation of the arginine pathway in patients with coronary artery disease.

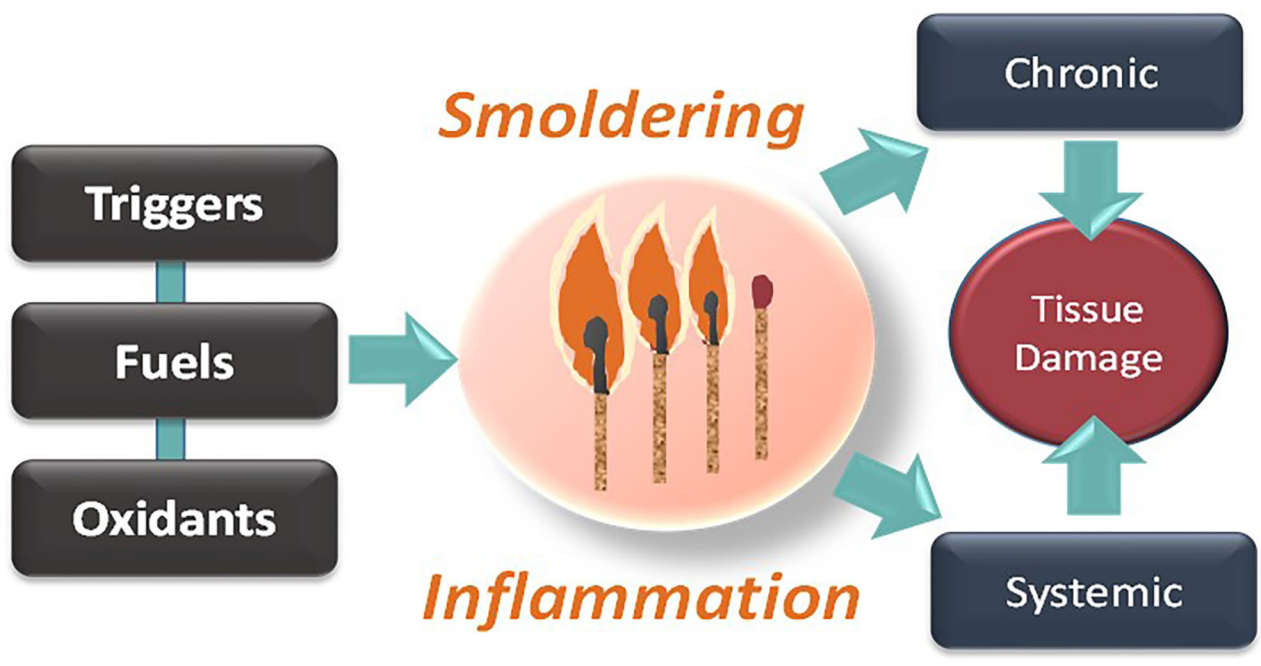

FIGURE 1 | Smoldering inflammation as a progressive disease requiring triggers, fuels, and pro-oxidative conditions, ultimately resulting in systemic tissue and chronic damage.

Alzheimer's disease (AD) is the most prevalent form of dementia in the elderly. A vast amount of literature indicates a role of inflammation in AD pathophysiology and several findings support the existence of a link between periodontitis, a chronic inflammatory oral disease and Alzheimer's disease (Heppner et al., 2015). Liccardo et al. provided an upgrade on the emerging evidence supporting a relationship between periodontitis and Alzheimer's disease.

But how do we protect ourselves from chronic inflammation? The above contributions highlight the need to counter clinical conditions that can determine the progression from acute to chronic inflammation. Among them, we should aim to lower cholesterol, reduce obesity, prevent gum disease, and stop smoking. Dietary changes are also likely to be important, including eliminating food and beverages high in fructose and other refined sugars while increasing our intake of polyphenols (Serino and Salazar, 2018), such as those contained in vegetables, fruits, and seeds. These alterations may represent an 'anti-inflammatory' lifestyle which may help reduce smoldering inflammation in chronic inflammatory conditions. Is this doable? In other words, does the multisource smoldering inflammation require many remedies? If so, then there might be no cure for this condition, and we have to side with Chekov on this one. In this scenario appears important to mention that results from the CANTOS trial have demonstrated that treatment with Canakinumab, a monoclonal antibody anti-IL-1β of patients with previous myocardial infarction and a high-sensitivity C-reactive protein level results in significantly reduced cardiovascular events. Moreover, patients with genetically-determined decreased IL-6 signaling showed a reduced risk of cardiovascular events and increased life-span (Rosa et al., 2019).

Early interventions, however, would help (e.g., a timely detection of any inflammatory focus). Pursuing this is feasible for easy-to-access areas of our body, such as skin, joints, and mouth. Moreover, preventing or eradicating the accumulation of visceral fat that is a consolidated fomite of chronic inflammation and atherosclerosis (Alexopoulos et al., 2014). Conversely, doing so for visceral organs is more complicated and requires a more articulated level of repeated inspections.

If chronic inflammation is, indeed, an enduring burning flame, then making an analogy to the fire of a match suggests another ineludible point (**Figure 1**). A match is composed of fuel (more specifically, antimony trisulfide) and an oxidant (an oxygen provider, i.e., potassium chlorate). We have described "antimony trisulfide" of different kinds (triggers and fuels), but we should not forget the importance of countering oxidative stress while cutting off the fuel and the trigger. Indeed, along with cytokines, reactive oxygen species can act as propagators of smoldering inflammation (Liu et al., 2020; Wiegman et al., 2020), morphing the phenomenon from local to systemic. We feel that this is another fertile and yet poorly explored terrain for future investigation.

We hope that articles harnessed in the current Research Topic help the readers with some new clues on how low-grade inflammation is initiated, maintained, and eventually resolved, at least to some extent.

AUTHOR CONTRIBUTIONS

GV wrote the article. NP, FR, and GR edited the article. GV, NP, and GR revised the article. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Intriguing Role of Interleukin 13 in the Pathophysiology of Asthma

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Approximately 5–10% of asthmatic patients worldwide suffer from severe asthma. Experimental and clinical studies have demonstrated that IL-13 is an important cytokine in chronic airways inflammation. IL-13 is involved in Th2 inflammation and has been identified as a possible therapeutic target in the treatment of asthma. Two different human monoclonal antibodies (mAbs) anti-IL-13 (tralokinumab and lebrikizumab) block binding and signaling of IL-13 to its receptors, IL-13Rα1 and IL-13Rα2. Several randomized, double-blind, placebo-controlled multicenter studies have evaluated the safety and efficacy of tralokinumab and lebrikizumab in the treatment of adult patients with severe asthma, but all have failed to meet their primary endpoints. No serious adverse events related to the treatment with these anti-IL-13 mAbs have been reported in these studies. These negative clinical results contrast with positive findings from blocking IL-13 signaling in experimental models of asthma, raising doubts about the transferrable value of some models. Interestingly, dupilumab, a mAb which blocks both IL-4 and IL-13 signaling reduces exacerbation rates and improves lung function in severe asthmatics. These results suggest that IL-4 and IL-13 share some, but not all functional activities in airway inflammation. Tralokinumab might show efficacy in a highly selected cohort of asthmatics characterized by overexpression of IL-13.

Keywords: asthma, biologics, chronic rhinosinusitis, interleukin 4, interleukin 13, nasal polyposis, tralokinumab

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INTRODUCTION

Bronchial asthma is a chronic inflammatory disorder of the airways characterized by reversible airflow obstruction, bronchial hyperreactivity (BHR), mucus overproduction, angiogenesis, and airway remodeling (Detoraki et al., 2010; Holgate et al., 2015). Asthma is a common disorder resulting in substantial morbidity, healthcare expenditure, and death (Lang and Polansky 1994; Lange et al., 1996). Worldwide, up to 300 million people are affected by asthma, making it one of the most common chronic diseases (World Health Organization. Global surveillance, prevention,

and control of chronic respiratory diseases. http://www.who.int/gard/publications/GARD/2007) and approximately 250,000 people die from asthma each year (Lozano et al., 2012). In the majority of patients, asthma can be controlled by combinations of inhaled glucocorticoids (ICS), short- or long-acting ß$_2$-adrenergic agonists (LABA), long-acting muscarinic antagonists, and leukotriene receptor antagonists, according to the Global Initiative for Asthma (GINA) guidelines (GINA http://www.ginasthma.org/pdf). However, in approximately 15% of patients, the disease is refractory to conventional treatments (Chachi et al., 2013; Chachi et al., 2017) and results in hospital admissions due to severe exarcerbations (Sekiya et al., 2016; Kerkhof et al., 2018).

As with many chronic inflammatory diseases, clinicians now realize that the traditional classification of asthma has been an oversimplification (Marone et al., 2005). Different asthma phenotypes, each with distinct pathophysiology, are now being defined as asthma endotypes (Wenzel 2012; Fahy 2015; Hinks et al., 2016; Terl et al., 2017). The heterogeneity of different forms of asthma reflects the involvement of different immune cell populations and inflammatory mediators (Bagnasco et al., 2017; Varricchi et al., 2017; Marone et al., 2019). Asthma can be classified according to two major endotypes: "T2-high" asthma is characterized by increased levels of type 2 inflammation mainly mediated by mast cells, eosinophils, basophils, T-helper 2 cells, group 2 innate lymphoid cells (ILC2s), and immunoglobulin E (IgE)-producing B cells (Fahy, 2015). Patients with T2-high asthma have eosinophilia and other signs of type 2 inflammation including high levels of IL-4 and IL-13 (Fahy, 2015; Kaur and Chupp, 2019). Increased blood and sputum levels of eosinophils, serum IgE, and the fraction of exhaled nitric oxide (FeNO) have been associated with the mechanisms of T2-high asthma (Gandhi et al., 2017; Robinson et al., 2017). Another feature is the high expression of the prostaglandin D_2 receptor chemoattractant receptor-homologous molecule ($CRTH_2$) on Th2 lymphocytes (Cosmi et al., 2000; Marone et al., 2019). "T2-low" asthma is less well characterized and may include several different endotypes (Wenzel, 2012; Choy et al., 2015; Fahy, 2015; Pepper et al., 2017). It has been suggested that Th1 and Th17 pathways and neurogenic inflammation may be involved (Ricciardolo et al., 2017; Samitas et al., 2017).

Levels of IgE, blood eosinophils and FeNO can be useful to guide the selection of monoclonal antibodies (mAbs) in the treatment of different forms of severe uncontrolled asthma. For example, several studies have demonstrated the safety and efficacy of mAbs blocking IL-5 (i.e., mepolizumab and reslizumab) for treatment of patients with severe eosinophilic asthma (Bel et al., 2014; Ortega et al., 2014; Bjermer et al., 2016; Khatri et al., 2019). Benralizumab, a mAb against IL-5Rα, expressed on the surface of human eosinophils (Varricchi et al., 2018a), is particularly effective in patients with severe asthma with high blood eosinophils (FitzGerald et al., 2016; FitzGerald et al., 2018). Because IgE and the high affinity receptor for IgE (FcεRI) play a central role in atopic asthma (Varricchi et al., 2018d; Borriello et al., 2019; Varricchi et al., 2019), a mAb anti-IgE (omalizumab) is indicated for the treatment of patients (aged ≥ 12 years) with moderate-to-severe uncontrolled allergic asthma (Samitas et al., 2015; Hew et al., 2016). Serum concentrations of IgE are used to guide this treatment.

IL-4 and IL-13 were among the first identified cytokines orchestrating Th2 inflammation (Macchia et al., 2015; McCormick and Heller 2015; Bagnasco et al., 2016). IL-4 and IL-13 are potent mediators of type 2 inflammation with both overlapping and distinct functions. Pascolizumab, a mAb selectively blocking IL-4, failed to produce positive effects (Hart et al., 2002). Two mAbs blocking IL-13 (i.e., anrukinzumab, lebrikizumab) have shown marginal effects in the treatment of asthmatic patients (Corren et al., 2011; Gauvreau et al., 2011; Hanania et al., 2015; Bagnasco et al., 2016; Hanania et al., 2016). Another mAb anti-IL-13 (i.e., tralokinumab, AstraZeneca) failed to reduce asthma exacerbation rate in severe uncontrolled asthmatics (Piper et al., 2013; Brightling et al., 2015; Panettieri et al., 2018; Russell et al., 2018). Considering the individual relevance of IL-4 and IL-13 in the pathogenesis of asthma, these results are surprising and intriguing. A simplistic explanation is that individual blockade of IL-4 or IL-13 is insufficient to inhibit the complex orchestration of allergic inflammation and clinical consequences in severe asthma. This hypothesis is indirectly supported by the efficacy of dupilumab, which inhibits both IL-4 and IL-13 signaling mediated by IL-4Rα, in patients with severe uncontrolled asthma (Castro et al., 2018; Rabe et al., 2018).

In this review we analyze the biological and immunological effects of IL-13 in the context of experimental models of asthma and in asthmatic patients. Despite promising findings in several experimental models of allergic inflammation, the results of multicenter studies evaluating the efficacy of anti-IL-13 mAbs in patients with asthma were surprisingly negative. Possible explanations of these discrepancies are discussed.

Biological and Immunological Effects of Interleukin 13

IL-13 is a pleiotropic cytokine originally cloned from activated human T-lymphocytes (Minty et al., 1993). The human *IL13* gene is located on chromosome 5q31-33 in the cluster of genes encoding IL-4, IL-3, IL-5, IL-9, and granulocyte-macrophage colony-stimulating factor (GM-CSF). The gene encoding IL-13 is upstream of the *IL4* gene, leading to the speculation that these genes arose as a duplication event during evolution. However, IL-13 has only 25% homology with IL-4 thus explaining why these cytokines share some, but not all functional properties. IL-13 can be produced by stimulated Th2 cells (de Vries 1998), B lymphocytes (Hajoui et al., 2004), $CD8^+$ cells (Dakhama et al., 2013), type 2 ILCs (Jia et al., 2016), alveolar macrophages

Abbreviations: ACQ, asthma control questionnaire; AHR, airway hypersensitivity; BAL, bronchoalveolar lavage; CRSwNP, chronic sinusitis with nasal polyps; DC, dendritic cell; ERK, extracellular signal-regulated kinase; DPP-4, dipeptidyl peptidase-4; FcεRI, high affinity receptor for IgE; FeNO, fraction of exhaled nitric oxide; FEV1, forced expiratory volume in the first second; ILC, innate lymphoid cell; JAK, Janus kinase; LABA, long-acting β2-agonist; ICS, inhaled glucocorticoid; mAb, monoclonal antibody; OVA, ovalbumin; PEF, peak expiratory flow; PGD2, prostaglandin D2; PRO, patient-reported outcomes; SABA, short-acting β2-agonist; STAT6, signal transducer and activator of transcription; TSLP, thymic stromal lymphopoietin; TYK2, tyrosine kinase 2; VCAM, vascular cell adhesion molecule.

(Hancock et al., 1998), human mast cells (Fushimi et al., 1998), and basophils (Ochensberger et al., 1996; Redrup et al., 1998; Borriello et al., 2015).

Figure 1 schematically illustrates the complex receptor system which mediates the signaling of IL-4 and IL-13. The IL-4Rα subunit is a component of both the type I and type II receptors. Type I receptors are composed of the IL-4Rα subunit complexed with common γ chain (γc); this receptor binds to IL-4 and is expressed on cells of hematopoietic stem cell origin. The type II receptor complex consists of IL-4Rα partnering with IL-13Rα1 and is found on many non-hematopoietic cells, such as bronchial epithelial cells, smooth muscle cells, fibroblasts, and keratinocytes (Akaiwa et al., 2001). IL-4 signals through both the type I and type II receptor complexes whereas IL-13 signals only through the type II complex, because IL-13 binds to IL-13Rα1, whereas IL-4 primarily binds to IL-4Rα (McKenzie et al., 1999). In addition, the two cytokines have different functions and signaling. IL-4Rα, γc, and IL-13Rα1 all contain proline rich regions that can bind the Janus kinases JAK1, JAK2, JAK3, and TYK2. In hematopoietic cells that express γc and the associated JAK3, IL-4 binding to type I receptor results in the activation of JAK1, JAK2, and JAK3 (Hershey, 2003; Bhattacharjee et al., 2013). IL-4 and IL-13 binding to type II receptor activate JAK1, JAK2, and TYK2. Activation of JAKs results in phosphorylation of cytoplasmic tyrosines leading to the recruitment of STAT6 to the receptor, followed by its phosphorylation and activation. The activation of STAT6 is the primary signaling event in the response to IL-4 or IL-13 (Cao et al., 2016). In certain experimental conditions STAT1 and STAT3 can also be activated by both IL-4 and IL-13 (Wang et al., 2004; Bhattacharjee et al., 2013; Pham et al., 2019). The cytoplasmic domain of human IL-13Rα1 contains two tyrosine residues, which might serve as docking sites for STAT3 (Hershey, 2003). Phosphorylated STAT6 and STAT3 monomers dimerize and then translocate to the nucleus, bind to specific DNA elements to regulate transcription (Bhattacharjee et al., 2013).

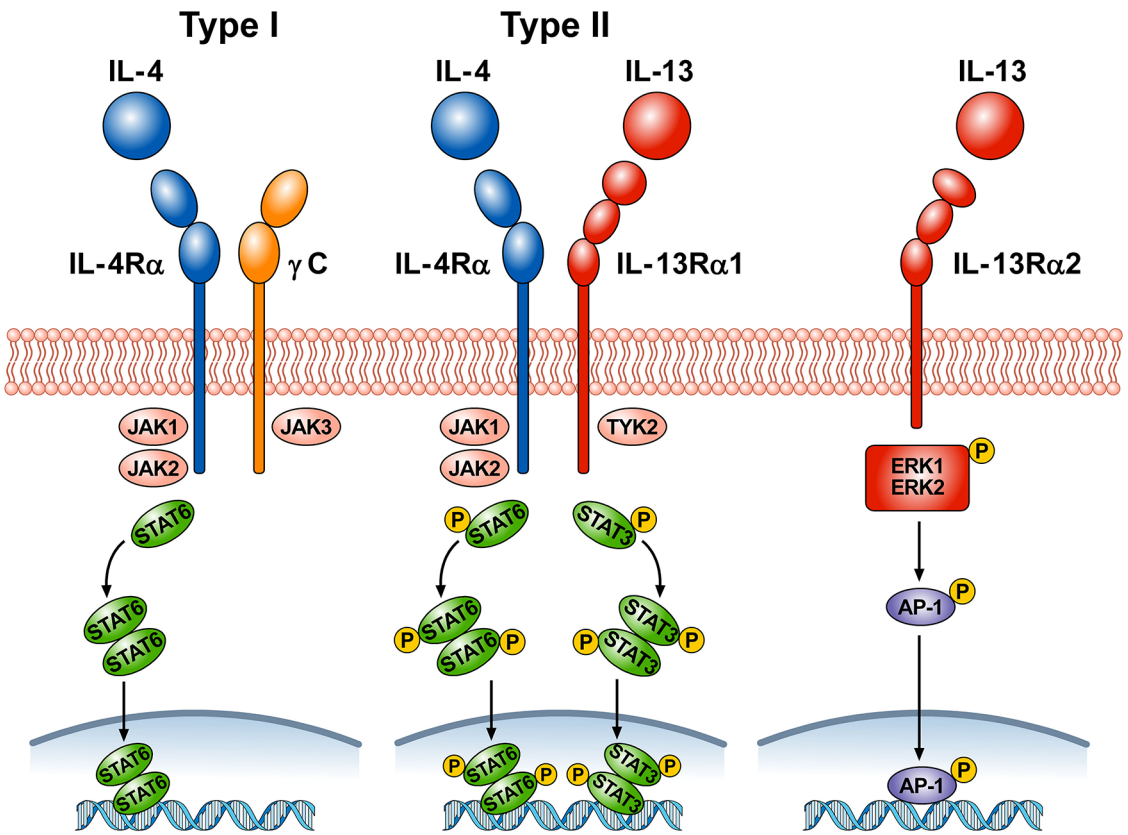

FIGURE 1 | Schematic representation of the three receptors that bind IL-4, IL-13, or both. Type I receptor is composed of the IL-4Rα subunit complexed with common γc. This receptor, expressed on hematopoietic cells, binds to IL-4. Ligand binding by type I receptor complex leads to activation of Janus family kinases (JAK1, JAK2, and JAK3) and subsequent phosphorylation of signal transducer and activator transcription 6 (STAT6). Type II receptor consists of IL-4Rα complexed with IL-13Rα1 and is found in many non-hematopoietic cells (e.g., bronchial epithelial cells, smooth muscle cells, fibroblasts, keratinocytes). Ligand binding type II receptor complex leads to activation of JAK1, JAK2, and tyrosine kinase 2 (TYK2) and subsequent phosphorylation of STAT6 and STAT3. Activation of JAKs leads to the recruitment of STATs to the receptors, followed by STAT phosphorylation and dimerization. Activated STAT dimers translocate to the nucleus, bind specific DNA elements, and initiate activation of downstream genes. IL-4 signals through both type I and type II receptors, whereas IL-13 signals only through type II receptor. IL-13 also binds to a third IL-13Rα2 receptor whose functions are largely unknown. Under certain circumstances, IL-13 signaling through IL-13Rα2 results in phosphorylation of ERK1/2 in a STAT6-independent manner and the formation of the dimeric transcription factor AP-1. Phosporylated AP-1 translocates to the nucleus and bind to specific DNA elements.

LaPorte and collaborators have examined in detail the molecular and structural basis of the IL-4/IL-13 receptor system (LaPorte et al., 2008). They demonstrated that IL-4 first binds to IL-4Rα to form a binary complex which then binds to the γc to form the functional ternary complex (type I receptor). IL-4 and IL-13 can also bind with high affinity to IL-4Rα and IL-13Rα1, respectively (type II receptor). The authors also compared the kinetics and potency of IL-4 and IL-13 signaling. IL-4 induced tyrosine phosphorylation of STAT6 more rapidly and more potently than IL-13. The latter observation was supported by previous experiments on cultured airway smooth muscle cells (Laporte et al., 2001). IL-4 is a central mediator for Th2 cell polarization, initiation of IgE synthesis, and recruitment of eosinophils (Chatila, 2004; Wynn, 2015). Although IL-13 has some redundancy in these effects, this cytokine has additional roles in mediating goblet cell hyperplasia, airway smooth muscle contractility, collagen deposition, and fibrosis (Gour and Wills-Karp 2015).

There is a distinct IL-13Rα2 subunit, to which only IL-13 binds (Chen et al., 2009). Initially this receptor was considered a decoy receptor (Ingram and Kraft, 2012) involved in removing IL-13 by internalization (Wood et al., 2003; Lupardus et al., 2010; Kasaian et al., 2011). Although the IL-13Rα2 lacks canonical JAK-STAT signaling activity (Kawakami et al., 2001), this hypothesis has come into question because several studies have shown that, under certain circumstances, IL-13Rα2 can mediate IL-13 signaling (Fichtner-Feigl et al., 2006; Fujisawa et al., 2009; He et al., 2013). In human airways, it was found that IL-13Rα2 is involved in IL-13 signaling through the transcription factor activator protein-1 (AP-1) to induce the activation of TGF-β (Fichtner-Feigl et al., 2006). Recently, it has been demonstrated that IL-13 induced phosphorylation of ERK1/2 and the downstream activation of AP-1-related genes in human nasal epithelial cells (Liu et al., 2018). The authors proposed that engagement of IL-13Rα2 by IL-13 activates

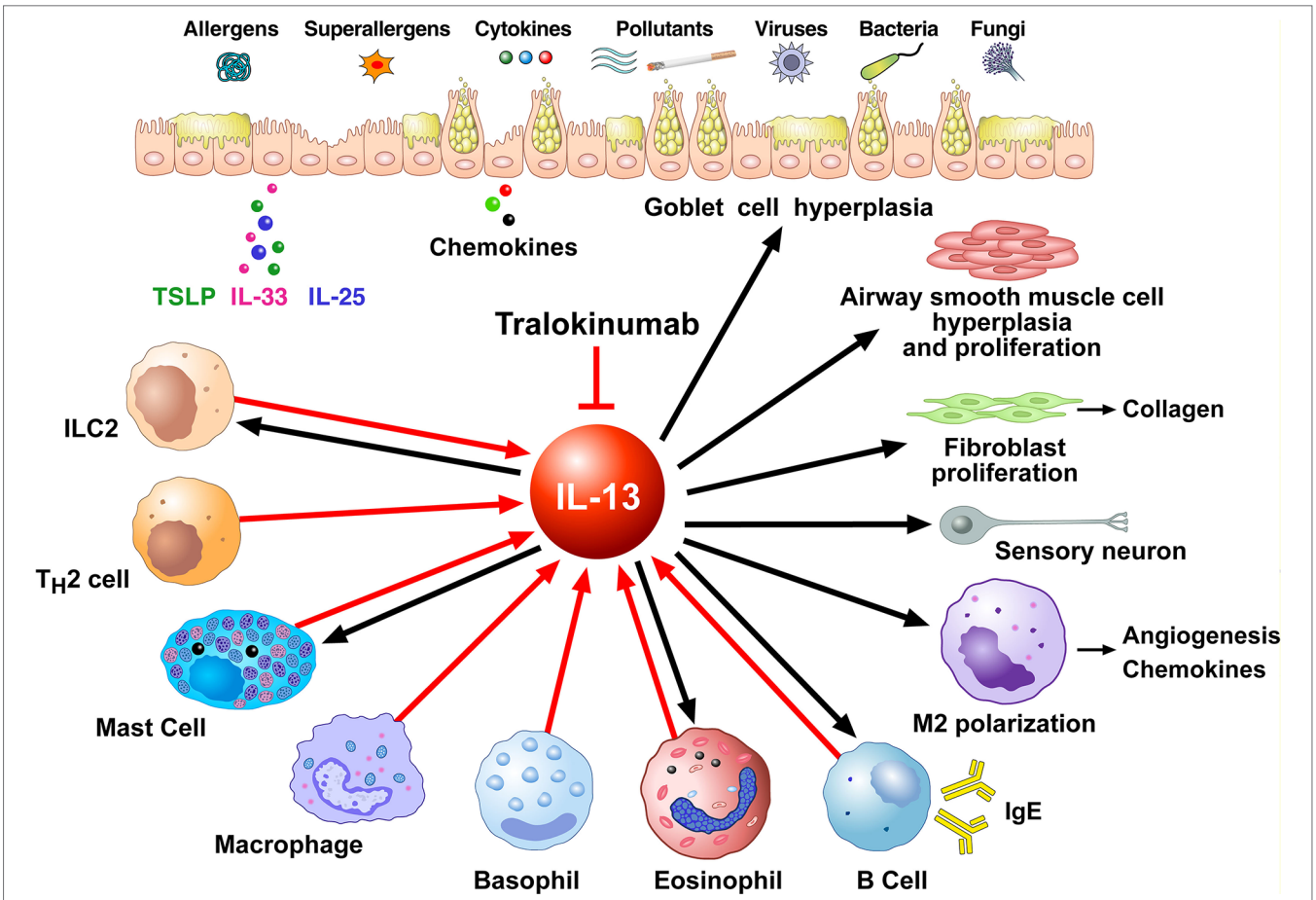

FIGURE 2 | Schematic representation of the cellular sources of IL-13 (red arrows) and its effects of immune and structural cells in asthma (black arrows). Different stimuli (allergens, superallergens, pollutants, viral and bacterial products, etc.) activate epithelial cells which release several cytokines (e.g., thymic stromal lymphopoietin, IL-33, IL-25). These mediators activate a variety of immune cells (ILC2, Th2 cells, mast cells, macrophages, basophils, eosinophils, B cells) which produce several cytokines including IL-13. IL-13 modulates the functions of IL-13^{+} ILC2, mast cells, macrophages, eosinophils, and B cells. This cytokine induces goblet cell hyperplasia and mucus production, airway smooth muscle cell hyperplasia and proliferation, fibroblast activation and collagen deposition, macrophage polarization (M2) and B cell activation and immunoglobulin E production. These effects of IL-13 are mediated by the engagement of type II IL-13 receptor (IL-13Rα1/IL-4Rα expressed on these cells). IL-13, as well as IL-4, can activate sensory neurons through the engagement of type II receptor. Tralokinumab, an anti-IL-13 human IgG_4 monoclonal antibody, binds IL-13, thus preventing it from binding to both IL-13Rα1 and IL-13Rα2 on target cells.

mitogen-activated protein ERK1/2 pathway and downstream AP-1-related gene *C-JUN*.

Figure 2 shows schematically that IL-13 is produced by several immune cells and has many diverse functions on a wide variety of cell types relevant to the pathogenesis of allergic disorders. IL-13 can be produced by activated ILC2 (Shimokawa et al., 2017; Wallrapp et al., 2018), Th2 cells (Finkelman et al., 2004; Wynn, 2015), mast cells (Burd et al., 1995; Fushimi et al., 1998; Varricchi et al., 2019), macrophages (Hancock et al., 1998), basophils (Gibbs et al., 1996; Ochensberger et al., 1996; Redrup et al., 1998; Patella et al., 2000; Genovese et al., 2003), eosinophils (Schmid-Grendelmeier et al., 2002; Varricchi et al., 2018a), and B cells (Hajoui et al., 2004). In human B cells IL-13 has similar effects as IL-4, including promoting B cell proliferation and inducing class switching to IgE and IgG_4 in combination with CD40/CD40L (Oettgen and Geha, 2001) and inducing expression of the low-affinity IgE receptor CD23 (Punnonen et al., 1993; Gould and Sutton, 2008). In macrophages IL-13 favors the M2 polarization (Martinez-Nunez et al., 2011; Bhattacharjee et al., 2013). IL-13 promotes survival, activation, and recruitment of eosinophils (Luttmann et al., 1996; Horie et al., 1997; Pope et al., 2001). In addition, IL-13 stimulates eosinophil trafficking from the peripheral blood to the site of inflammation by inducing the production of IL-5 and eosinophil chemokines such as eotaxins (Webb et al., 2000; Rosenberg et al., 2007) IL-13 promotes FcεRI expression and proliferation of human mast (Nilsson and Nilsson, 1995; Kaur et al., 2006).

IL-13 also has important effects on non-hematopoietic cells, including endothelial cells, smooth muscle cells, fibroblasts, epithelial cells, and sensory neurons. IL-13 is a potent inducer of vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells, an important aspect in the recruitment of eosinophils (Bochner et al., 1995). In addition, IL-13 increases the expression of $ß_1$ integrin and VCAM-1 on human lung fibroblasts (Doucet et al., 1998) and increases muscular contraction in response to acetylcholine (Laporte et al., 2001; Grunstein et al., 2002). Furthermore, IL-13 enhances proliferation and cholinergic-induced contraction of smooth muscle cells (Wills-Karp, 2001) and induces collagen synthesis in human fibroblasts contributing to airways remodeling. In epithelial cells, IL-13 is a potent inducer of eotaxin (Li et al., 1999). Moreover, IL-13 induces mucus overproduction and goblet cell metaplasia (Kuperman et al., 2002; Kondo et al., 2006). IL-13 induces vascular endothelial growth factors (VEGFs) (Corne et al., 2000) which are pro-angiogenic factors relevant in bronchial asthma (Detoraki et al., 2009; Detoraki et al., 2010; Varricchi et al., 2018b). Recently, it has been demonstrated that IL-13 and IL-4 directly activate mouse and human sensory neurons which express IL-13Rα1 and IL-4Rα (Oetjen et al., 2017).

Interleukin 13 in Experimental Models of Asthma

Seminal studies in animal models of allergic asthma demonstrated that selective neutralization of IL-13 reduced airway hypersensitivity (AHR), bronchoalveolar lavage (BAL) eosinophils, and mucus overproduction (Grunig et al., 1998; Wills-Karp et al., 1998). Furthermore, IL-13 delivery to the airways caused all of these effects (Grunig et al., 1998; Wills-Karp et al., 1998). Overexpression of IL-13 in the lung of mice caused mucus hypersecretion, subepithelial fibrosis, eotaxin production, and eosinophilic infiltration (Zhu et al., 1999). Interestingly, mice with targeted deletion of IL-13 failed to develop allergen-induced AHR, despite the presence of eosinophilic pulmonary infiltration (Walter et al., 2001). Mice lacking STAT6 were protected from pulmonary effects of IL-13 (Kuperman et al., 2002). Importantly, reconstitution of STAT6 in epithelial cells only was sufficient for IL-13-induced AHR and mucus production in the absence of inflammation and fibrosis. Administration of anti-IL-13 in a mouse model of chronic asthma inhibited eosinophil recruitment in the airways, goblet cell hyperplasia, and subepithelial fibrosis, but only marginally inhibited AHR (Kumar et al., 2004). In another study, blockade of IL-13 with sIL-13Rα2-human IgG fusion protein inhibited AHR associated with brief allergen exposure, but did not modify AHR associated with chronic airway remodeling (Leigh et al., 2004).

An IL-13 vaccine prepared by inserting a murine IL-13 peptide into a viral carrier protein, induced sustained and intense anti-IL-13 IgG antibodies (Ma et al., 2007), associated with inhibition of ovalbumin (OVA)-induced acute airway allergic responses. In a more recent study, the same experimental approach suppressed BAL IL-13 concentration and eosinophils, subepithelial collagen deposition, and mucus hyperproduction (Ma et al., 2013). Interestingly, while IL-13 vaccine inhibited AHR development, it did not revert AHR. The latter findings suggest that IL-13 may be crucial in the development, but not in the maintenance of airway hyperresponsiveness.

Il13ra2 gene silencing or blockade of IL-13Rα2 signaling led to marked downregulation of TGF-β1 production and collagen deposition in a model of lung fibrosis (Fichtner-Feigl et al., 2006). In IL-13Rα2-deficient mice, AHR and airway inflammation (i.e., mucus production and BAL eosinophils) were attenuated compared to wild type mice following house dust challenge (Chen et al., 2013). More recently, it was reported that, in IL-4Rα-deficient mice, IL-13, but not IL-4, was required for development of OVA-mediated AHR and goblet cell hyperplasia (Kirstein et al., 2016). Munitz and collaborators studying *Il13ra1* $^{-/-}$ mice found that IL-13Rα1 is critical for baseline IgE production, AHR, mucus production, and eotaxin production. By contrast, Th2 and IgE responses to antigen were IL-13Rα1-independent (Munitz et al., 2008).

In a novel mouse model of non-allergic asthma overexpression of the activator protein-1 (AP-1) subunit Fra2, caused airway inflammation with IL-13 overexpression, BAL eosinophilia, mucus hyperproduction, AHR, and peribronchial collagen deposition (Gungl et al., 2018). Administration of anti-IL-13 antibody markedly decreased STAT6 phosphorylation in the lung, BAL eosinophilia, and goblet cell hyperplasia. However, peribronchial collagen deposition and bronchial smooth muscle width were not affected by anti-IL-13 administration. The interesting results obtained in this experimental model may be more reflective of severe asthma which exhibit poor response to mAb anti-IL-13.

Genetic deletion of the IL-33 receptor in a mouse model of experimental asthma increased TSLP production, which

stimulated the emergence of IL-13$^+$ ILC2s and lung mast cells leading to airway hyperresponsiveness (Verma et al., 2018).

Collectively, the results derived from different experimental models indicate that IL-13 plays an important role in the development of several aspects of asthma. However, it is becoming evident that IL-13 neutralization is not sufficient to reverse certain aspects of airway inflammation once they are established.

Interleukin 13 Expression in Asthmatic Patients

Increased concentration of IL-13 have been found in the blood (Alasandagutti et al., 2017), sputum, bronchial mucosa (Berry et al., 2004), and BAL fluid (Prieto et al., 2000) of asthmatic patients compared to healthy individuals. Increased IL-13 expression in asthma was confirmed by IL-13 mRNA overexpression (Truyen et al., 2006) and by *ex vivo* stimulation of sputum T cells (Boniface et al., 2003). Following allergen challenge, IL-13 is increased in BAL (Huang et al., 1995; Kroegel et al., 1996). *Ex vivo* BAL T cells express IL-13 mRNA (Bodey et al., 1999); expression is inversely related to forced expiratory volume in 1 s (FEV_1) (Barcelo et al., 2006). Several studies have shown that IL-13 is expressed in bronchial biopsies in patients with asthma (Kroegel et al., 1996; Naseer et al., 1997; Berry et al., 2004; Saha et al., 2008). IL-13$^+$ ILC2 were increased in the circulation of asthmatic patients with levels correlating with asthma severity (Jia et al., 2016). High production of IL-13 by cord blood CD4$^+$ T cells is a predictor of development of atopic disorders (Martinez 2002).

Polymorphisms in the Interleukin 13/Interleukin 4 Receptor Complex Associated With Asthma

Polymorphisms have been identified both in the IL-13 promoter (IL-13-1112 T) and the coding region (IL-13+Arg 130Gen, IL-13+2044G > A) in asthmatic patients (Hershey et al., 1997; Graves et al., 2000; Heinzmann et al., 2000; Arima et al., 2002; Cameron et al., 2006). One of the polymorphisms (Arg 130 Gln) (A/G) identified occur in the region critical for receptor-ligand interactions. Multiple polymorphism in IL-4R gene have also been identified and associated with asthma (Hershey et al., 1997; Kruse et al., 1999; Ober et al., 2000). Recently, three meta-analyses of IL-13 polymorphisms in adults and children suggested that the IL-13+1923 C/T polymorphism is associated with increased risk of asthma (Liu et al., 2014; Mei and Qu, 2017; Xu et al., 2017).

Tralokinumab and Lebrikizumab for the Treatment of Severe Uncontrolled Asthma

Several anti-IL-13 mAbs (anrukinzumab, lebrikizumab, tralokinumab) have provided an opportunity to investigate the role of this cytokine in the pathophysiology of severe asthma, as well as assessing treatment response. A phase 1 study evaluated the pharmacokinetics, safety, and tolerability of tralokinumab in asthmatic patients receiving three different i.v. doses: 1 mg/kg, 5 mg/kg, or 10 mg/kg (Singh et al., 2010). Despite the small sample size, pharmacokinetics were linear over the dose range studied. The half-life was found to be 2–3 weeks and tralokinumab exhibited an acceptable safety profile.

A phase 2a, randomized, double-blind, placebo-controlled, parallel-group, multicenter study investigated the effects of different dose regimens of tralokinumab in 194 adults with moderate-to-severe asthma inadequately controlled with standard therapy (Piper et al., 2013). Three dose regimens were evaluated: 47 patients received s.c. tralokinumab 150 mg, 51 patients received tralokinumab 300 mg, 48 subjects received tralokinumab 600 mg, and 48 patients received placebo. The primary endpoint was the change from baseline in mean Asthma Control Questionnaire score (ACQ-6) at week 13. Secondary endpoints were change in FEV_1, pre-bronchodilator lung function, patient-reported outcomes (PROs), rescue β_2-agonist use, and safety outcomes. Mean ACQ-6 score improved in all treatment groups, from baseline to week 13. These changes in ACQ-6 persisted through week 24 and were greater in active patients with higher IL-13 sputum concentrations compared with subjects with lower IL-13 sputum concentrations or subjects receiving placebo. Improvement in FEV_1 were higher in patients with peripheral eosinophil counts ≥ 300 cells/ml. Pulmonary function improvements were higher in tralokinumab patients with higher sputum IL-13 (≥ 10 pg/ml^{-1}) compared to tralokinumab patients with lower sputum IL-13 (≤10 pg/ml^{-1}) and patients receiving placebo. However, there was no significant difference in asthma exacerbation rate. The authors defined asthma exacerbations as either a progressive increase of asthma symptoms (cough, wheeze, chest tightness, and/or shortness of breath) or a reduction of >20% in peak expiratory flow or FEV_1 from baseline that did not resolve after the initiation of rescue medications and resulted in an administration of systemic glucocorticoids.

The phase 2b clinical trial run by Brightling and colleagues evaluated safety and tolerability profile and the reduction in exacerbation rate and FEV_1 improvement (Brightling et al., 2015). The authors randomized 452 severe asthmatic patients, all with two-to-six asthma exacerbations in the previous year, to receive tralokinumab (300 mg s.c. either every 2 weeks or every 2 weeks for 3 months and then every 4 weeks) or placebo as add-on therapy for 1 year. At the end of the study, they reported no changes in the annual exacerbation rate at week 52 (primary endpoint) in patients treated with tralokinumab, either every 2 or 4 weeks, *versus* placebo or in time to first exacerbation. The authors defined asthma exacerbation as an increase in asthma symptoms resulting in use or increase in dose of systemic glucocorticoids for three or more consecutive days. Similarly, secondary endpoints such as improvements in prebronchodilator FEV_1, ACQ-6, and AQLQ(S) were not significant in patients treated with tralokinumab compared with placebo. They found a significant improvement in FEV_1 in patients treated with tralokinumab every 2 weeks. In this study measurement of serum dipeptidyl peptidase-4 (DPP-4) and periostin concentrations were included as a predictive candidate biomarkers before the study was unmasked. Subgroup analyses of patients receiving tralokinumab every 2 weeks with airway reversibility at baseline, but not receiving oral glucocorticoids, showed some clinical improvements in the subgroup who had raised serum DPP-4

and periostin (Brightling et al., 2015). These preliminary results suggested that certain subpopulations of patients with severe asthma might respond to tralokinumab treatment.

A phase 2 multicenter, double-blind, randomized, placebo-controlled trial evaluated the effects of tralokinumab on eosinophilic airway inflammation in uncontrolled moderate-to-severe asthma (MESOS) (Russell et al., 2018). In this study, participants aged 18–75 years were randomly assigned to receive tralokinumab (300 mg s.c. every 2 weeks) or placebo. The primary outcome measure was change from baseline to week 12 in bronchial biopsy eosinophil count. Secondary outcome measures included change in blood and sputum eosinophil counts. Exploratory outcomes included FeNO and blood IgE concentrations. Tralokinumab did not affect bronchial, peripheral blood, or eosinophil counts compared to placebo at week 12. FeNO concentrations and total blood IgE were significantly reduced. The authors concluded that IL-13 is not crucial for eosinophilic airway inflammation control in patients with moderate-to-severe asthma.

Two large phase 3 clinical trials, STRATOS 1 and STRATOS 2, explored the use of periostin and DPP-4 as biomarkers of IL-13-driven inflammatory patterns in patients aged 12–75 years with severe uncontrolled asthma treated with tralokinumab (300 mg s.c. every 2 weeks for 52 weeks) or placebo (Panettieri et al., 2018). The primary endpoint was the annualized asthma exacerbation rate reduction at week 52 in the all-comers population for STRATOS 1 and in the biomarker-positive population for STRATOS 2. The results of both trials confirmed that tralokinumab did not improve the annual asthma exacerbation rate in the all-comers population with severe asthma. In contrast to the preliminary results of the phase 2 trial (Brightling et al., 2015), periostin and DPP-4 were not shown to predict response to tralokinumab. In both trials, tralokinumab-treated participants had a small increase in blood eosinophil counts from baseline, whereas placebo-treated participants did not. A recent meta-analysis of six randomized clinical trials suggested that tralokinumab was well tolerated and modestly improved FEV_1 but did not reduce asthma exacerbations in severe uncontrolled asthma (Zhang et al., 2019).

Given the need to reduce oral glucocorticoid administration in patients with severe asthma, treatments that may allow tapering of glucocorticoids without loss of disease control are needed. A 40-week, randomized, double-blind trial (TROPOS) evaluated the oral glucocorticoid-sparing potential of tralokinumab in patients with severe, uncontrolled asthma requiring maintenance glucocorticoid treatment plus ICS/LABA (Busse et al., 2019). One hundred forty patients were randomized to tralokinumab (300 mg s.c. every 2 weeks) or placebo. The primary endpoint was percentage change from baseline in average glucocorticoid dose at week 40, while maintaining asthma control. Secondary endpoints included patients with a prescribed maintenance glucocorticoid dose of ≤ 5 mg, those with greater than 50% reduction in prescribed maintenance glucocorticoids dose and annual asthma exacerbation rate. An asthma exacerbation was defined as worsening of asthma that required a temporary increase in systemic glucocorticoids for ⩾3 days or that resulted in an emergency-room or urgent-care visit that led to a temporary increase in systemic glucocorticoids for ⩾3 days to treat symptoms or an inpatient hospitalization due to asthma. There were no significant between-group differences for primary and secondary endpoints. Reporting of adverse events and serious adverse events were similar for the tralokinumab and placebo groups.

A randomized, double-blind, placebo controlled study examined the effects of another anti-IL-13 mAb, lebrikizumab (250 mg s.c. once monthly for 6 months), on change in prebronchodilator FEV_1 from baseline to week 12 in 219 adults with uncontrolled asthma (Corren et al., 2011). Lebrikizumab treatment was associated with greater improvement in percent change in FEV_1 in patients with high pretreatment levels of serum periostin compared to patients with low periostin levels. In two replicate studies (LUTE and VERSE) in patients with moderate-to-severe uncontrolled asthma, lebrikizumab (37.5, 125 or 250 mg s.c. every 4 weeks) reduced asthma exacerbation rate by 60% compared to placebo in periostin-high patients and by 5% in periostin-low patients (Hanania et al., 2015). In these studies the authors defined asthma exacerbation as new or increased asthma symptoms that led to treatment with systemic glucocorticoids or to hospitalization. Two replicate, phase 3 trials (LAVOLTA1 and LAVOLTA2) explored the use of periostin and eosinophilia (≥ 300 cells/μl) as biomarkers of IL-13-driven inflammatory patterns in patients with severe uncontrolled asthma treated with lebrikizumab (37.5 mg or 125 mg s.c. once every 4 weeks for 52 weeks or placebo) (Hanania et al., 2016). The primary endpoint was the reduction in the rate of asthma exacerbations over 52 weeks in biomarker-high patients (periostin ≥ 50 ng/ml or blood eosinophils ≥ 300 cells/μl. In contrast to the preliminary results of phase 2 trials (Corren et al., 2011; Hanania et al., 2015), lebrikizumab did not consistently show significant reduction in asthma exacerbations in biomarker-high patients.

Anti-Interleukin 13 in Nasal Polyposis

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a common and relevant comorbidity for severe asthma (Heffler et al., 2013). The prevalence of CRSwNP is greater in asthmatics compared to the general population (Settipane and Chafee, 1977) and it increases with the severity of asthma (Pearlman et al., 2009; Lin, 2011), with the highest prevalence rates in non-atopic, late-onset, severe asthmatics (Amelink et al., 2013). More than 60% of patients with CRSwNP have asthma (Ragab et al., 2004; Guida et al., 2010). Moreover, CRWwNP is one of the significant determinants of poor asthma control (Heffler et al., 2013) and a hallmark of refractory eosinophilic asthma (Amelink et al., 2013). The clinical evidence of a strong relationship between CRSwNP and severe asthma raises the possibility of a shared pathogenesis. In particular both diseases seem to be dependent on eosinophilic inflammation mediated by epithelial cytokines such as thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 secreted as a consequence of epithelial damage (Boita et al., 2016; Metha 2016; Varricchi et al., 2018c). The latter activation is mediated by ILC2 which produce several type 2 cytokines such as IL-13 and IL-5 (Aron and Akbari 2017; Poposki et al., 2017). Recently, it has been reported that there were significant increases in IL-13, IL-13Rα1, and IL-13Rα2

mRNA and protein concentrations in nasal polyp epithelium (Liu et al., 2018). Moreover, IL-13 treatment resulted in mucus overproduction and impairment of ciliary function of human nasal epithelial cells. Therefore, an anti-IL-13 strategy, such as tralokinumab, may be helpful in the management of patients with nasal polyps. Unfortunately, there are no clinical trials of treatment of CRSwNP with anti-IL13 mAbs. Interestingly, patients treated with dupilumab, a mAb against IL-4Rα, the common receptor for both IL-4 and IL-13 had a significant reduction in polyp size and improvement in symptoms and nasal and olfactory function (Bachert et al., 2016).

CONCLUDING REMARKS AND PERSPECTIVES

Three phase 2 clinical trials (Piper et al., 2013; Brightling et al., 2015; Russell et al., 2018) and three phase 3 clinical trials (Panettieri et al., 2018; Busse et al., 2019) have shown that tralokinumab did not lower the annual exacerbation rate and did not improve ACQ-6 scores compared to placebo in severe uncontrolled asthmatic patients. These negative findings parallel the results of two replicate, phase 3 trials with lebrikizumab (Hanania et al., 2016). These negative results are surprising given the wide spectrum of pro-inflammatory and pro-fibrogenic activities of IL-13 in experimental models of asthma (Chu et al., 1998; Laporte et al., 2001; Komai et al., 2003; Kanoh et al., 2011) and in asthmatic patients (Huang et al., 1995; Kroegel et al., 1996; Boniface et al., 2003; Truyen et al., 2006; Saha et al., 2008; Jia et al., 2016).

There are several possible explanations of these negative findings. First, it is likely that IL-13 is not the main cytokine involved in the complex network of severe asthma pathogenesis, meaning blocking it alone is ineffective. Second, as shown in several trials (Brightling et al., 2015; Hanania et al., 2016; Panettieri et al., 2018), the biomarkers (e.g., periostin, DPP-4, peripheral eosinophil count) used to identify responders to anti-IL-13 therapy are not optimal. Third, in both experimental models (Walter et al., 2001) and clinical studies (Brightling et al., 2015; Hanania et al., 2016; Panettieri et al., 2018) blocking IL-13 appears to have no effect on reducing tissue or blood eosinophilia, the pathophysiological feature most closely linked to asthma exacerbations. Fourth, perhaps the route of administration (i.e., s.c.) and the size of the anti-IL-13 mAb particles are not ideal. For instance, it has been reported the preliminary efficacy of a nebulized inhaled anti-IL-13 mAb antigen-binding fragment in macaque model of asthma (Lightwood et al., 2018). Finally, we cannot exclude the possibility that some of these negative findings could be due to the inclusion in clinical trials of patients with Th2-low asthma.

Interestingly, the initial attempts to develop cytokine therapies for asthma focusing on antagonizing IL-4 were also unsuccessful (Hart et al., 2002; Steinke 2004), as was an attempt to block the combined receptor (Wenzel et al., 2007; Burmeister Getz et al., 2009). By contrast, dupilumab, which binds to IL-4Rα and consequently blocks both IL-4 and IL-13 signaling, decreases asthma exacerbations and improves respiratory symptoms in patients with persistent asthma (Castro et al., 2018; Rabe et al., 2018). These observations suggest that only the effective simultaneous blockade of signaling from two main cytokines (i.e., IL-4 and IL-13) is effective in the treatment of severe asthma. Using allergic preclinical models, it has been demonstrated that the combined blockade of the IL-13 and IL-33 pathways leads to a greater inhibition of type 2 inflammation over inhibition of either pathway alone (Ramirez-Carrozzi et al., 2017). Similarly, co-blockade of IL-13 and IL-25 attenuated AHR, eosinophil infiltration in the lung, and mucus hyperproduction in a mouse model of OVA-induced asthma (Zhang et al., 2017). Recently, a novel dual antagonist anti-TSLP/IL-13 bispecific antibody has been described (Venkataramani et al., 2018). It will be interesting to see whether combinatorial blockade of multiple cytokines, including IL-13, may yield additional efficacy over single-axis therapies alone.

It is intriguing that IL-13 blockade modulates several aspects of different experimental models of allergic asthma (Laporte et al., 2001; Walter et al., 2001; Komai et al., 2003; Munitz et al., 2008; Chen et al., 2013; Ma et al., 2013; Chachi et al., 2017). Moreover, recent results demonstrate that anti-IL-13 antibody improves bronchial hyperresponsiveness and mucus production in a mouse model of non-allergic asthma (Gungl et al., 2018). These positive experimental results contrast with negative results in the treatment of asthmatic patients with different anti-IL-13 mAbs (tralokinumab and lebrikizumab) (Piper et al., 2013; Brightling et al., 2015; Hanania et al., 2016; Panettieri et al., 2018; Russell et al., 2018; Busse et al., 2019). These findings highlight that the results from murine studies do not always predict clinical effectiveness.

In conclusion, despite several efforts, attempts to demonstrate a benefit of anti-IL-13 in patients with severe asthma remain unproven. While this may be because the right predictive biomarkers or patient phenotypes have not yet been identified, it is perhaps more likely that there is enough redundancy in the pathophysiology of severe asthma to persist without IL-13. This is not to say, at this stage, that another clinical indication might not be found for IL-13-blocking drugs in future.

AUTHOR CONTRIBUTIONS

All authors contributed to reviewing the current literature and writing of the manuscript and approved the final version of the paper. Conceptualization: GM, GS, GV. Original draft preparation: GM, GS, GV. Final editing: GM, FG, VP, AP, EH, SL, GS, GV.

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REVIEW
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Interleukin-5 in the Pathophysiology of Severe Asthma

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Interleukin-5 (IL-5) exerts a central pathogenic role in differentiation, recruitment, survival, and degranulation of eosinophils. Indeed, during the last years, significant advances have been made in our understanding of the cellular and molecular mechanisms underlying the powerful actions of IL-5 finalized to the induction, maintenance, and amplification of eosinophilic inflammation. Therefore, IL-5 is a suitable target for add-on biological therapies based on either IL-5 inhibition (mepolizumab, reslizumab) or blockade of its receptor (benralizumab). These modern treatments can result in being definitely beneficial for patients with severe type 2 (T2)-high eosinophilic asthma, refractory to conventional anti-inflammatory drugs such as inhaled and even systemic corticosteroids.

Keywords: IL-5, eosinophils, T2-high asthma, mepolizumab, reslizumab, benralizumab

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INTRODUCTION

Asthma is a chronic and heterogeneous airway disorder, characterized by recurrent respiratory symptoms including wheezing, cough, and chest tightness, which are caused by usually reversible airflow limitation due to bronchial inflammation and remodeling (Holgate et al., 2015; Pelaia et al., 2015). In particular, many patients with severe asthma express a type 2 (T2)-high phenotype featured by eosinophilic inflammation (Bousquet et al., 1990; Schleich et al., 2014). Indeed, airway eosinophilic infiltration is quite frequent in both allergic and non-allergic asthma, and can also occur in severe and fatal disease (Huber and Koessler, 1922; Houston et al., 1953; Varricchi et al., 2016; Haldar, 2017). T2-high asthma is characterized by accumulation of eosinophils within the airways, where these cells produce and release cytokines, chemokines, growth factors, cytotoxic proteins, and lipid mediators, which together play a relevant role in the pathobiology of bronchial inflammation and remodeling (Bochner and Gleich, 2010). Detection of high eosinophil counts in both peripheral blood and induced sputum is a common feature of T2-high asthma. In this regard, it is noteworthy that airway eosinophilia can occur in more than half asthmatic subjects, and high eosinophil levels are associated with recurrent asthma exacerbations and severe bronchial obstruction (Bousquet et al., 1990).

The most important biological factor responsible for eosinophil differentiation, growth, activation, survival, and recruitment to airways is interleukin-5 (IL-5) (Stirling et al., 2001; Fulkerson and Rothenberg, 2013; Varricchi and Canonica, 2016). Therefore, this cytokine exerts key functions in the pathogenesis of eosinophilic asthma, which is often therapeutically responsive to corticosteroids because of its effective ability to induce eosinophil apoptosis (Zhang et al., 2000). However, severe eosinophilic asthma may be resistant to both inhaled and systemic

corticosteroids, also because of an excessive bronchial amount of IL-5, which can thereby overcome the pro-apoptotic effects of these drugs on eosinophils (Dunican and Fahy, 2017). Hence, patients with severe T2-high eosinophilic asthma, refractory to corticosteroids, may experience an inadequate control of respiratory symptoms and frequent disease exacerbations, thus being characterized by relevant unmet needs. Moreover, in these subjects, IL-5-dependent eosinophilia can also contribute to the development of clinically significant comorbidities such as chronic rhinosinusitis with nasal polyps. Indeed, these upper airway disorders originate from cellular and molecular mechanisms, which appear to be very similar to those underlying type 2 inflammation in asthma (Heffler et al., 2018, 2019; Ahern and Cervin, 2019).

For all such reasons, in severe T2-high asthma, IL-5 represents a pivotal pathogenic factor and a highly valuable target for add-on biological therapies of corticosteroid-resistant, difficult-to-treat eosinophilic phenotypes (Varricchi and Canonica, 2016; Brussino et al., 2018). In particular, several monoclonal antibodies have been developed against either IL-5 (mepolizumab, reslizumab) or its receptor (benralizumab), thereby making it possible to break down the main pathobiological pathway implicated in eosinophilic asthma (Egan et al., 1999; Gnanakumaran and Babu, 2003; Kolbeck et al., 2010; Pelaia et al., 2016; Bagnasco et al., 2017; Pelaia et al., 2017; Varricchi et al., 2017a,b; Bagnasco et al., 2018a,b; Pelaia et al., 2018a,b,c, 2019).

Taking together the above considerations, it is very clear that IL-5 plays a central role as the most important pathogenic mediator responsible for eosinophilic asthma, as well as a crucial therapeutic target for anti-asthma biological treatments. Therefore, the aim of the present review article is to discuss the pathobiological interactions between IL-5 and T2-high eosinophilic asthma, the mechanism of action of IL-5, and the relevance of both this cytokine and its receptor as targets of selective anti-eosinophil monoclonal antibodies.

IL-5 AND EOSINOPHILIC ASTHMA

The main cellular sources of IL-5 include T helper-2 (Th2) lymphocytes and group 2 innate lymphoid cells (ILC2) (**Figure 1**; Woodruff et al., 2009; Brusselle et al., 2013; Walker et al., 2013; Smith et al., 2016; Yanagibashi et al., 2017). Th2 cells produce and secrete IL-5 upon a complex activation process triggered by inhaled allergens and driven by dendritic cells (Lambrecht et al., 2019). In this regard, the presence of interleukin-4 (IL-4) is essential, because of its requirement for Th2 cell commitment and activation *via* stimulation of key transcription factors such as STAT6 and GATA3 (Lambrecht and Hammad, 2015). IL-5 release from ILC2 is dependent on GATA3 activation induced by epithelial innate cytokines including IL-25, IL-33, and especially thymic stromal lymphopoietin (TSLP) (**Figure 1**; Lambrecht and Hammad, 2015). In addition to ILC2 and Th2 cells, other cellular sources of IL-5 include invariant natural killer (NK) T cells, mast cells, and eosinophils themselves (**Figure 1**; Shakoory et al., 2004; Sakuishi et al., 2007; Hogan et al., 2008). In particular, by releasing IL-5 activated mast cells implement a bidirectional cross-talk with eosinophils (Galdiero et al., 2017). Such functional interactions between mast cells and eosinophils, also supported by physical contacts involving these two cell types, harbor the so-called "allergic effector unit" (Minai-Fleminger et al., 2010; Galdiero et al., 2017).

IL-5 is a powerful pro-inflammatory cytokine that is responsible for maturation, proliferation, activation, and migration of eosinophils (**Figure 1**). The close pathogenic link between IL-5 and eosinophilic inflammation has been clearly demonstrated using both animal and human experimental models of asthma (Greenfeder et al., 2001). In particular, IL-5 is responsible for airway eosinophilia and bronchial hyperresponsiveness induced by allergen challenge in sensitized guinea pigs (Mauser et al., 1993). Moreover, in the lungs of these animals, an eosinophilic inflammatory response can be experimentally evoked by recombinant human IL-5 (Lilly et al., 1996). Similar to guinea pigs, upon allergen challenge, an IL-5-dependent influx of eosinophils was also detected in bronchoalveolar lavage fluid (BALF) and lung tissue of sensitized mice (Kung et al., 1994, 1995). Such results have been further corroborated by demonstrating that bronchial eosinophilia and airway hyperresponsiveness, induced by multiple allergen challenges, were abrogated in sensitized IL-5-deficient mice (Foster et al., 1996; Kopf et al., 1996). In experimental monkey models of asthma, IL-5 was capable of inducing bronchial eosinophilia and the consequent airway hyperresponsiveness (Mauser et al., 1995). Furthermore, it has been shown in both rabbits and humans that delivery of recombinant IL-5 to airway smooth muscle enhanced the contractile response to acetylcholine (Hakonarson et al., 1999), and this effect was probably mediated by the release of eosinophil granule proteins (Elbon et al., 1995). In atopic patients experiencing both early and late asthmatic reactions, the bone marrow responds to antigen challenge by enhancing the production of eosinophils, which resulted in being associated with an increase in IL-5 mRNA levels (Wood et al., 2002). In addition, IL-5 prolonged eosinophil survival in allergen-challenged atopic asthmatics (Ohnishi et al., 1993).

In allergic asthmatic subjects, the eosinophilopoietic actions of IL-5 take place in both bone marrow and bronchial mucosa ("*in situ* eosinophilopoiesis"), where this cytokine promotes eosinophil differentiation and maturation from CD34+ hematopoietic progenitor cells (Wood et al., 2002; Dorman et al., 2004; Bhalla et al., 2018). In fact, elevated IL-5 levels and high cell counts of eosinophil progenitors and mature eosinophils can be found in induced sputum from patients with allergic asthma (Dorman et al., 2004). Furthermore, in comparison to both healthy controls and subjects with mild asthma, higher serum IL-5 concentrations were detected in patients with severe disease (Greenfeder et al., 2001). IL-5 synergizes with eotaxins, thus contributing to recruit eosinophils to asthmatic airways (Fulkerson and Rothenberg, 2013). Indeed, high levels of IL-5 and eotaxins were found in induced sputum from patients experiencing acute asthma exacerbations (Park et al., 2003). A synergic action is also exerted by IL-5 in conjunction with IL-18 (Kandikattu et al., 2019). In particular, concomitant increases of serum levels of IL-5 and IL-18 were found in patients with asthma, and the concentrations of these

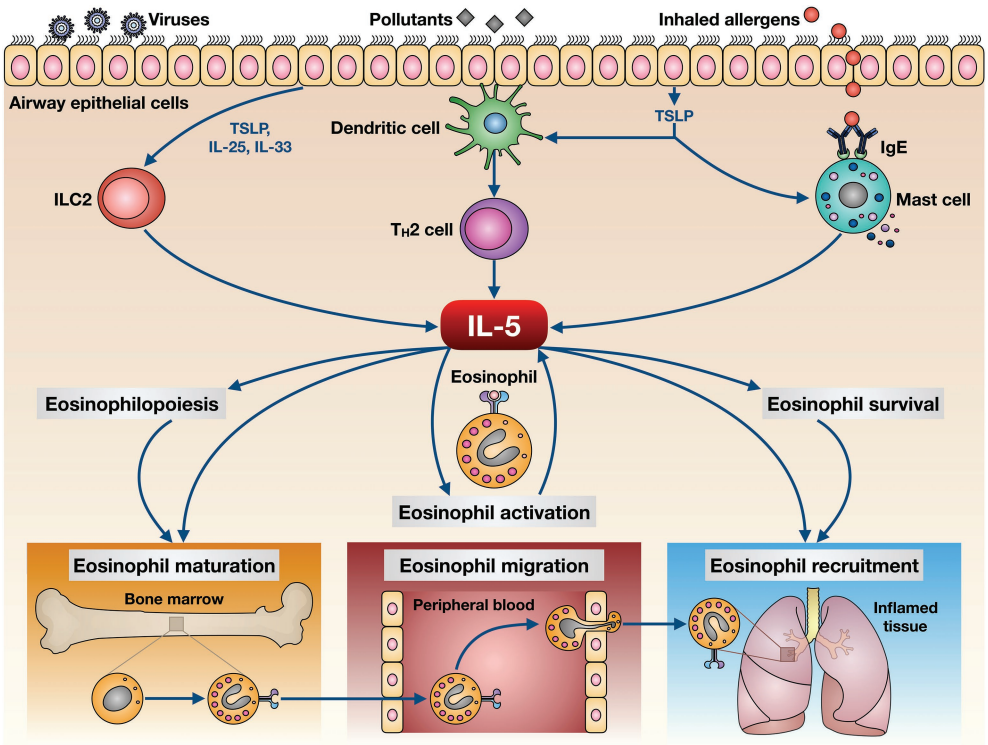

FIGURE 1 | Biological actions exerted by IL-5 on eosinophils. IL-5 is produced by several cellular elements, including Th2 lymphocytes, group 2 innate lymphoid cells (ILC2), mast cells, and eosinophils. These cells release IL-5 upon activation triggered by multiple environmental stimuli such as inhaled allergens, respiratory viruses, and airborne pollutants. IL-5 exerts pleiotropic effects on eosinophils, thereby promoting their maturation, activation, survival, migration from bloodstream, and recruitment to airways.

two cytokines correlated with disease exacerbations (Kandikattu et al., 2019). IL-5 and IL-18 strongly cooperate to induce eosinophil development and functional activation. IL-5 also inhibits eosinophil apoptosis, and sputum IL-5 levels were reported to be negatively correlated with apoptotic eosinophils in subjects with either asthma exacerbations or stable disease (Xu et al., 2007; Ilmarinen et al., 2014). Moreover, in T2-high asthma IL-5 induces eosinophil adhesion to and migration in the extracellular matrix by favoring the interaction of eosinophils with periostin, a matricellular protein whose enhanced expression is associated with eosinophil trafficking toward bronchi (Johansson, 2017). IL-5 is also involved in the pathobiology of late-onset, non-allergic eosinophilic asthma (Brusselle et al., 2013). In this case, ILC2 and not Th2 lymphocytes are mainly responsible for IL-5 production (Walker et al., 2013). Differently from blood and airway pro-inflammatory eosinophils, the lung resident subsets of homeostatic anti-inflammatory and anti-allergic eosinophils seem to be partially independent from IL-5, at least in mice (Marichal et al., 2017).

With regard to the pathobiology of asthma, in addition to promoting the development and amplification of eosinophilic inflammation, IL-5 is also implicated in the induction of airway remodeling (Kay et al., 2004). Indeed, in murine models of asthma, it has been shown that IL-5 gene deletion was associated with a parallel suppression of both lung eosinophilia and bronchial remodeling (Cho et al., 2004). On the other hand, IL-5 transgenic mice were reported to be characterized by an enhanced airway fibrotic response to repeated allergen challenges (Tanaka et al., 2004). The results of these animal studies have been further corroborated by examining the bronchial biopsies taken from asthmatic patients treated with an anti-IL-5 monoclonal antibody (Flood-Page et al., 2003). In particular, it was demonstrated through confocal microscopy that anti-IL-5 treatment decreased the thickness of reticular basement membrane by reducing the deposition of extracellular matrix proteins such as procollagen III, tenascin, and lumican (Flood-Page et al., 2003).

IL-5: MECHANISM OF ACTION

The biological effects of IL-5 are mediated by its selective interaction with the IL-5 receptor (IL-5R), consisting of a specific α subunit (IL-5Rα) and a non-specific βc heterodimer, which can be recognized also by interleukin-3 (IL-3) and granulocyte-macrophage colony stimulating factor (GM-CSF) (**Figure 2**; Rossjohn et al., 2000; Murphy and Young, 2006). IL-5 binds as a homodimeric protein to IL-5Rα, which is highly expressed on eosinophil surface (Varricchi et al., 2016), thus recruiting the βc dimer and inducing the assembly of the IL-5/IL-5Rα/βc ternary complex (Broughton et al., 2012). When IL-5 is absent, IL-5Rα is complexed with the intracellular tyrosine kinase Janus kinase

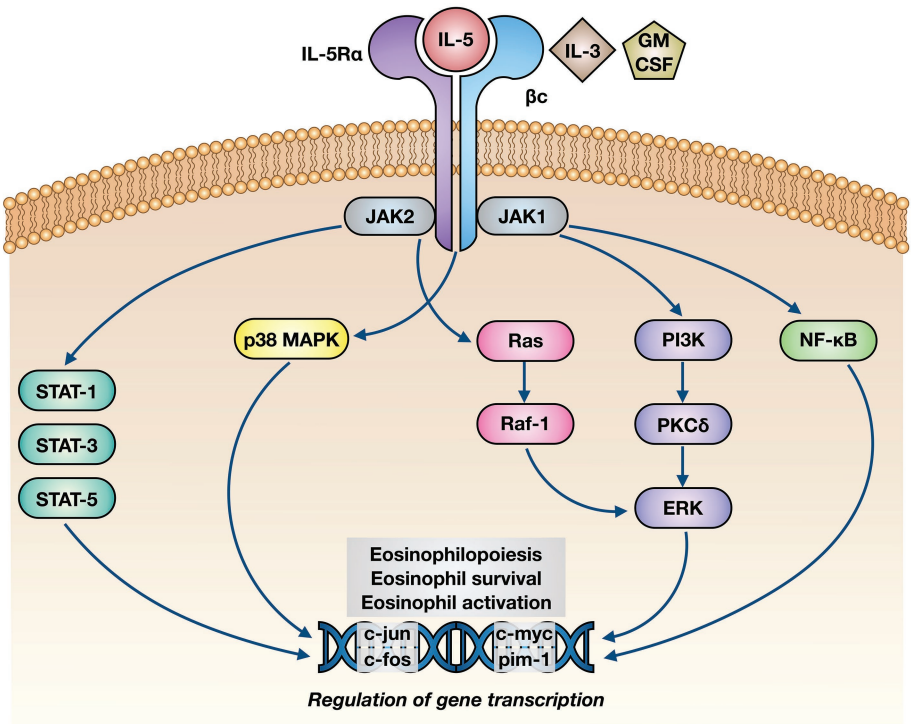

FIGURE 2 | Molecular mechanisms of action underlying the effects of IL-5 on eosinophils. IL-5 binds to the α subunit of IL-5 receptor (IL-5Rα), thereby inducing its interaction with βc subunits and the following activation of a complex intracellular signaling network, consisting of JAK1/2-STAT1/3/5 modules, p38 and ERK MAP kinases, and NF-κB transcription factor. The consequent stimulation of specific target genes leads to eosinophil maturation, survival, and activation.

(JAK)2, whereas the βc subunit is associated with JAK1 (Kouro and Takatsu, 2009). When IL-5 is present, it binds to IL-5Rα and drives the constitution of a functional IL-5Rα/βc receptor complex, which is responsible for the activation of an intricate network of signaling pathways (**Figure 2**; Johanson et al., 1995; Ishino et al., 2008; Molfino et al., 2012). In particular, binding of IL-5 to IL-5Rα sequentially activates JAK2 and signal transducers and activators of transcription (STAT)1, 3, and 5, which in turn stimulate the transcriptional functions of many genes involved in eosinophil proliferation, including pim-1 and cyclin D3 (Pazdrak et al., 1995; Stout et al., 2004). Moreover, JAK2 is engaged in active cooperation with Lyn and Raf-1 kinases, and such functional interactions lead to inhibition of eosinophil apoptosis (Pazdrak et al., 1998); the inhibitory effect of IL-5 on eosinophil apoptosis is also mediated by NF-κB-dependent induction of the anti-apoptotic protein Bcl-xL (Schwartz et al., 2015; Amruta and Kandikattu, 2018). Raf-1 also stimulates eosinophil degranulation (Pazdrak et al., 1998).

Other signal transduction modules activated by IL-5 include further intracellular kinases such as phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinases (MAPK) (**Figure 2**). In particular, *via* activation of extracellular signal-regulated kinases (ERK)1/2 and protein kinase C (PKC), PI3K mediates IL-5-induced interaction of eosinophils with intercellular adhesion molecule-1 (ICAM-1) (Sano et al., 2005). Ras-Raf-1-mediated activation of the ERK subfamily of MAPK drives c-fos gene transcription, which is involved in several eosinophil functions including cell maturation, survival, and proliferation, as well as stimulation of the production of the powerful eosinophil chemoattractant leukotriene C4 (Adachi and Alam, 1998; Bates et al., 2000; Pelaia et al., 2005; Takatsu and Nakajima, 2008; Thompson-Souza et al., 2017). Furthermore, through a NF-κB-dependent mechanism, p38 MAPK up-regulates eosinophil biosynthesis of pro-inflammatory cytokines, and also stimulates eosinophil recruitment within the context of allergic inflammatory responses (Adachi et al., 2000; Ip et al., 2005; Pelaia et al., 2005).

Therefore, because of the pivotal role played by IL-5 in the pathophysiology of T2-high asthma, this cytokine and its receptor represent key molecular targets for current biological therapies aimed to improve the control of severe and difficult-to-treat eosinophilic disease (Varricchi et al., 2016; Bagnasco et al., 2018a,b; McGregor et al., 2019; Siddiqui et al., 2019).

IL-5 AND ITS RECEPTOR: MOLECULAR TARGETS FOR BIOLOGICAL THERAPIES OF SEVERE ASTHMA

In clinical practice, three monoclonal antibodies, namely mepolizumab, reslizumab, and benralizumab, are currently available, which make it possible to effectively interfere with the pathogenic IL-5/IL-5R pro-eosinophilic axis. While mepolizumab

and reslizumab are selective IL-5 inhibitors, benralizumab is an IL-5 receptor antagonist (**Figure 3**).

Mepolizumab (SB-240563) is a humanized IgG1/k monoclonal antibody that specifically binds with high affinity to IL-5, thereby impeding its linkage to IL-5Rα (**Figure 3**; Gnanakumaran and Babu, 2003; Walsh, 2015; Fainardi et al., 2016; Varricchi et al., 2017a). In particular, mepolizumab was developed by incorporating specific murine antibody fragments targeting human IL-5 into a human IgG1 heavy chain (Hart et al., 2001). The relevant benefits induced by mepolizumab in patients with severe refractory eosinophilic asthma have been clearly documented by many randomized controlled trials (RCT) (Pelaia et al., 2017; Varricchi et al., 2017a). Initially, the efficacy of mepolizumab was demonstrated by Nair et al. and Haldar et al. in a few patients with severe eosinophilic asthma experiencing frequent disease exacerbations (Haldar et al., 2009; Nair et al., 2009), These two seminal studies were the first ones to show that mepolizumab was able to significantly decrease asthma exacerbations, and this effect was concomitant with a sharp reduction of both sputum and blood eosinophils (Haldar et al., 2009; Nair et al., 2009). In addition to such results, using chest CT (computed tomography) scan, Haldar et al. also documented that mepolizumab decreased both thickness and total area of bronchial walls (Haldar et al., 2009). These observations corroborated previous findings published by Flood-Page et al., who reported that mepolizumab was capable of reducing the amount of extracellular matrix proteins deposited within the sub-epithelial reticular basement membrane of airway mucosa; this effect was associated with decreased BALF concentrations of transforming growth factor-β1 (TGF-β1) (Flood-Page et al., 2003). Therefore, it can be inferred that the potential anti-remodeling effect of mepolizumab was probably a consequence of the depleting action exerted on eosinophils, which are important cellular sources of TGF-β1, a prominent growth factor involved in the pathobiology of the airway structural changes occurring in asthma (Makinde et al., 2007).

Later, Pavord et al. performed the phase 2b/3 DREAM (Dose Ranging Efficacy And safety with Mepolizumab in severe asthma) trial, thus confirming, in a much larger study population, that mepolizumab lowered sputum and blood eosinophil levels, and also significantly decreased the asthma exacerbation rate (Pavord et al., 2012). Subsequently, two further studies named MENSA (MEpolizumab as adjunctive therapy iN patients with Severe Asthma) and SIRIUS (SteroId ReductIon with mepolizUmab Study) were conducted by Ortega et al. and Bel et al., respectively. Both trials demonstrated that, in patients with severe eosinophilic asthma, mepolizumab decreased the number of asthma exacerbations, improved symptom control and quality of life, and also induced a slight FEV_1 (forced expiratory volume in 1 s) increase (Bel et al., 2014; Ortega et al., 2014), Moreover, the SIRIUS trial provided convincing evidence about the oral corticosteroid-sparing action of mepolizumab, consisting of a 50% decrease in prednisone intake (Bel et al., 2014). More recently, the phase IIIb MUSCA study, performed by Chupp et al., confirmed the ability of mepolizumab to improve health-related quality of life (Chupp et al., 2017). All these studies also showed that mepolizumab is characterized by a very good safety and tolerability profile. The main RCT referring to mepolizumab have been summarized in **Table 1**. In addition to RCT, mepolizumab is also undergoing evaluation within the context of real-life studies carried out in daily clinical practice. In this regard, preliminary data suggest that in a real-world setting mepolizumab can result in being even more effective than in RCT, and such findings might

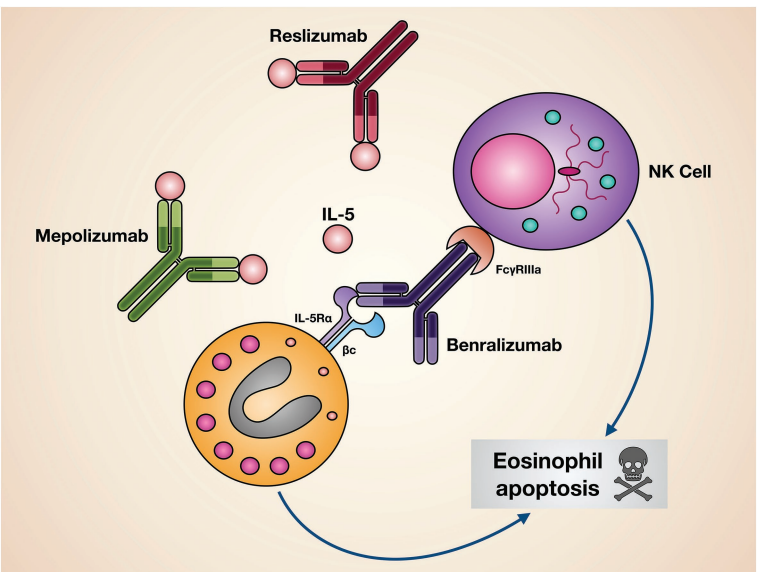

FIGURE 3 | Mechanisms of action of biological drugs targeting IL-5 or its receptor. Mepolizumab and reslizumab interact with IL-5, thus inhibiting its biological effects on eosinophils. Benralizumab blocks *via* its Fab fragments IL-5Rα, thereby neutralizing IL-5 bioactivity. Moreover, through its Fc constant region benralizumab binds to the FcγIIIRa receptor expressed by natural killer cells, enabling them to induce eosinophil apoptosis.

TABLE 1 | Mepolizumab: main randomized clinical trials.

Authors	Inclusion criteria	*N*	Main results
Flood-Page et al. (2003)	Mild atopic asthmatics	11	↓ Blood and BALF eosinophils = FEV_1, = PEF, = airway hyperresponsiveness
Haldar et al. (2009)	Eosinophilic asthma	61	↓ Blood and sputum eosinophils = FEV_1, = FeNO, = airway hyperresponsiveness ↓ Exacerbations ↑ QoL
Nair et al. (2009)	Prednisone-dependent eosinophilic asthma	9	↓ Blood and sputum eosinophils ↓ Exacerbations
Pavord et al. (2012)	Severe eosinophilic asthma	462	↓ Blood and sputum eosinophils = FEV_1, = FeNO, = AQLQ, = ACQ ↓ Exacerbations
Ortega et al. (2014)	Severe eosinophilic asthma	385	↑ FEV_1 ↓ Exacerbations, ↓ Hospitalizations ACQ-5 and SGRQ improvement
Bel et al. (2014)	Severe eosinophilic asthma	135	↓ Blood and sputum eosinophils ↓ Exacerbations, ↓ OCS intake ACQ-5 improvement
Chupp et al. (2017)	Severe eosinophilic asthma	274	↑ FEV_1, ↑ $FEF_{25–75}$ ACQ-5 and SGRQ improvement

BALF, Bronchoalveolar lavage fluid; FEV_1, Forced expiratory volume in 1 s; PEF, Peak expiratory flow; FeNO, Exhaled fraction of nitric oxide; QoL, Health-related quality of life; AQLQ, Asthma quality of life questionnaire; ACQ, Asthma control questionnaire; SGRQ, St. George's Respiratory Questionnaire; OCS, Oral corticosteroids; $FEF_{25–75}$, Forced expiratory flow at 25–75% of forced vital capacity.

depend on the higher blood eosinophil counts characterizing real-life patients when compared to asthmatics enrolled in RCT (Pelaia et al., 2018a; Bagnasco et al., 2018b).

Reslizumab (SCH55700) is a humanized IgG4/κ monoclonal antibody which includes in its structure the complementarity-determining regions of the rat monoclonal IgG2a antibody JES1-39D10, that specifically interact with the epitope encompassing amino acids 89–92 of human IL-5, thereby preventing its binding to IL-5Rα (**Figure 3**; Zhang et al., 1999). In regard to add-on biological therapy of severe eosinophilic asthma, the efficacy and safety of reslizumab have been evaluated in several RCT (Pelaia et al., 2016; Varricchi et al., 2017b). The first phase 2 study was performed by Kips et al., who showed that reslizumab lowered blood and sputum eosinophil counts, and also induced a transient FEV_1 increase (Kips et al., 2003). A subsequent, larger phase 2 trial, carried out by Castro et al., demonstrated that reslizumab significantly increased FEV_1, and also elicited a non-significant trend toward a better asthma control, especially in highly eosinophilic patients with concomitant nasal polyposis (Castro et al., 2011), Later, two phase 3 studies were conducted by Castro et al., who demonstrated the effectiveness of reslizumab in decreasing by 50–59% the annual rate of asthma exacerbations in severe asthmatics with blood eosinophil counts >400 cells/ml (Castro et al., 2015); reslizumab also improved asthma symptom control and enhanced FEV_1 (Castro et al., 2015). The beneficial effects of reslizumab on lung function were further confirmed by another phase 3 trial carried out by Bjermer et al., who reported that reslizumab not only increased FEV_1, but also improved airflow limitation at level of peripheral airways, as shown by significant increases in $FEF_{25–75}$ (forced expiratory flow at 25–75% of forced vital capacity) (Bjermer et al., 2016). More recently, an additional phase 3 trial performed by Brusselle et al. highlighted that reslizumab was able to reduce asthma exacerbations and improve

TABLE 2 | Reslizumab: main randomized clinical trials.

Authors	Inclusion criteria	*N*	Main results
Kips et al. (2003)	Severe asthmatics	18	↓ Blood and sputum eosinophils Transient FEV_1 increase
Castro et al. (2011)	Poorly controlled eosinophilic asthma	61	↓ Blood and sputum eosinophils ↑ FEV_1, ↑ FVC ACQ-5 improvement
Castro et al. (2015)	Severe eosinophilic asthma	953	↓ Blood eosinophils ↑ FEV_1 ↓ Exacerbations AQLQ, ACQ-7, ASUI improvement
Bjermer et al. (2016)	Severe eosinophilic asthma	315	↓ Blood eosinophils ↑ FEV_1, ↑ $FEF_{25–75}$ ACQ-5, ACQ-6, AQLQ, ASUI improvement
Brusselle et al. (2017)	Severe eosinophilic asthma	477	↓ Exacerbations ↑ FEV_1

FEV_1, Forced expiratory volume in 1 s; FVC, forced vital capacity; PEF, Peak expiratory flow; ACQ, Asthma control questionnaire; AQLQ, Asthma quality of life questionnaire; ASUI, Asthma symptom utility index; $FEF_{25–75}$, forced expiratory flow at 25–75% of forced vital capacity.

lung function, especially in patients with eosinophilic late-onset asthma (Brusselle et al., 2017). Taken together, the results of the above studies evidenced a good safety and tolerability profile of reslizumab. The main RCT referring to reslizumab have been summarized in **Table 2**.

TABLE 3 | Benralizumab: main randomized clinical trials.

Authors	Inclusion criteria	*N*	Main results
Bleecker et al. (2016)	Severe asthma	797	↑ FEV_1 ↓ Exacerbations ACQ-6 and AQLQ improvement
FitzGerald et al. (2016)	Severe eosinophilic asthma	866	↑ FEV_1 ↓ Exacerbations ACQ-6 and AQLQ improvement
Ferguson et al. (2017)	Severe eosinophilic asthma	106	↓ Blood eosinophils ↑ FEV_1 = ACQ-6, = AQLQ
Nair et al. (2017)	Severe eosinophilic asthma	145	↓ Exacerbations ↓ OCS intake
Busse et al. (2019)	Severe eosinophilic asthma	1,576	Long-term safety and tolerability

FEV_1, Forced expiratory volume in 1 s; ACQ, Asthma control questionnaire; AQLQ, Asthma quality of life questionnaire; OCS, Oral corticosteroids.

Benralizumab (MEDI-563) is a humanized afucosylated IgG1/κ monoclonal antibody, developed *via* hybridoma technology, whose Fab fragments contain murine amino acid sequences which selectively recognize the isoleucine-61 residue of the domain 1 of human IL-5Rα, located near IL-5 binding site (Ishino et al., 2004; Koike et al., 2009; Kolbeck et al., 2010). As a consequence, the interaction of benralizumab with its recognition site on IL-5Rα impedes IL-5 binding to target cells (**Figure 3**), thus preventing hetero-dimerization of IL-5Rα and βc subunits, as well as the subsequent activation of IL-5-dependent signaling pathways. Furthermore, through the constant Fc region benralizumab binds to the FcγRIIIa membrane receptor expressed by natural killer cells (**Figure 3**), which upon FcγRIIIa activation release the pro-apoptotic proteins granzyme B and perforin, responsible for eosinophil apoptosis implemented *via* antibody-dependent cell-mediated cytotoxicity (ADCC), a mechanism which is markedly amplified by afucosylation (Shields et al., 2002; Ghazi et al., 2012).

Several phase 3 RCT have recently shown that, as add-on treatment of severe eosinophilic asthma, benralizumab is characterized by an excellent pattern of efficacy, safety, and tolerability (Pelaia et al., 2018b,c; Gonzalez et al., 2019). In particular, CALIMA and SIROCCO trials showed that benralizumab significantly decreased the annual rate of severe eosinophilic exacerbations of asthma, and also improved asthma symptom control and enhanced FEV_1 (Bleecker et al., 2016; FitzGerald et al., 2016). Benralizumab-induced improvement in

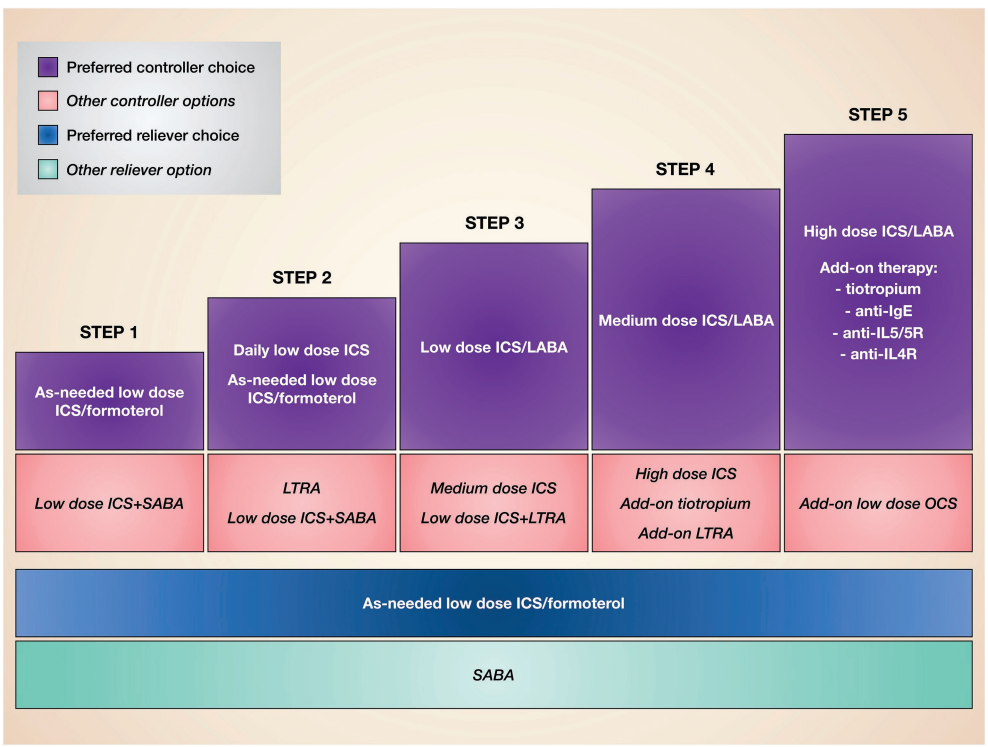

FIGURE 4 | Stepwise therapy of asthma. Current asthma treatment is based on a stepwise approach, consisting of progressive therapeutic increases until disease control is achieved.

lung function was also confirmed by BISE study (Ferguson et al., 2017). In addition, the ZONDA study demonstrated that benralizumab was able to significantly lower the daily intake of oral corticosteroids (Nair et al., 2017). Moreover, the BORA trial showed that a long-term use of benralizumab was associated with a very good safety and tolerability profile (Busse et al., 2019). Furthermore, it is noteworthy that benralizumab appears to be very effective in both allergic and non-allergic severe asthma (Chipps et al., 2018). The main RCT referring to benralizumab have been summarized in **Table 3**. The latter findings have also been recently confirmed by preliminary real-life observations, which suggest that in daily clinical practice the therapeutic actions of benralizumab may result in being even more rapid and effective with respect to RCT (Pelaia et al., 2019).

In regard to IL-5 receptor blockade finalized to the treatment of eosinophilic asthma, a potential alternative approach to the use of monoclonal antibodies can be represented by the development of small molecule antagonists (Uings and McKinnon, 2002). Within such a context, an isothiazolone compound was identified, which appeared to be able to selectively interfere with IL-5/IL-5R interaction (Devos et al., 1994; Uings and McKinnon, 2002). However, to our knowledge this small molecule IL-5R antagonist has not yet reached the stage of clinical investigation.

CONCLUDING REMARKS

Our very strong awareness of the pivotal pathobiological role played by IL-5 in T2-high eosinophilic asthma makes it critical to carefully characterize asthmatic patients on the basis of their inflammatory substrate, as well as in consideration of the clinical and functional responses to standard treatments. Indeed, the most relevant unmet needs are experienced by both allergic and non-allergic asthmatics who are not well controlled by corticosteroids, also because of the prominent pro-eosinophilic action of IL-5, which probably overwhelms the potential efficacy of conventional anti-inflammatory drugs. Therefore, under such circumstances, IL-5 and its receptor may represent valuable therapeutic targets. In this regard, several RCT and some preliminary real-life studies have clearly shown that mepolizumab, reslizumab, and benralizumab are safe and effective as add-on biological therapies for patients with difficult-to-treat eosinophilic asthma. Indeed, such biologics are currently included within the step 5 of GINA (Global Initiative for Asthma) guidelines (**Figure 4**; Global Initiative for Asthma, 2019). Therefore, the only limitation of these monoclonal antibodies depends on their high cost (Anderson and Szefler, 2019). Although the use of anti-eosinophilic biological treatments for severe asthma can significantly decrease the intake of oral corticosteroids, the number of emergency visits and hospitalizations, as well as the loss of work- and school-days, and their cost-effectiveness should be improved by price reductions eventually provided by manufacturers (Anderson and Szefler, 2019). Hopefully, lower costs of mepolizumab, reslizumab, and benralizumab could make these drugs more affordable by health care systems of economically weak countries.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Chronic Adipose Tissue Inflammation Linking Obesity to Insulin Resistance and Type 2 Diabetes

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Obesity is one of the major health burdens of the 21st century as it contributes to the growing prevalence of its related comorbidities, including insulin resistance and type 2 diabetes. Growing evidence suggests a critical role for overnutrition in the development of low-grade inflammation. Specifically, chronic inflammation in adipose tissue is considered a crucial risk factor for the development of insulin resistance and type 2 diabetes in obese individuals. The triggers for adipose tissue inflammation are still poorly defined. However, obesity-induced adipose tissue expansion provides a plethora of intrinsic signals (e.g., adipocyte death, hypoxia, and mechanical stress) capable of initiating the inflammatory response. Immune dysregulation in adipose tissue of obese subjects results in a chronic low-grade inflammation characterized by increased infiltration and activation of innate and adaptive immune cells. Macrophages are the most abundant innate immune cells infiltrating and accumulating into adipose tissue of obese individuals; they constitute up to 40% of all adipose tissue cells in obesity. In obesity, adipose tissue macrophages are polarized into pro-inflammatory M1 macrophages and secrete many pro-inflammatory cytokines capable of impairing insulin signaling, therefore promoting the progression of insulin resistance. Besides macrophages, many other immune cells (e.g., dendritic cells, mast cells, neutrophils, B cells, and T cells) reside in adipose tissue during obesity, playing a key role in the development of adipose tissue inflammation and insulin resistance. The association of obesity, adipose tissue inflammation, and metabolic diseases makes inflammatory pathways an appealing target for the treatment of obesity-related metabolic complications. In this review, we summarize the molecular mechanisms responsible for the obesity-induced adipose tissue inflammation and progression toward obesity-associated comorbidities and highlight the current therapeutic strategies.

Keywords: obesity, insulin resistance, diabetes, low-grade inflammation, adipose tissue inflammation, innate immune system, adaptive immunity, inflammatory triggers

INTRODUCTION

Overweight and obesity are the consequence of a chronic imbalance between energy intake and energy expenditure, culminating in the excess of fat accumulation in AT. Since 1980, the global incidence of overweight and obesity has risen to the extent that almost one-third of the world population is now considered being overweight or obese (Chooi et al., 2019).

Obesity is a heterogeneous condition deriving from genetic and lifestyle interactions (Albuquerque et al., 2015; Hopkins and Blundell, 2016; MacLean et al., 2017; Schwartz et al., 2017) and is correlated with several pathological dysfunctions with important repercussions for individual and community health (Kyrou et al., 2018). Lifestyle and behavioral interventions (e.g., increased physical activity and decreased caloric intake) are fundamental parts for weight control (Butryn et al., 2011; Greenway, 2015). A gradual weight loss up to 16% of the original body weight is sufficient to improve β-cell function and insulin sensitivity in AT, liver, and skeletal muscle (Magkos et al., 2016). The improved glycemic control after weight loss is due, in part, to the dysregulated expression of genes involved in cholesterol flux, lipid synthesis, ECM remodeling, and oxidative stress (Magkos et al., 2016). In the light of the foregoing, obesity is the most frequent metabolic disorder in the world and the primary risk factor for IR and diabetes mellitus (Boles et al., 2017).

Diabetes mellitus refers to a group of conditions in which the body cannot use and store glucose correctly (American Diabetes Association, 2018). The proportion of individuals affected by diabetes mellitus has risen dramatically over the previous three decades, making it one of the major causes of death in the world. More than 300 million people are expected to develop T2D as a complication of obesity by 2025 (Ncd Risk Factor Collaboration [Ncd-RisC], 2016).

T2D is the most prevalent form of diabetes mellitus (American Diabetes Association, 2018), a chronic disease characterized by increased plasma glucose levels due to insulin secretion deficiencies (i.e., β-cell dysfunction) and IR (i.e., decreased target tissue capacity to react regularly to insulin) (American Diabetes Association, 2018).

Obesity is a risk factor for IR and a complete understanding of the mechanisms linking obesity to IR will enhance our knowledge of T2D pathogenesis and the capacity to manage obesity-related disorders (Choi and Cohen, 2017; Maksymets et al., 2018). For this purpose, several studies have been conducted on human and transgenic animal models demonstrating a correlative and causative association between dietary excess and activation of the innate and adaptive immune system in organs that control systemic energy homeostasis (Lumeng et al., 2007b; Lumeng and Saltiel, 2011; Lackey and Olefsky, 2016).

The initial mechanistic evidence supporting the inflammatory origin of obesity and diabetes comes from human and animal studies carried out in the early 1990s. In these studies, AT from obese rodents and humans show inflammatory modifications and enhanced secretion of pro-inflammatory cytokine TNF-α able to induce IR by inactivating the IRS-1 (Hotamisligil et al., 1993, 1995; Uysal et al., 1998). The pivotal role of TNF-α is significantly supported by evidence establishing that TNF-α neutralization in obese mice improves insulin sensitivity and glucose metabolism (Hotamisligil et al., 1993).

Low-grade chronic AT inflammation (also noted as meta-inflammation) is strongly and consistently associated with excess body fat mass and is characterized by infiltration and activation of pro-inflammatory macrophages and other immune cells that produce and secrete pro-inflammatory cytokines and chemokines (Chawla et al., 2011; Burhans et al., 2018).

Macrophages change not only their number during obesity (i.e., up to 40% of all AT cells in this context) but also their location and inflammatory phenotype (Weisberg et al., 2003). While in normal weight subjects the macrophages show anti-inflammatory properties, the polarization of AT macrophages (ATMs) in obese AT shifts to a pro-inflammatory phenotype (Lumeng et al., 2007a; Castoldi et al., 2016; Boulenouar et al., 2017). In obesity, macrophages surround dead adipocytes (i.e., forming crown-like structures) and secrete an array of pro-inflammatory cytokines that lead to local and systemic inflammation and IR (Lumeng et al., 2007a; Haase et al., 2014).

The inflammatory triggers are still almost unknown; however, obesity-induced AT remodeling provides a plethora of intrinsic signals (e.g., adipocyte death, hypoxia, and mechanical stress) capable of initiating an inflammatory response (Reilly and Saltiel, 2017). The role of inflammation in T2D pathogenesis and associated metabolic complications has led to a growing interest in targeting inflammatory mediators or pathways to prevent and treat T2D (Shoelson et al., 2006; McLaughlin et al., 2016).

In this review, we address the primary role played by the loss of immune regulation in the AT inflammation and the development of obesity-associated disorders, providing details on molecular aspects. We highlight the cellular and molecular triggers for obesity-induced inflammation and finally give some insights into the new anti-inflammatory therapeutic strategies.

Abbreviations: AMPK, AMP-activated kinase; AT, adipose tissue; ATMs, adipose tissue macrophages; CRP, C-reactive protein; DAMPs, danger-associated molecular patterns; DCs, dendritic cells; ECM, extracellular matrix; ER, endoplasmic reticulum; Flt3L, Fms-like tyrosine kinase 3 ligand; HbA1c, hemoglobin A1c; HFD, high-fat diet; HIF-1, Hypoxia-inducible factor-1; HREs, hypoxia-response elements; IFN-γ, Interferon-γ; IKKβ, IκB kinase β; IL-18, Interleukin-18; IL-1R, IL-1 receptor; IL-1β, Interleukin-1β; IL-6, Interleukin-6; ILC2s, innate lymphoid cells; iNKTs, Invariant natural killer T cells; IR, insulin resistance; IRS-1, insulin receptor substrate-1; IRSs, insulin receptor substrates; IκBs, inhibitors of κB; JNK, C-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MCP-1, Monocyte chemotactic protein-1; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NKs, natural killer cells; NKTs, Natural killer T cells; NLRs, nod-like receptors; PAMPs, pathogen-associated molecular patterns; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PPARγ, Peroxisome proliferator-activated receptor γ; RA, Rheumatoid arthritis; RhoA, Ras homolog gene family, member A; T2D, Type 2 diabetes; TGF-β, Transforming growth factor-β; Th1, T helper 1 cells; TLRs, Toll-like receptors; TNF-α, Tumor necrosis factor-α; TNFi, Tumor necrosis factor inhibitors; Tregs T, regulatory cells; TZDs, Thiazolidinediones; UPR, unfolded protein response; WAT, white adipose tissue.

MOLECULAR PATHWAYS LINKING OBESITY-INDUCED INFLAMMATION AND IR

Insulin is an anabolic hormone secreted by β-cells that plays a crucial role not only in carbohydrate metabolism but also in protein and lipid anabolic regulation, cell growth, and proliferation (Fu et al., 2013). Blood glucose concentrations stimulate insulin synthesis and release; its effects on whole-body metabolism result from its binding to the cell membrane receptor, which is activated by autophosphorylation of specific tyrosine residues. The activated insulin receptor phosphorylates and recruits intracellular proteins, also known as IRSs. Downstream of IRS proteins, PI3K mediates insulin functions mainly by activating PKB and protein kinase C cascades (i.e., stimulating glucose uptake, glycogen synthesis and inhibiting hepatic gluconeogenesis). Insulin signaling also exerts mitogenic effects, most of which are mediated by PKB cascade and Ras/mitogen activated protein kinase (MAPK) pathway activation (Ramalingam et al., 2013).

Obesity association with T2D has long been recognized, and the primary reason is the ability of obesity to promote IR, the main pathophysiological aspect of T2D (Kahn and Flier, 2000).

IR is a metabolic complication in which the three major insulin-sensitive tissues (skeletal muscle, liver, and AT) become less responsive to insulin action. IR is characterized by serious failures in glucose uptake, glycogen synthesis, and, to a lesser extent, glucose oxidation (Ormazabal et al., 2018). In this scenario, the β-cells compensate for IR by increasing insulin secretion and restoring blood glucose concentration within the normal range. A further decline in insulin sensitivity makes the β-cells exhausted, and this results in persistent hyperglycemia and T2D (Shulman, 2000).

A number of studies have been performed to identify the causal factors responsible for obesity-induced IR. One of the most accepted theories considers chronic systemic inflammation induced by obesity as a preponderant mechanism (Weisberg et al., 2003; Xu et al., 2003; Luft et al., 2013). This theory is strongly supported by many findings and clinical evidence; for instance, inflammatory markers such as CRP, TNF-α, and interleukin 6 (IL-6) are elevated in obese and insulin-resistant subjects (Dandona et al., 1998; Kern et al., 2001; Vozarova et al., 2001; Phosat et al., 2017; Uemura et al., 2017). The first evidence of an association between IR and inflammation has been hypothesized when, following the administration of anti-inflammatory agents, an improvement in glucose homeostasis has been observed in T2D patients (Williamson, 1901; Reid et al., 1957; Yuan et al., 2001; Hundal et al., 2002). Further studies in the mid-1990s have shown that the white AT (WAT) of obese rodents and humans exhibited changes in the levels of pro-inflammatory molecules (e.g., TNF-α) (Hotamisligil et al., 1993, 1995; Uysal et al., 1998). Such inflammatory mediators modulate IR either directly by affecting insulin signaling or indirectly by stimulating inflammatory pathways (Tilg and Moschen, 2008). Other studies have shown that hypoxia, which occurs in AT during obesity, is directly responsible for IR induction in both human and murine models (Regazzetti et al., 2009; Yin et al., 2009).

Animal and human studies have identified WAT as the primary site where obesity-related chronic inflammation is initiated and exacerbated (Weisberg et al., 2003; Xu et al., 2003). AT remodeling during obesity provides a plethora of intrinsic and extrinsic signals capable of triggering an inflammatory response (Chawla et al., 2011; Huh et al., 2014; Reilly and Saltiel, 2017). These triggers, discussed later in the review, converge on the activation of the JNK and NF-κB signaling pathways (Nakatani et al., 2004; Shoelson et al., 2006; Bluher et al., 2009; Lee and Lee, 2014). The activation of these signaling pathways increases the production of pro-inflammatory cytokines, endothelial adhesion molecules, and chemotactic mediators that promote the infiltration of monocytes in AT and the differentiation into pro-inflammatory M1 macrophages (Shoelson et al., 2006). Infiltrating macrophages produce and secrete many inflammatory mediators that promote local and systemic pro-inflammatory status and impair insulin signaling (Haase et al., 2014).

The effects of these cytokines are mediated by stimulation of IκB kinase β (*IKK*β) and *JNK1*, expressed in myeloid and insulin-targeted cells (McLaughlin et al., 2016).

JNK is one of the most investigated signal transducers in obesity models of IR. It is activated after exposure to many inflammatory stimuli including cytokines, free fatty acids, and activation of cellular pathways, such as UPR (Aguirre et al., 2000; Ozcan et al., 2004). Once activated, JNK starts a pro-inflammatory gene transcription and inhibits insulin signaling pathway through inhibitory serine–threonine phosphorylation of IRS-1, thereby decreasing PI3K/PKB signaling (Tanti et al., 1994; Gual et al., 2005). In obese mice (ob/ob and diet-induced obesity), JNK activity is increased in AT compared to control mice. The role of *Jnk1* in adipocytes has been investigated using tissue-specific *Jnk1*-deficient mice. These mice are protected against the development of IR when fed a HFD. This effect is tissue specific because *Jnk1* deficiency in adipocytes does not affect muscle insulin sensitivity (Hirosumi et al., 2002; Sabio et al., 2008).

Obesity is also associated with the activation of NF-κB inflammatory pathway. In physiological conditions, NF-κB proteins are retained in the cytoplasm of myeloid and insulin-targeted cells by a family of inhibitors called inhibitors of κB (IκBs) (McLaughlin et al., 2017). Activation of IKK kinase complex (that contains IKKα and IKKβ subunits) induces proteasomal degradation of IκBα, leading to NF-κB nuclear translocation. This culminates in the increased expression of several NF-κB target genes [e.g., *IL-6*, *TNF*α, interferon-γ (*IFN*-γ), transforming growth factor-β (*TGF*-β), monocyte chemotactic protein-1 (*MCP-1*), and interleukin-1β (*IL-1*β)], which further exacerbate IR progression (Shoelson et al., 2006; Panahi et al., 2018). IKKβ deficiency in adipocytes totally prevents the expression of *IL-6* and *TNF*-α induced by free fatty acid, while its activation inhibits the expression of anti-inflammatory cytokines such as adiponectin and leptin (Jiao et al., 2011). Therapeutic approaches capable of targeting these pathways and improving insulin sensitivity in obese subjects will be further discussed below in this review.

Macrophages represent another important cell type in mediating the obesity-induced inflammation in the AT. During obesity, macrophages infiltrate the AT and secrete many pro-inflammatory cytokines (Weisberg et al., 2003). These mediators have local effects on adipocytes and resident immune cells (e.g., neutrophils, B cells, and T cells) and circulate in the periphery, where they affect the liver and skeletal muscle insulin sensitivity (Weisberg et al., 2003; Xu et al., 2003; Haase et al., 2014; McLaughlin et al., 2017).

Myeloid cells activate another molecular pathway, called inflammasome, in obesity (Lee and Lee, 2014). Macrophages and other innate immune cells may trigger inflammatory responses by detecting pathogen- or danger-associated molecular patterns (PAMPs or DAMPs) using a broad variety of pattern-recognition receptors such as TLRs and NLRs (Pedra et al., 2009; Vandanmagsar et al., 2011). Compelling evidence shows that NLRP3 (the most studied member of the NLR family) activation by DAMPs (generated by nutrient excess in obesity) plays a key role behind the chronic inflammation characteristic of obesity and IR (Stienstra et al., 2010, 2011, 2012; Zhou et al., 2010; Koenen et al., 2011; Vandanmagsar et al., 2011; Lee et al., 2013).

NLRP3 is present in several tissues and cell types (Vandanmagsar et al., 2011). It is unclear which cell compartments in AT express the inflammasome components; however, immunostaining of AT sections of obese mice confirmed a strong co-localization of NLRP3 with macrophage marker F4/80 in crown-like structures (Vandanmagsar et al., 2011). Once activated, NLRP3 interacts with procaspase-1 through an adaptive protein forming the NLRP3 inflammasome (Schroder et al., 2010; Davis et al., 2011; Shao et al., 2015). This results in the processing and activation of caspase-1, which mediates the maturation and secretion of IL-1β and IL-18 by macrophages (Shoelson et al., 2003; Schroder et al., 2010; Davis et al., 2011; Shao et al., 2015). The primary role played by NLRP3 inflammasome is also supported by evidence that genetic ablation of *NLRP3*$^{-/-}$ prevents the obesity-induced inflammasome activation in AT and protects against HFD-induced IR (Vandanmagsar et al., 2011). Caloric and exercise-mediated weight loss in obese people with T2D reduces *NLPR3* and *IL-1*β gene expression in abdominal subcutaneous AT and improves systemic insulin sensitivity (Vandanmagsar et al., 2011).

Inflammasome-activated IL-1β is a major cytokine produced by macrophages (Sims and Smith, 2010). Its enhanced production in pancreatic islets and insulin-sensitive tissues is associated to T2D (Hotamisligil et al., 1993; Donath and Shoelson, 2011). In obesity, chronic rise in circulating nutrients such as glucose and free fatty acids (FFAs) resulted in over-expression of IL-1β in pancreatic β-cells (Maedler et al., 2002; Böni-Schnetzler et al., 2008; Fei et al., 2008; Böni-Schnetzler et al., 2009). It is now clear that IL-1β is a key cytokine in the etiology of T2D since it has been implicated in IR, β-cell dysfunction, and death (Eizirik and Mandrup-Poulsen, 2001; Donath et al., 2008, 2019). IL-1β alters the insulin sensitivity of AT by suppressing insulin signaling; exposure to IL-1β of murine and human adipocytes decreases insulin-stimulated glucose uptake and lipogenesis (Lagathu et al., 2006; Jager et al., 2007; Fève and Bastard, 2009), reduces glucose transporter type 4 (GLUT4) expression, and inhibits GLUT4 translocation to the plasma membrane (Jager et al., 2007; Ballak et al., 2015).

The pro-apoptotic effects of IL-1β on β-cells derive from a complex network of signaling events triggered by IL-1β binding to its cognate receptor, whose expression is higher in β-cells than in other tissues (Böni-Schnetzler et al., 2009). Once cytokine binds its receptor, the co-receptor is recruited, and this results in the formation of the heterodimer receptor transmembrane complex. Both receptors and co-receptors share a cytoplasmic motif, the Toll/IL-1 receptor (IL-1R) domain, which is required to initiate intracellular signaling by recruitment of different adaptor proteins and kinases, including the myeloid primary response differentiation-88 protein and the interleukin-1-associated kinase receptor. This leads to activation of MAPK and NF-κB signaling pathways. The activation of these two signaling pathways causes variations in gene expression, therefore triggering the apoptotic cell death program in β-cells (Donath et al., 2008, 2019). The pro-apoptotic effects mediated by NF-kB depend on the cell type, nature, and duration of the stimulus. Indeed, NF-kB activation in β-cells is more marked, rapid, and sustained than in other cell types (Ortis et al., 2006). MAPKs also take part in β-cell apoptosis through transcription-independent mechanisms, such as regulating B-cell lymphoma 2 protein activity (Donath et al., 2008, 2019). The combined use of IFN-γ and IL-1β induces the activation of an additional mechanism, the so-called non-canonical NF-kB pathway, also implicated in the pro-apoptotic effects of IL-1β on β-cells (Meyerovich et al., 2016).

IL-1β is also implicated in cardiovascular and microvascular long-term complications (nephropathy, retinopathy, and polyneuropathy) of diabetes (Herder et al., 2013, 2015; Agrawal and Kant, 2014; Stahel et al., 2016; Donath et al., 2019). Endothelial cell damage is a crucial and an early manifestation of diabetic-associated vascular complications (van den Oever et al., 2010; Gilbert, 2013; Liu et al., 2014). Among the multiple and potential mechanisms that contribute to this phenomenon, a crucial role is played by chronic low-grade inflammation. IL-1β has been reported to cause endothelial cell damage in isolated mesenteric rat micro-vessels (Vila and Salaices, 2005; Shashkin et al., 2006). Vallejo et al. (2014) have shown that the deleterious effects of IL-1β on endothelial cells are due to the IL-1R-mediated activation of NADPH oxidase, which stimulates the production of superoxide anion (Vallejo et al., 2014). Over-activation of NADPH oxidase has also been associated with excess ROS production and the development of atherosclerosis in diabetic vasculopathy (Olukman et al., 2010; Gray et al., 2013).

IL-18 is another pro-inflammatory mediator activated by inflammasome and produced and released by human AT (Wood et al., 2005). IL-18 plasma levels are increased in obese people and in individuals with T2D (Moriwaki et al., 2003; Evans et al., 2007) while being restored in subjects who have lost weight following bariatric surgery (Schernthaner et al., 2006). It is a powerful pro-inflammatory cytokine that increases the maturation of T and NKs, as well as the production of other pro-inflammatory cytokines, exacerbating the obesity-induced systemic inflammation (Weisberg et al., 2003).

Likewise, IL-6 has been suggested to be involved in the development of obesity-related and T2D-related IR (Fève and Bastard, 2009). IL-6 leads to impaired insulin signaling, and this occurs primarily by inhibition of insulin-stimulated tyrosine phosphorylation of IRSs both in the liver and in AT (Senn et al., 2002; Klover et al., 2003; Lagathu et al., 2003; Rotter-Sopasakis et al., 2004; Fève and Bastard, 2009). Nonetheless, conflicting results have been reported for IL-6 action on skeletal muscle (Fève and Bastard, 2009). Carey et al. (2006) have shown that IL-6 increases GLUT4 translocation on plasma membrane and promotes insulin-stimulated glucose uptake in myotubes (Carey et al., 2006). Nevertheless, it has also been shown that in murine skeletal muscle cells, IL-6 is capable of reducing insulin-stimulated glucose uptake through JNK activation (Nieto-Vazquez et al., 2008). In pancreas, IL-6 impairs insulin secretion and has pro-apoptotic effects on β-cells (Ellingsgaard et al., 2008). An opposite effect is carried out on α-cells; IL-6 prevents α-cells apoptosis and induces the secretion of glucagon-like peptide-1. This could be considered an adaptive mechanism to compensate for β-cell failure (Ellingsgaard et al., 2008, 2011; Akbari and Hassan-Zadeh, 2018). Such findings support the tissue-specific effect of IL-6 on glucose homeostasis, which depends on several factors, such as concentrations, targets, and signaling pathways activated.

The IL-6 signaling cascade involves activation of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway (Fève and Bastard, 2009; Dodington et al., 2018). It serves as a crucial downstream mediator for a variety of hormones, cytokines (Gadina et al., 2018), and growth factors, including growth hormone, leptin, IL-6, and IFN-γ (Dodington et al., 2018). There are four identified members in the JAK kinase family (JAKs 1-3 and Tyk2), which associate with cytokine and growth factor receptors. JAK-mediated signaling leads to the activation of seven STAT family members (STATs 1-4, 5A, 5B, and 6). STAT proteins have cell- and tissue-specific distribution that influences their specificity and function (Schindler and Darnell, 1995; Richard and Stephens, 2011, 2014). The regulation of tissue-specific genes and the ability to have cell-specific tasks appear to be important physiological roles of the JAK/STAT pathway (Richard and Stephens, 2011). JAK/STAT signaling in the peripheral metabolic organs modulates a multitude of metabolic processes, including adiposity, energy expenditure, glucose tolerance, and insulin sensitivity (Dodington et al., 2018). This signaling pathway mediates the action of several hormones that have profound effects on adipocyte development and function. Adipocytes also produce hormones that utilize this pathway (Richard and Stephens, 2011). The expression of several STATs is modulated during adipogenesis (Richard and Stephens, 2011). Additional functions of JAK/STAT signaling in adipocytes include the transcriptional regulation of genes involved in insulin action and lipid and glucose metabolism (Richard and Stephens, 2011). JAK2, STAT3, and STAT5 are essential for signaling through both the growth hormone and leptin receptors and have been characterized in WAT (Dodington et al., 2018). As the major upstream kinases required for STAT activity, it is not surprising that JAK proteins also play important roles in the control of AT function (Gurzov et al., 2016). Adipose-specific *Jak2* KO mice have demonstrated defective lipolysis, increased body weight and adiposity compared to controls, leading to IR (Nordstrom et al., 2013; Shi et al., 2014; Corbit et al., 2017). Similarly, loss of either *Stat3* or *Stat5* in AT contributes to increased weight gain, adiposity, and impaired lipolysis (Dodington et al., 2018). There is a controversy over the effects of adipocyte JAK2/STAT5 on insulin sensitivity. Some studies have shown IR (Shi et al., 2014) while others have demonstrated enhanced whole-body insulin sensitivity in the absence of JAK2 or STAT5 (Nordstrom et al., 2013; Corbit et al., 2017). This inconsistency might be due to a variety of factors including tissue specificity and cell stage-dependent expression of the *cre* transgene, mouse genetic background, physiologic status, and other environmental factors in which the experiments were performed (Dodington et al., 2018). Although the direct role of STAT1 in the anti-adipogenic action of IFN-γ was not investigated, experiments using pharmacological inhibitors show that the JAK-STAT1 pathway plays a key role in the ability of IFN-γ to induce IR, decline triglyceride stores, and down-regulate expression of lipogenic genes in mature human adipocytes (Richard and Stephens, 2014). The increased IFN-γ levels and JAK-STAT1 signaling in obesity contribute to AT dysfunction and IR (Gurzov et al., 2016).

Emerging evidence demonstrates that the highly conserved and potent JAK/STAT signaling pathway is dysregulated in metabolic diseases, including obesity and T2D (Gurzov et al., 2016; Dodington et al., 2018). Studies show that many STAT activators play an important role in the regulation of adipocyte gene expression and exhibit differential expression in the condition of obesity and/or IR (Richard and Stephens, 2014). Obesity increases levels of IL-6 in WAT that, in turn, chronically activate intracellular JAK-STAT3 signaling. Chronic JAK-STAT3 signaling induced by IL-6 leads to the increased expression of suppressor of cytokine signaling-3 that not only negatively regulates IL-6 signaling but also hinders insulin action, eventually resulting in obesity and IR (Wunderlich et al., 2013). JAK/STAT signaling can have both physiological and pathological roles depending on the context. It is difficult to speculate how JAK/STAT inhibition will affect individuals with obesity and diabetes (Dodington et al., 2018). This complexity highlights the need for validation of the relative contribution of STAT proteins in human samples. Further studies will also be required to reveal the complex roles of the JAK-STAT pathway in adipocytes, obesity, and IR. Manipulation of this pathway within AT is a novel therapeutic approach for the treatment of obesity and diabetes.

Systemic inflammation is characterized by high circulating levels of inflammatory mediators and immune cells that infiltrate insulin-dependent tissues (Weisberg et al., 2003). As has already been discussed in the review, WAT is the main site where low-grade systemic inflammation begins (Weisberg et al., 2003; Xu et al., 2003). Accumulation of lipids that occurs in AT during obesity triggers an inflammatory response that results in an increased secretion of several inflammatory cytokines (Haase et al., 2014; Raciti et al., 2017). Such molecules can also activate JNK and NF-κB signaling pathways in the liver and skeletal

muscle, thus inhibiting systemic insulin signaling (Hotamisligil et al., 1993; Ciccarelli et al., 2016).

Obesity-induced inflammation initiates in WAT and then spreads to other tissues, resulting in low-grade systemic inflammation. In obesity, both liver and skeletal muscle exhibit signs of local inflammation (**Figure 1**).

Skeletal muscle is the principal organ for insulin-stimulated glucose uptake (i.e., capable for 80% of glucose disposal in human), and muscle IR plays a key part in T2D etiology (DeFronzo and Tripathy, 2009; Honka et al., 2018). Obesity contributes to the development of chronic muscle inflammation, characterized by increased pro-inflammatory M1 macrophage infiltration (Fink et al., 2013, 2014). These macrophages secrete many cytokines, which have been shown to trigger inflammatory pathways within myocyte, culminating in decreased insulin signaling (Varma et al., 2009; Pillon et al., 2012; Patsouris et al., 2014).

In the liver, obesity leads to increased infiltration and pro-inflammatory activation of two major macrophage groups: Kupffer cells (i.e., resident specialized hepatic macrophages) and monocyte-derived recruited hepatic macrophages (Tencerova et al., 2015). Further work indicates that, during obesity, the number of Kupffer cells remains unaffected, whereas the accumulation of monocyte-derived recruited macrophages increases several times (Morinaga et al., 2015). It has been demonstrated that ATM-released inflammatory mediators lead to hepatic IR by reducing insulin signaling (Morinaga et al., 2015). These inflammatory mediators also contribute to liver steatosis by promoting lipogenesis and toxic ceramide biosynthesis (Schubert et al., 2000; Obstfeld et al., 2010).

ROLE OF INNATE AND ADAPTIVE IMMUNITY IN OBESITY

Recently, as specified above, particular attention has been paid to the role played by macrophages in AT inflammation. Nevertheless, many other immune cells (both innate and adaptive immune systems) are involved in the development of local and systemic inflammation and IR.

Macrophages

During obesity, different types of both innate and adaptive immune cells accumulate in AT (Lackey and Olefsky, 2016). Macrophages are the most abundant and constitute up to 40% of all AT cells in obesity (Lumeng et al., 2007a; Lee et al., 2018).

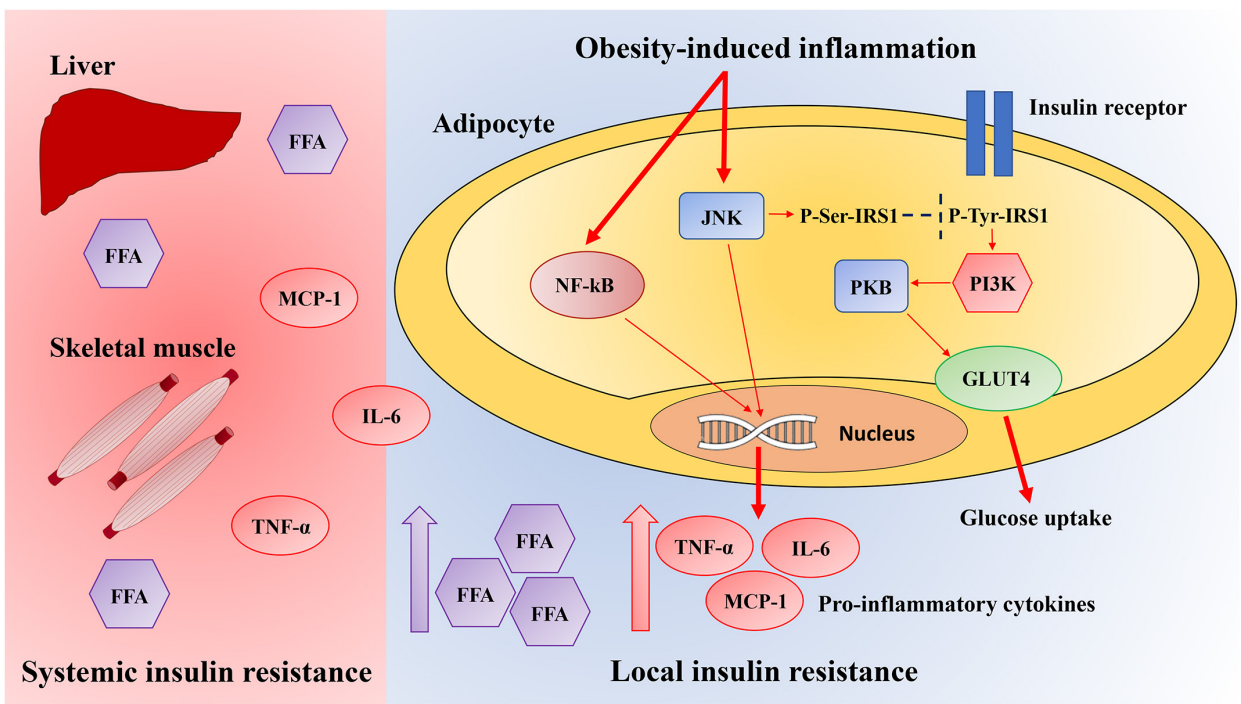

FIGURE 1 | Pathways linking local obesity-induced inflammation to systemic insulin resistance. Obesity results in the activation of the inflammatory signaling pathways mediated by JNK and nuclear factor-kappa B (NF-κB). Once activated, these pathways induce the production of several pro-inflammatory cytokines in adipocytes, which contribute to insulin resistance and pro-inflammatory macrophages infiltration. Activation of the JNK signaling pathway starts the transcription of pro-inflammatory genes and inhibits the insulin signaling pathway through the insulin receptor substrate-1 (IRS-1) inhibitory serine phosphorylation, which reduces the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) signaling pathway. Instead, NF-κB signaling pathway activation culminates in the increased expression of several NF-κB target genes such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and monocyte chemotactic protein-1 (MCP-1), which leads to serine phosphorylation of IRS-1, therefore preventing insulin signaling. The inflammatory mediators including free fatty acids (FFA), IL-6, TNF-α, and MCP-1 also spread through systemic circulation and activate JNK and NF-κB signaling pathways in the liver and skeletal muscle, inhibiting systemic insulin signaling. GLUT4, glucose transporter type 4.

They are suggested to be the major source of pro-inflammatory cytokines (Samuel and Shulman, 2012), which can cause IR (Xu et al., 2003).

Obesity is associated with the recruitment of M1-polarized macrophages, which secrete pro-inflammatory cytokines such as TNF-α and IL-1β (Han and Levings, 2013). Inflamed AT is characterized by the combination of an increase in total macrophages and an increased ratio of M1 to M2 (anti-inflammatory) macrophages, which comes along obesity and is linked with the development of IR (Lumeng et al., 2007a; Reilly and Saltiel, 2017). However, we should consider that macrophage inflammation in response to obesity is not identical to the classic M1 activation state observed in inflammation associated with acute infection. As ATMs express a different set of surface markers, the pro-inflammatory activation in the setting of obesity has been referred to as metabolic activation, or Me, rather than M1 (Reilly and Saltiel, 2017). The pro-inflammatory macrophages in obese AT also upregulate the expression of genes that encode proteins involved in lipid metabolism. Hence, they can be also distinguished from classically activated macrophages (Xu et al., 2013; Henao-Mejia et al., 2014; Reilly and Saltiel, 2017).

Neutrophils

While ATMs are the pivotal effector innate immune cells causing IR, alterations in several other innate immune cell types in obese AT contribute to the initiation and/or progression of AT inflammation (Lee et al., 2018).

Neutrophils are the leukocyte subpopulation (Chmelar et al., 2013) and granulocytes involved in innate immunity (Asghar and Sheikh, 2017; Kane and Lynch, 2019). They comprise up to 90% of all granulocytes in the blood but are relatively rare in AT of lean mice (Talukdar et al., 2012; Kane and Lynch, 2019). However, neutrophils are among the first responders recruited to AT in mice as early as 3 days after the initiation of HFD. Talukdar et al. (2012) reported that the early recruitment of neutrophils was prolonged over 90 days on HFD (Talukdar et al., 2012; Chmelar et al., 2013). We should take into account that another study already showed that this migration is transient (Elgazar-Carmon et al., 2008).

Neutrophils stimulate AT inflammation by producing TNF-α and MCP-1 (Dam et al., 2016; Trim et al., 2018). Neutrophils also produce elastase, which impairs glucose uptake in AT (Wang et al., 2009) and promotes IR by degrading IRS-1 (Talukdar et al., 2012; McLaughlin et al., 2017; Lee et al., 2018). The activity of elastase is also increased in the AT of HFD mice, corresponding to the number of infiltrated neutrophils (Talukdar et al., 2012; Chmelar et al., 2013). Genetic deletion of elastase attenuates macrophage influx into the AT of obese mice and results in improved insulin sensitivity (Talukdar et al., 2012; Hotamisligil, 2017).

Dendritic Cells

Dendritic cells are specialized antigen-presenting cells that link the innate and adaptive immunity (Bertola et al., 2012; McLaughlin et al., 2017; Chung et al., 2018) by presenting antigens to T cell receptors (Steinman, 2008).

Dendritic cells accumulate in AT of mice fed an HFD and in the subcutaneous AT of obese humans (Cho et al., 2016). They likely induce the pro-inflammatory microenvironment through macrophage recruitment and IL-6 production (Stefanovic-Racic et al., 2012). Blocking their accumulation improves insulin sensitivity in obese mice (Cho et al., 2016).

DCs inhibit healthy expansion of AT, and depletion of these cells improves glucose homeostasis in mice (Hotamisligil, 2017). Adipose-recruited DCs have been shown to be associated with the deregulation of chemerin, a particular adipokine (Ghosh et al., 2016; Lu et al., 2019).

Altogether, these studies suggest a pathogenic role for DCs in the development of obesity in mice and humans. Mice with deletion of Fms-like tyrosine kinase 3 ligand (*Flt3L*), that lack DCs, revealed reduced macrophage number in the AT and liver as well as improved insulin sensitivity in diet-induced obesity. Administration of recombinant Flt3L to these mice reversed this phenotype (Chung et al., 2018).

Mast Cells

Mast cells are innate immune cells (Liu et al., 2009) that originate from $CD34^+$, $CD13^+$, and $CD17^+$ multipotent hematopoietic stem cells (Zelechowska et al., 2018). AT is a reservoir of mast cells (Zelechowska et al., 2018). There is a significant increase in the number of mast cells in the WAT of mice and humans with obesity (Liu et al., 2009) and/or T2D (Lackey and Olefsky, 2016).

Mast cells promote AT low-grade inflammation in obesity (Liu et al., 2009). They mediate the macrophage infiltration (Liu et al., 2009). Interestingly, mast cells are regulated by IL-6 and IFN-γ but not via TNF-α (Liu et al., 2009; Sun et al., 2012). IL-6 and IFN-γ play a crucial role in the ability of mast cells to regulate metabolism, and they may mediate diet-induced obesity and diabetes (Zelechowska et al., 2018). Immature mast cells that infiltrate into AT the non-obese stage progressively mature and promote obesity and diabetes progression (Hirai et al., 2014).

Mast cells tend to degranulate (Zelechowska et al., 2018), resulting in the secretion of a large amount of pro-inflammatory and immunomodulatory mediators, such as histamine, cytokines, and chemokines (Sun et al., 2012; Zelechowska et al., 2018). Hence, they have a key role in allergic responses and AT homeostasis (Sun et al., 2012). Mast cell deficiency is associated with improved insulin sensitivity (McLaughlin et al., 2017).

B Cells

Lymphocytes account for up to 10% of non-adipocytes cells in human AT and include T cells, B cells, NKs, NKTs, and ILC2s (McLaughlin et al., 2017).

B cells are an important component of the adaptive immunity that release immunoglobulins or antibodies to recognize the cognate antigen. This feature differs from the cell-mediated immunity where T cells recognize processed antigenic peptides presented by antigen-presenting cells (Sun et al., 2012). B cells in AT are phenotypically different from B cells found in other tissues, as B cells in AT have unique genetic markers (Dam et al., 2016). They are present across all known AT depots but are less well characterized than T cells (Kane and Lynch, 2019).

B cells have also been shown to be pathogenic in obesity (Kane and Lynch, 2019). B cells accumulate in the AT of obese mice relative to lean mice and become more inflammatory, producing chemokines that promote the recruitment of neutrophils, T cells, and monocytes (Kane and Lynch, 2019). B cells promote pro-inflammatory activation of ATMs and T cells (Winer et al., 2011; Lee et al., 2018).

B cells modulate IR by accumulating in the AT of obese mice (Winer et al., 2009). It has been reported that B cell accumulation precedes T cell accumulation during the development of obesity (Lau et al., 2012). B cells might contribute to AT inflammation by producing immunoglobulin G antibodies or pro-inflammatory cytokines. The B cells from obese mice release a more inflammatory repertoire of cytokines (Michelsen et al., 2004; Winer et al., 2011; Ding et al., 2012).

Obese mice with B cell deficiency reduce IR (DeFuria et al., 2013; Hotamisligil, 2017). Transfer of B cells from obese donor mice causes impaired insulin sensitivity and glucose homeostasis in the recipients (Winer et al., 2011; Hotamisligil, 2017). By contrast, there are also tolerance-promoting B regulatory cells in AT, and their number is decreased in models of obesity (Nishimura et al., 2013; Hotamisligil, 2017).

T Cells

$CD3^+$ T cells constitute the largest AT immune-cell population next to macrophages, and their abundance is increased in HFD obese mice (Lee et al., 2018). T cells can be divided into two subtypes depending on the markers on their surface, CD4 and CD8 T cells (Pennock et al., 2013).

Obesity is associated with an increase in the number of $CD8^+$ T cells in AT (Henao-Mejia et al., 2014), and these cells promote macrophage differentiation and chemotaxis (Nishimura et al., 2009).

$CD4^+$ T cells identify major histocompatibility complex class II, presented on the surface of antigen-presenting cells like DCs, macrophages, and B cells (Dam et al., 2016; Trim et al., 2018). $CD4^+$ T cells are subclassified into pro-inflammatory T helper 1 (Th1) and Th17 cells, anti-inflammatory Th2 cells, and T regulatory cells (Tregs). The number of $CD3^+CD4^+$ Th1 cells is increased in obesity, and they stimulate AT inflammation by secreting IFN-γ. In contrast, the number of $CD3^+CD4^+$ Th2 cells is declined in obese AT (Winer et al., 2009; Lee et al., 2018).

Treg cells ($CD3^+CD4^+FOXP3^+$) are a small subset of $CD4^+$ T lymphocytes that inhibit inappropriate inflammation. Treg population in lean AT is characterized by high peroxisome proliferator-activated receptor γ (*PPARγ*) expression. These Tregs play a critical role in maintaining AT inflammatory tone and insulin sensitivity (Lee et al., 2018). The decline in the numbers of AT Treg cells during obesity contributes to increased AT inflammation (Zhou et al., 2010; Henao-Mejia et al., 2014) and IR (Feuerer et al., 2009; Lee et al., 2018).

Invariant natural killer T cells (iNKTs) are lipid-antigen-reactive T cells restricted by the major histocompatibility complex-like molecule CD1d (Lee et al., 2018). iNKT cells form a subset of lymphocytes in normal AT. The number of iNKTs is reduced in obesity (Lee et al., 2018). Furthermore, mice lacking iNKTs shows increased weight gain, larger adipocytes, and IR on HFD. This is associated with increased infiltration of macrophages into AT (Lynch et al., 2012).

Collectively, the network of T and B cells has crucial effects to influence macrophage infiltration. Thus, pro-inflammatory macrophages are the final effector cells that induce IR (Lee et al., 2018).

OBESITY-INDUCED AT INFLAMMATION TRIGGERS

There is a limited understanding of how obesity-induced inflammation in AT is triggered. However, potential mechanisms identified include dysregulation of fatty acids homeostasis, increased adipose cell size and death, local hypoxia, mitochondrial dysfunction, ER stress, and mechanical stress (**Figure 2**) (Heilbronn and Liu, 2014; Reilly and Saltiel, 2017). These mechanisms are recognized as the link between chronic caloric excess and AT inflammation or as factors that may perpetuate chronic tissue inflammation (Burhans et al., 2018). The list of potential mechanical links mentioned here is not complete, and it is likely that the triggers leading to AT inflammation have not yet been identified.

Dysregulated Fatty Acids Homeostasis

Saturated fatty acids promote inflammatory activation of macrophages, partially mediated by indirect binding to TLR4 and TLR2 (Konner and Bruning, 2011), resulting in the activation of NF-κB and JNK pathways (Shi et al., 2006; Milanski et al., 2009).

Once these pathways have been stimulated, many chemokines (e.g., MCP-1 and TNF-α) are produced and released from adipocytes, resulting in inflammatory macrophage infiltration (Reilly and Saltiel, 2017). In obesity, in addition to an increased intake of saturated fatty acids, *TLR4* and *TLR2* expression are increased in the AT, further supporting the role of these receptors in obesity-associated inflammatory signaling (Husam et al., 2008; Vitseva et al., 2008). In regard to this, acute lipid infusion is enough to stimulate AT inflammation and systemic IR in wild-type mice, and these effects are prevented in *TLR4*-deficient mice (Shi et al., 2006). Based on these findings, TLR4 appears to be an interesting candidate for linking dietary fatty acids with AT inflammation and IR (Poggi et al., 2007). Despite saturated fatty acids, unsaturated omega-3 and -9 fatty acids have beneficial effects and alleviate AT inflammation (Oliveira et al., 2015).

Adipocyte Hypertrophy, Hypoxia, and Death

WAT plays a major role in regulating systemic energy homeostasis, which acts as a safe reservoir for fat storage. In response to changes in nutritional status, AT expands by increasing the number (hyperplasia) and size of the adipocytes (hypertrophy) (Sun et al., 2011; Longo et al., 2018; Hammarstedt et al., 2018). Cross-sectional studies have demonstrated that the size of visceral adipocytes is negatively correlated with insulin sensitivity (O'Connell et al., 2010; Hardy et al., 2011), and these findings allow proposing adipocyte size as an IR determinant (O'Connell et al., 2010).

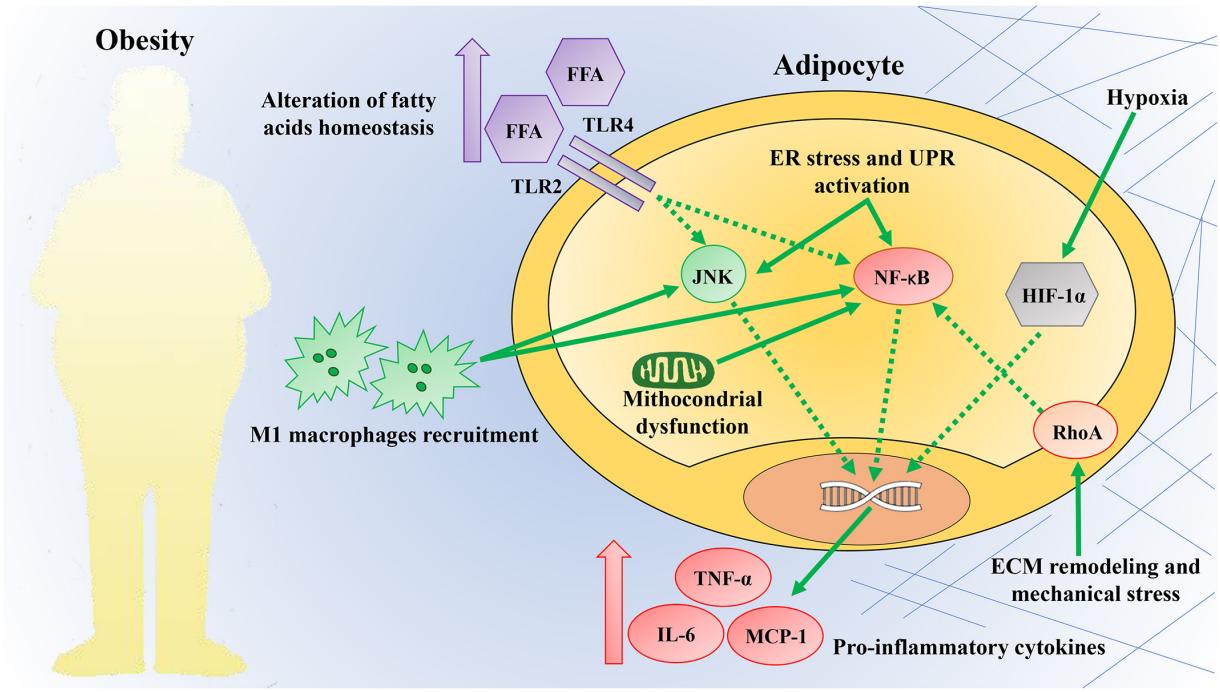

FIGURE 2 | Obesity triggers inflammation. Obesity provides a plethora of intrinsic and extrinsic signals capable of triggering an inflammatory response in AT. These mechanisms are commonly considered the link between chronic caloric excess and adipose tissue inflammation. Some of these mechanisms include dysregulation of fatty acid homeostasis, increased adipose cell size and death, local hypoxia, mitochondrial dysfunction, endoplasmic reticulum (ER), and mechanical stress. These triggers converge on the activation of the c-Jun N-terminal kinase (JNK) and nuclear factor-kappa B (NF-κB) pathways, commonly considered signaling hubs. The activation of these pathways increases the production of pro-inflammatory cytokines and promotes the infiltration of pro-inflammatory M1 macrophages. TLR2, Toll like receptor 2; TLR4, Toll like receptor 4; FFA, free fatty acids; UPR, unfolded protein response; HIF-1α, hypoxia-inducible factor-1α; RhoA, ras homolog gene family, member A; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; MCP-1, monocyte chemotactic protein-1; ECM, extracellular matrix.

Thus, the evidence indicating that adipocyte hypertrophy certainly contributes to AT inflammation is quite convincing at the present. Increased adipocyte size is characterized by a higher rate of adipocyte death and macrophage recruitment. Larger adipocytes exhibit an altered chemoattractant and immune-related proteins secretion that may promote pro-inflammatory macrophage infiltration (Jernas et al., 2006; Heilbronn and Liu, 2014). Most of these infiltrated macrophages surround necrotic adipocytes and form crown-like structures. In obese rodents as well as humans, necrosis-related factors further attract monocytes in AT where they uptake the lipids released by dead adipocytes (Cinti et al., 2005; Murano et al., 2008; Choe et al., 2016). As described above, the recruited monocytes have a pro-inflammatory phenotype and secrete cytokines and reactive oxygen species in neighboring adipocytes that interfere with insulin signaling (Shapiro et al., 2011). An increase in the number of dead adipocytes has been recognized to prevent normal AT functions and cause inflammation (Choe et al., 2016).

During adipocyte hypertrophy, angiogenesis is initiated to supply oxygen to the expanding tissue. If the AT expansion is very rapid, the vasculature cannot fulfill the oxygen requirement and hypoxia occurs (Gealekman et al., 2011; Sun et al., 2011; Trayhurn, 2013).

Hypoxia is a strong metabolic stressor. Current evidence reveals that hypoxia develops as AT expands because of a relative under perfusion of the enlarged AT or increased oxygen utilization (Gealekman et al., 2011; Sun et al., 2011; Trayhurn, 2013; Lee et al., 2014).

Cellular hypoxia can initiate inflammation by activating hypoxia-inducible factor-1 (*HIF-1*) gene program. Activated HIF-1α translocates to the nucleus where it recognizes and binds the HREs on DNA. The binding to HREs promotes not only the expression of many genes involved in the angiogenesis but also inflammation (Trayhurn, 2013; Fiory et al., 2019). These include vascular endothelial growth factor, insulin-like growth factor 2, transforming growth factor α, as well as nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 and inflammatory cytokines such as interleukin-33 and 18 (Shi and Fang, 2004). It has been shown that adipocyte-specific *HIF-1α* deletion prevents obesity-induced inflammation and IR (Lee et al., 2014).

Mitochondrial Dysfunction

Mitochondria are present in almost all eukaryotic cells and are responsible for cellular energy production, calcium signaling, and apoptosis (Osellame et al., 2012). Alterations in mitochondrial functions are capable of causing inflammation, oxidative stress, cell death, and metabolic dysfunction (Hock and Kralli, 2009; Kim et al., 2016). A number of studies in obese mice and human subjects have shown that mitochondrial dysfunction is strongly associated with pathological conditions such as inflammation, IR,

and T2D (Silva et al., 2000; Petersen et al., 2003, 2004; Morino et al., 2005; Woo et al., 2019). Alterations in mitochondrial functions and reductions in mitochondrial DNA content have been reported in obese (ob/ob) and diabetic (db/db) mice (Choo et al., 2006; Koh et al., 2007). A comparable decrease in mitochondrial activity has also been observed in human AT from obese individuals (Yin et al., 2014).

The mitochondrial dysfunction leads to inflammation through modulating redox-sensitive inflammatory mechanisms such as NF-κB or direct inflammasome activation (Vaamonde-García et al., 2012; López-Armada et al., 2013). The activation of both pathways induces an upregulation of inflammatory cytokines and adhesion molecules secretion, resulting in a substantial amplification of the inflammatory response (Escames et al., 2011; López-Armada et al., 2013).

Petersen et al. (2003, 2004) reported that the mitochondrial dysfunction also contributes to ectopic fat accumulation (i.e., accumulation of intracellular fatty acid metabolites such as fatty acyl-CoA and diacylglycerols), which blocks insulin signaling.

Woo et al. (2019) suggest a new hypothesis that adipocyte mitochondrial dysfunction causes AT inflammation and systemic IR by inducing fatty acids accumulation in adipocytes, and resulting in adipocyte hypertrophy and hypoxia.

ER Stress

ER is a cellular organelle that exhibits high sensitivity to cellular nutrients and energy status (Hummasti and Hotamisligil, 2010). Many genetic and environmental hits can alter the functions of ER and therefore contribute to ER stress (Hummasti and Hotamisligil, 2010). Several studies have shown that the incorrect functioning of the UPR (i.e., ER-stress mitigation system in eukaryotes) is associated with chronic metabolic diseases including obesity, IR, and T2D (Ozcan et al., 2006; Boden et al., 2008; Sharma et al., 2008; Gregor et al., 2009). It has been shown in mice that obesity results in increased ER stress, particularly in the liver and AT. Indeed, the expression of most ER stress markers and chaperones is strongly BMI-related and associated with AT insulin sensitivity (Sharma et al., 2008). Additionally, a weight-loss gastric bypass surgery has been shown to enhance insulin sensitivity and decrease ER stress in obese (Gregor et al., 2009).

Inflammation is the predominant mechanism by which ER stress negatively affects metabolic homeostasis. The primary mechanisms by which ER stress establishes inflammatory mechanisms in AT involve the activation of NF-κB, JNK, and apoptosis signaling pathways.

In response to ER stress, the three UPR branches are activated. The activation of two branches is mediated by protein kinase RNA (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6). This activation stimulates NF-κB signaling pathway, resulting in the subsequent inhibition of insulin action via IRS-1 phosphorylation. In addition, the branch mediated by inositol-requiring enzyme 1 results in the activation of the JNK signaling pathway (Hotamisligil, 2010; Hummasti and Hotamisligil, 2010). There is also crosstalk between the three branches. For example, spliced X-box binding protein 1, as well as activating transcription factor 4, induces the production of the inflammatory cytokines IL-6, interleukin-8, and MCP-1 by human endothelial cells (Hotamisligil, 2010).

A further important function of UPR is to activate pro-apoptotic signaling pathways in order to prevent the release and accumulation of misfolded proteins, which may have adverse effects on cellular functions (Hotamisligil, 2010). However, ER stress-induced apoptosis may also contribute to increased inflammatory signaling and other aspects of metabolic diseases. For instance, adipocyte death in obesity has been suggested as a potential trigger for the recruitment of macrophages and other inflammatory cells (Cinti et al., 2005), as described in the review. Evidence also indicates that ER stress is essential for β-cell development and survival (Harding et al., 2001; Scheuner et al., 2001; Zhang et al., 2002).

In 2016, we have reported that UPR hyper-activation by glucose insult leads to a pro-inflammatory phenotype in preadipocytes. Cells exposed to hyperglycemia release an increased amount of pro-inflammatory cytokines, chemokines and IL-12 lymphokine, which can trigger inflammation by affecting inflammatory cells. However, such effects are prevented by a chemical chaperone such as 4-phenyl butyric acid (Longo et al., 2016). ER stress pharmacological inhibition can reverse metabolic dysfunction also in other tissues, including liver and brain (Ozcan et al., 2006; Longo et al., 2016).

Meta-inflammation and ER dysfunction are emerging as critical mechanisms. If these mechanisms are targeted therapeutically, they can enhance multiple metabolic parameters, as shown in preclinical and clinical studies (Hummasti and Hotamisligil, 2010).

Dynamics of the ECM and Mechanical Stress

The protein composition and dynamics of the ECM are crucial for the adipocyte function. ECM remodeling is essential for the expansion and contraction of adipocytes to accommodate changes in energy stores (Rutkowski et al., 2015). During a positive energy balance, ECM accumulation occurs in AT, which contributes to fibrosis and impairs its role as a nutrient storage organ (Lee et al., 2014).

Abnormal accumulation of ECM components in AT has been shown to cause obesity-associated IR (Lin et al., 2016). Excessive ECM deposition in AT is suggested for triggering adipocyte necrosis, which attracts pro-inflammatory macrophages and causes AT inflammation and metabolic dysfunction. In addition, excess ECM deposition causes adipocyte death and AT inflammation by activation of integrins and CD44 signaling pathways (Lin et al., 2016).

Lipid accumulation occurring in obesity may also cause ECM instability and induce various mechanical stresses on these cells. The mechanisms governed by these mechanical stresses in adipocytes have not yet been fully explained, but certain pathways such as RhoA, and NF-κB have been evaluated. RhoA signaling, for instance, inhibits adipogenesis through *PPARγ* suppression and stimulates the secretion of pro-inflammatory cytokines (McBeath et al., 2004; Li et al., 2010). Meanwhile, Li et al. (2010) have shown that the elevated density of ECM

proteins reduces insulin signaling and increases MCP-1 secretion by activating the NF-κB signaling pathway (Li et al., 2010).

As mentioned above, some of the potential mechanisms involved in AT inflammation have been identified; however, it is likely that there are still unknown triggers. The temporal sequence of events leading to AT inflammation, as well as the contribution of each mechanism described above, has not yet been fully established. In our opinion, adipocyte hypertrophy may be the primary and initial event causing AT inflammation. In obesity, adipocytes respond to excess energy by storing lipids inside and undergoing dramatic changes in size (hypertrophy). Hypertrophy is associated with hypoxia, cellular and tissue stress, and cell death due to the activation of both necrotic and apoptotic mechanisms. Hypertrophic adipocytes are also characterized by excessive lipolysis, resulting in increased release of FFAs acting on TLR4, as previously indicated. All the above mechanisms promote adipocyte dysfunction, characterized by an altered cytokine secretion pattern. These mechanisms play a dual role; they are able both to trigger individually inflammatory responses and to induce downstream processes, amplifying and eliciting chronic systemic inflammation and thus promoting systemic IR. The temporal sequence of events suggested here and the relevance that we attribute to adipocyte hypertrophy in the initiation of AT inflammation needs to be further verified.

INFLAMMATION AS A THERAPEUTIC TARGET FOR METABOLIC DISEASES

The role of chronic inflammation, particularly in the AT, in the pathogenesis of T2D and associated complications, is now well established. The association between obesity, AT inflammation, and metabolic disease makes inflammatory pathways an appealing target to treat metabolic disorders. Inflammation is recognized as the pathologic mediator of these frequently common comorbidities. Several anti-inflammatory approaches have been tested in clinical studies of obese individuals with IR, but clinical trials to confirm the therapeutic potential are still ongoing (Goldfine and Shoelson, 2017). The number of available drugs that can target different components of the immune system and improve different metabolic aspects is increasing rapidly (Donath, 2014).

Based on the mechanism of action, therapeutic approaches to target inflammation in IR and T2D can be divided into (i) pharmacologic approaches that directly target inflammation and (ii) diabetes drugs with anti-inflammatory properties.

Pharmacologic Approaches That Directly Target Inflammation: Salsalate

Salsalate is an analog of salicylate that belongs to the non-steroidal anti-inflammatory drug classes. Independent studies have shown that salsalate can improve glycemic control in T2D patients. The mechanism of action of salsalate in reverse hyperglycemia in obese mice is through the inhibition of NF-κB pathway and has been identified in 2001 by Shoelson (Yuan et al., 2001).

Goldfine then translated this initial finding to the clinical study and showed that salsalate decreases fasting glucose and triglyceride levels, increases adiponectin levels and glucose utilization in diabetic patients during hyperinsulinemic–euglycemic clamp, and improves insulin clearance (Goldfine et al., 2008). These observations have been confirmed in two multicenter, randomized, placebo-controlled trials in subjects with T2D (Goldfine et al., 2010, 2013). In the first study, treatment with this drug improves insulin sensitivity and decreases HbA1c levels by 0.5% relative to placebo over 14 weeks in a group of patients with T2D (Goldfine et al., 2010). In the second study, 48 weeks of salsalate administration in a larger patient population (283 participants; placebo, n = 137; salsalate, n = 146) leads to a smaller decrease in the levels of HbA1c (–0.33%) and serum triglycerides (Goldfine et al., 2013). This treatment also decreases levels of glycation end products (Barzilay et al., 2014).

Other studies also suggest that metabolic improvement, induced by salsalate treatment, is mediated through AMPK activation (Hawley et al., 2012). Although the effects on glycemic control are modest, the salsalate is not expensive and has a very safety profile.

Pharmacologic Approaches That Directly Target Inflammation: TNF-α Inhibitors

In 1993, a preclinical study clearly showed the role of TNF-α in the pathophysiology of IR in the AT (Hotamisligil et al., 1993), and this finding has raised the hypothesis that TNF-α blockade has potential therapeutic benefits. However, the results of clinical studies have so far been disappointing. For instance, TNF-α neutralizing antibodies have been shown to be effective for the treatment of many other inflammatory diseases, and some patients have shown slight improvements in glycemic control (Ofei et al., 1996; Feldmann, 2002; Reilly et al., 2013). However, prospective studies in T2D patients have been confusing. In spite of valuable effects in mice, a human clinical trial showed that anti-TNF-α therapy leads to no improvements in insulin sensitivity in patients with T2D (Ofei et al., 1996; Moller, 2000; Paquot et al., 2000). In contrast, a study performed in obese subjects without T2D showed that an inhibition of TNF-α for 6 months is able to reduce fasting glucose and increase adiponectin levels (Stanley et al., 2011).

Pharmacologic Approaches That Directly Target Inflammation: IL-1β Antagonists

IL-1β is a strong mediator of the obesity-induced inflammation and participates in the pathogenesis of T2D, mediating the adverse consequences of hyperglycemia on pancreatic β-cells (Maedler et al., 2002). Antagonism of IL-1R for 13 weeks, in a proof-of-concept study of patients with T2D, shows an improved glycemic control and secretory function of the pancreatic β-cells and the reduced markers of systemic inflammation (Larsen et al., 2007). The follow-up study on the same population proves that 39 weeks after the last IL-1R antagonist administration, β-cell insulin secretion is still increased and CRP decreased (Larsen et al., 2009). The long-term effects are probably due to the block

of IL-1β auto induction mechanism (Böni-Schnetzler et al., 2008). Further studies have also noted that the use of antibodies directed against IL-1β has potential benefits in the treatment of T2D, as it significantly reduces HbA1c levels (Cavelti-Weder et al., 2012; Sloan-Lancaster et al., 2013).

Pathological activation of IL-1β also contributes to the development of other T2D-associated diseases, such as Crohn's disease, gout, and RA (Donath et al., 2019). Recently, a multicenter randomized controlled trial, specifically designed to evaluate the glycemic outcome, enrolled participants, with RA and T2D (followed up for 6 months). Thirty-nine participants were randomized to IL-1R antagonist (anakinra) or TNF inhibitors (TNFi) to assess the efficacy of these drugs in controlling glucose alterations of T2D (Ruscitti et al., 2019). After 3 and 6 months of treatment, anakinra showed a significant improvement in metabolic alteration (reduction of HbA1c by more than 1%), whereas TNFi showed no enhancement. Regarding RA, there has been a gradual reduction in disease activity in both groups. In conclusion, results of this research indicate a specific effect of IL-1 inhibition in subjects with RA and T2D, reaching the therapeutic targets of both disorders and improving the main outcome of enrolled participants. A clearer reduction of HbA1c, comparing this to the previous study on T2D (Larsen et al., 2007), can be explained based on the theory that pathogenic mechanisms of T2D could be exaggerated in the context of RA. On this basis, IL-1 pathway can be considered a shared pathogenic mechanism, and a single treatment that manages both diseases appears to be a promising option for improving the care of RA and T2D patients (Giacomelli et al., 2016).

Diabetes Drugs With Anti-inflammatory Properties: Thiazolidinediones

Thiazolidinediones (TZDs) are antidiabetic drugs that improve insulin sensitivity and glycemia, as they function as agonists for PPARγ nuclear receptor (Yki-Järvinen, 2004). TZDs have also anti-inflammatory effects; they repress NF-κB action and reduce the expression of its target genes (Pascual et al., 2005).

The inhibition of NF-κB pathway reduces ATM content (Esterson et al., 2013; Koppaka et al., 2013), restores the M2 macrophages phenotype (Chawla, 2010), and stimulates the recruitment of the anti-inflammatory regulatory T cells in the AT (Cipolletta et al., 2012).

Furthermore, the ability of TZDs to reduce circulating inflammatory mediators (such as CRP and MCP-1) seems to be independent of glycemic control (Pfützner et al., 2005). Therefore, TZDs act through different mechanisms and the anti-inflammatory properties of these drugs are not definitely established.

Diabetes Drugs With Anti-inflammatory Properties: Metformin

The mechanism of metformin action is not completely explained, but it decreases glycemia by reducing hepatic glucose production and raising glucose uptake in peripheral tissues (Inzucchi et al., 1998). In addition to its clear metabolic effects, metformin has also anti-inflammatory properties; for instance, it directly inhibits the production of reactive oxygen species in the mitochondria and can reduce the production of many cytokines (Wheaton et al., 2014). Emerging evidence supports the novel hypothesis that metformin can exhibit immune-modulatory features. The effects of metformin on immune cells (T cells, B cells, monocytes/macrophages, neutrophils) involved in the pathogenesis of autoimmune and inflammatory diseases have been extensively reviewed by Ursini et al. (2018). Inside the immune cells, metformin temporarily inhibits the complex I of the mitochondrial electron transport chain, contributing to an increased AMP/ATP ratio (Rena et al., 2017). Decreased ATP concentration causes AMPK activation, and among several targets, AMPK inhibits the mammalian target of rapamycin (mTOR) (Zhou et al., 2001). mTOR is crucial for cellular metabolism, cytokine responses, antigen presentation, macrophage polarization, and cell migration (Weichhart et al., 2015) through its interaction with the STAT3 pathway (Saleiro and Platanias, 2015). Metformin can also regulate other pathways relevant to immune cells, including NF-kB (Hattori et al., 2006; Chaudhary et al., 2012) and JNK (Wu et al., 2011). Indeed, other studies have proved that metformin is able to inhibit TNF-α-induced activation of the NF-κB axis and IL-6 production (Huang et al., 2009) through PI3K-dependent AMPK phosphorylation. Metformin, in a dose-dependent manner, reduces IL-1β production in lipopolysaccharide-activated macrophages, and the effect is independent of AMPK activation (Kelly et al., 2015). Moreover, metformin concurrently decreases circulating inflammatory proteins, including CRP, in impaired glucose tolerance and T2D patients (De Jager et al., 2005; Haffner et al., 2005). The anti-inflammatory effects of metformin, like TZDs, appear to be independent of glycemic control (Caballero et al., 2004). In murine models, the attenuation of the inflammatory state has been shown to be effective in improving the obesity-induced IR; however, there are ongoing clinical trials in humans to confirm the therapeutic potential of metformin. This issue represents an essential step in proving the translational relevance of these observations.

T2D is a heterogeneous disorder, and the absence of clinical biomarkers, showing whether the treatments have anti-inflammatory effects in the AT, is a potential issue complicating the analysis (Donath, 2016). The identification and profiling of these biomarkers in T2D patients would allow us to predict those that should respond to an anti-inflammatory therapy.

CONCLUSION

The global obesity epidemic results in a higher incidence of metabolic disorders. The mechanisms underlying the association between obesity and IR have not yet been fully explained. Therefore, further well-designed clinical and basic research studies are needed to establish this relationship. From our point of view, inflammation occurring in the AT during obesity is the primary mechanism for developing local and systemic IR.

AT is the primary whole-body regulator of lipid and glucose homeostasis and is no longer considered merely a storage tissue.

Obesity leads to severe adipocyte disorders by altering the amount and activity of almost all resident immune cells. The imbalance of immunological phenotypes is correlated with the development of persistent local inflammation during which several biologically active molecules are released. These molecules affect distal tissues and organs, such as skeletal muscle and liver.

The inflammatory nature of obesity opens new prospects in the development of therapeutic strategies for the treatment of its related metabolic complications. However, there are still a lot of issues that need to be addressed.

Anti-inflammatory strategies have proven to be effective in improving obesity-induced IR in murine models. However, clinical studies are still ongoing to confirm the therapeutic potential in obese and insulin-resistant individuals. Another issue is the modest effects of anti-inflammatory therapies observed in these studies. Targeting only one inflammatory molecule may not be sufficient to have a beneficial effect; therefore, we could hypothesize the combined use of more anti-inflammatory therapies. In addition, a recent study showed that acute and transient inflammation is essential for healthy AT expansion and remodeling in obesity (Asterholm et al., 2014). This finding raises further questions on the effectiveness of anti-inflammatory therapies in the treatment of obesity-induced metabolic disorders. Inflammation is a finely regulated mechanism, and all defects in its balance can cause AT dysfunction.

In the era of personalized and precision medicine, increasing our knowledge of the obesity-induced inflammation mechanisms might enable us to overcome the limitations of the traditional anthropometric indices of obesity. These anthropometric indices are not correlated with obesity-induced metabolic complications and additional clinical parameters need to be identified for risk assessment (Longo et al., 2019). From our point of view, given the strong association between inflammation and obesity complications, circulating inflammatory biomarkers may be used for the risk assessment of these diseases in the future. The identification and evaluation of these biomarkers in obese patients will allow the prediction of those who will develop obesity-associated metabolic complications.

AUTHOR CONTRIBUTIONS

FB and CM conceived the idea and edited the manuscript. FZ, ML, JN, GR, and AD wrote the manuscript. FZ and ML prepared the figures. All authors reviewed the manuscript.

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MINI REVIEW
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Pathogenesis of Chronic Plaque Psoriasis and Its Intersection With Cardio-Metabolic Comorbidities

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Psoriasis is a chronic, systemic immune-mediated disease characterized by development of erythematous, indurated, scaly, pruritic plaques on the skin. Psoriasis is frequently associated to comorbidities, including psoriatic arthritis, cardiovascular diseases, diabetes mellitus, obesity, non-alcoholic fatty liver disease, and inflammatory bowel diseases. In this review, we discuss the pathophysiological relationship between psoriasis and cardio-metabolic comorbidities and the importance of therapeutic strategies to reduce systemic inflammation in patients with moderate-to-severe psoriasis. Pathogenesis of psoriasis and its comorbidities share both genetic predisposition and inflammatory pathways, which include the TNFα and the IL-23/IL-17 pathways. These pathways are selectively addressed by biological treatments, which have substantially changed the outcomes of psoriasis therapy and affect positively comorbidities including reducing cardiovascular risk, allowing a more comprehensive approach to the patient.

Keywords: psoriasis, pathogenesis, treatment, cardio-metabolic comorbidities, inflammation

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INTRODUCTION

Psoriasis is a chronic, inflammatory disease involving skin of genetically predisposed individuals. It affects approximately 2% of the general population, with >50% of patients presenting in the first three decades of life (Bowcock, 2005; Griffiths and Barker, 2007; Chandran and Raychaudhuri, 2010). There is a wide spectrum of cutaneous manifestations of psoriasis, with individual lesions varying from pinpoint to large plaques, or even generalized erythroderma (Griffiths and Barker, 2007). The most common and well-recognized morphological presentation of psoriasis is the plaque type. These lesions reflect skin inflammation, epidermal hyperplasia, and angiogenesis, as consequence of a dysregulation of skin immune responses (Griffiths and Barker, 2007; Lowes et al., 2014). However, altered immunity can operate systemically and signs of inflammation can readily be detected at areas outside the skin. As a result, in psoriatic patients, inflammation is widespread, and, in most cases, different comorbid conditions co-exist (Binus et al., 2012; Armstrong et al., 2013a; Takeshita et al., 2017; Boehncke, 2018). Among them, cardio-vascular diseases (CVD) are importantly associated to psoriasis, together with diabetes mellitus and metabolic disorders, including obesity, hypertension, dyslipidemia, and non-alcoholic fatty liver disease (NAFLD) (Armstrong et al., 2012; Langan et al., 2012; Armstrong et al., 2013a; Armstrong et al., 2013b;

Coto-Segura et al., 2013; Armstrong et al., 2015; Candia et al., 2015; Takeshita et al., 2017). Also inflammatory bowel diseases (IBD) and kidney diseases, as well as infections, depression, and cancer are often comorbid conditions that can be developed in psoriatic patients (Bernstein et al., 2005; Binus et al., 2012; Wan et al., 2013). To date, it is still controversial whether the chronic inflammatory nature of psoriasis is a contributing factor or an independent risk factor for the development of these comorbidities. Consistently, inflammatory arthritis, that frequently affects small joints of psoriatic patients, could be considered either as an extracutaneous manifestation of psoriasis or as a separate entity, and thus a comorbidity (Gladman et al., 2005). Among the comorbid conditions, cardiovascular and metabolic diseases are of particular importance, as they may considerably reduce life expectancy of psoriatic patients, especially of those affected by the most severe forms of the disease (Armstrong et al., 2013a; Samarasekera et al., 2013).

This review summarizes the evidence on the pathophysiological relationship between psoriasis and its comorbidities, with emphasis on cardio-metabolic comorbid conditions. The ability of biologic therapies to reduce systemic inflammation and to ameliorate comorbidities, including reducing cardiovascular risk, in patients with psoriasis will be also discussed.

PATHOGENESIS OF CHRONIC PLAQUE PSORIASIS

Primary cause of psoriasis is a dysregulation of immune responses, which manifests in individuals carrying one or more psoriasis susceptibility genes, either skin specific or related to immune functions, and after their exposure to certain environmental triggers (Nestle et al., 2009; Perera et al., 2012; Di Meglio et al., 2014). The latter include physical trauma (Koebner phenomenon) and infections, which trigger innate immune responses by promoting the formation and the release of nucleic acid/autoantigen complexes by injured skin cells. In particular, complexes formed by the cathelecidin LL37 and self-DNA/RNA fragments activate plasmacytoid dendritic cells (pDCs), a subset of DC releasing high IFN-α and TNF-α (Nestle et al., 2005; Lande et al., 2007) pDC recruitment in psoriatic skin is determined by the chemokine chemerin, and correlates with the massive presence in the mid-papillary dermis of other innate immune cells, such as neutrophils, macrophages, monocytes, and mast cells (Albanesi et al., 2009). Local production of IFN-α and other type I IFNs induces keratinocyte immune activation and maturation of myeloid DCs (mDCs), with consequent beginning of the adaptive immune response phase. As a consequence, a IL-23/IL-17 inflammatory environment is established in psoriatic skin, with DC and macrophage-derived IL-23 promoting the type 17 helper (Th17) and cytotoxic (Tc17) cell effector functions (Lowes et al., 2013; Girolomoni et al., 2017). In parallel, mDCs induce the IL-12/IFN-γ cytokine axis, which is responsible for the strong type II IFN transcriptomic signature and the high frequency of Th1 and Tc1 cells in both psoriasis plaques and peripheral blood of patients (Schlaak et al., 1994; Austin et al., 1999). The innate lymphoid cells (ILC), a class of immune cells bearing lymphoid morphology, but no immune cell lineage markers (Spits and Cupedo, 2012; Bernink et al., 2013), together with γδ-T cells, an innate-like T-cell population involved in surveillance of epithelial surfaces, are also critical contributors to plaque development by releasing considerable levels of IL-17 and IL-22 (Villanova et al., 2014). Also mast cells and neutrophils can represent an innate sources of IL-17 in psoriatic skin (Lin et al., 2011).

Following the massive expansion of effector immune cells in both the epidermis and dermis, very high levels of IL-17 and IL-22 are produced. These two cytokines, together, mediate most of the epidermal hyperplasia by impairing differentiation of keratinocytes, and inducing their premature maturation and aberrant cornification (Nograles et al., 2008). IL-17 also functions by activating keratinocytes to produce neutrophil- and T-cell-recruiting chemokines, namely CXCL1/CXCL2/CXCL8 and CCL20, respectively, as well as antimicrobial peptides (AMP), including LL37 and S100 family members (Albanesi et al., 1999; Wilson et al., 2007). Thus, IL-17 is central in a pathogenic loop linking T cells and keratinocytes. On the other hand, the T-cell-derived IFN-γ and TNF-α activate a plethora of inflammatory pathways in resident skin cells, in particular keratinocytes and endothelial cells (Albanesi and Pastore, 2010; Albanesi et al., 2018). Each cytokine regulates distinct responses with a certain degree of synergism in getranscription factor regulating gene ene expression induction/inhibition. Most of the effects induced by IFN-γ are potentiated by TNF-α, which intracellularly activates NF-κB, a transcription factor regulating gene expression frequently in collaboration with the signal transducer and activator of transcription (STAT)1 induced by IFN-γ. TNF-α induces expression of ICAM-1 on resident skin cells, permitting the adhesion and extravasation of circulating leukocytes. Moreover, TNF-α stimulates the production of several chemokines active on immune cells, as well as pro-inflammatory cytokines, in particular IL-6 and IL-1, which sustain Th17 expansion (Albanesi et al., 2018; Chiricozzi et al., 2018). Importantly, TNF-α, together with IL-17, induces IL-36γ in psoriasis lesions, which in turn promotes expression of AMP and chemokines recruiting neutrophils and Th17 cells, as well as interferes with terminal differentiation and cornification process of the epidermis (Carrier et al., 2011). Interestingly, transcriptional profiling studies conducted on lesional psoriatic skin showed that the IFN-γ-signature predominates, even though IL-17 and TNF-α also potently induce a vast panel of genes (Nograles et al., 2008; Chiricozzi et al., 2014). Although studies demonstrated a central role of IL-22 in psoriasis pathogenesis by activating STAT3-dependent genes involved in differentiation and proliferation processes, this cytokine induces a limited panel of genes compared to IL-17, as detected in human lesional psoriatic skin (Chiricozzi et al., 2014). Importantly, intrinsic or genetic alterations of keratinocytes in the activation of key signaling pathways induced by pro-inflammatory cytokines (i.e., STAT1 and STAT3, NF-κB, ERK1/2, and Act1) may be

responsible for the typical unbalance between proliferation and differentiation processes, as well as inflammatory signatures observed in psoriatic epidermis (Harden et al., 2015; Capon, 2017).

Collectively, a pathogenic cross-talk between DCs, T cells, and keratinocytes, sustained by type I IFNs, IL-23, IL-12, IFN-γ, IL-17, TNF-α, and IL-22, and possibly supported by other immune cell players, causes keratinocyte production of pro-inflammatory molecules, as well as concurs to derange proliferative and differentiative programs of the epidermis. This becomes a self-amplifying loop, where these products and altered homeostasis act back on T cells and DC to perpetuate the cutaneous inflammatory processes.

COMORBIDITIES OF CHRONIC PLAQUE PSORIASIS

Since 1897, when Strauss reported an association between psoriasis and diabetes, emerging epidemiologic studies find additional associations between psoriasis and inflammatory diseases, apart from well-known psoriatic arthritis (PsA) (Strauss, 2009). The association between psoriasis and inflammatory diseases is stronger in the most severe forms of psoriasis (Takeshita et al., 2017). Comorbidity psoriasis burden includes mainly CVD, metabolic disorders, such as diabetes, dyslipidemia, and metabolic syndrome, inflammatory bowel disease, and kidney disease. The prevalence of traditional CV risk factors such as hypertension, obesity, diabetes, dyslipidemia, metabolic syndrome, and cigarette smoking is increased in patients with psoriasis compared to controls (Gisondi et al., 2010).

Patients with psoriasis are more frequently overweight or obese (**Figure 1**). Obesity, but also BMI, hip circumference and waist-hip ratio are independent risk factors for psoriasis. The risk was found to increase with obese severity, as higher body mass index (BMI) (Kumar et al., 2013). A meta-analysis of 16 observational studies found a pooled OR of 1.66 for the association between the two diseases (95% CI 1.46–1.89) (Armstrong et al., 2012). A cross-sectional study found a direct correlation between psoriasis severity and obesity (Duarte et al., 2013). As obesity, also hypertension is prevalent among psoriatic patients compared to those who are not affected. A meta-analysis of 24 studies showed a pooled OR of 1.58 (95% CI 1.42–1.76) for the association between hypertension and psoriasis (Armstrong et al., 2013b). Poor controlled and sever hypertension appear to increase with more severe disease (Langan et al., 2012). Psoriasis is an independent risk factor for diabetes, with higher risk with greater severity of psoriasis (Takeshita et al., 2015). Diabetic patients with psoriasis appear to be more likely to suffer from micro and macro-vascular complications, compared to patients without psoriasis. The pooled OR for psoriasis associated with diabetes in a meta-analysis of 44 studies was 1.76 (95% CI

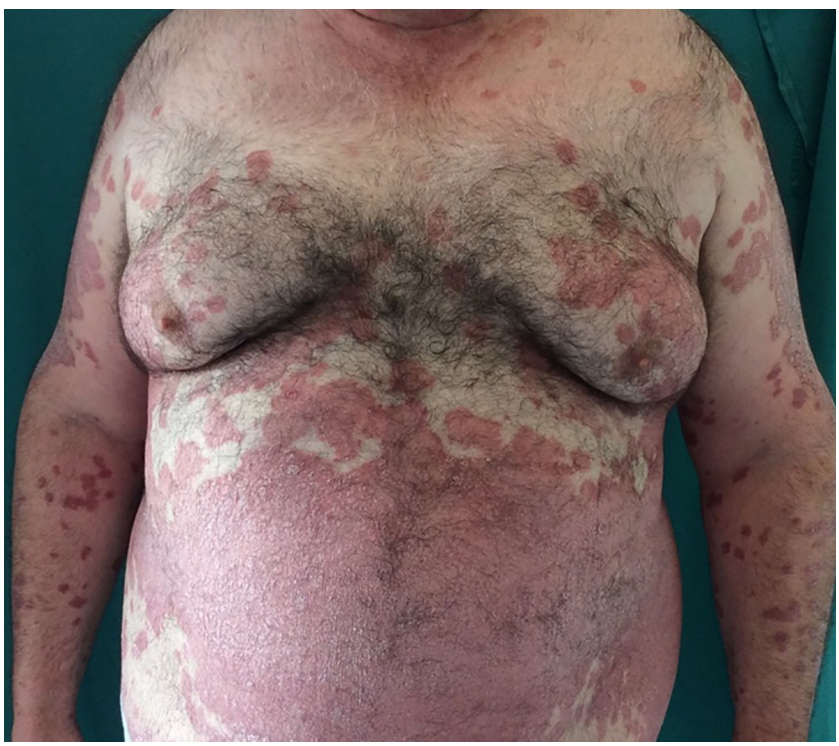

FIGURE 1 | Man affected by psoriasis and central obesity.

1.59–1.96) (Coto-Segura et al., 2013). Atherogenic lipid profile and reduced high density lipoprotein (HDL) were reported among patients with psoriasis, compared to patients without psoriasis. Dyslipidemia may be more prevalent in psoriatic patients. In a systematic review, most of the studies found significant association between psoriasis and dyslipidemia, with OR ranging between 1.04 and 5.55 (Ma et al., 2013). Higher odds of dyslipidemia were reported in severe psoriasis, compared to patients with mild disease. According to some studies, dyslipidemia may be a risk factor for developing psoriasis (Wu et al., 2014). Metabolic syndrome comprises a group of well-known cardiovascular (CV) risk factors, including central obesity, hypertension, insulin resistance, and dyslipidemia. A cross-sectional study reported that the prevalence of metabolic syndrome correlated directly with psoriasis body surface area (Langan et al., 2012). A meta-analysis of 12 studies found a pooled OR of 2.26 (95% CI 1.70–3.01) for the association with psoriasis (Armstrong et al., 2013c). The analysis of the separate components of metabolic syndrome showed the strongest association between obesity, suggesting that the adiposity is the main factor in the association between psoriasis and metabolic syndrome.

Although both CV risk factors and CVD are prevalent among psoriatic patients, psoriasis is an independent risk factor for the latters. A large cohort study found that psoriasis is an independent risk for myocardial infarction (MI), also considering other traditional CV risk factors (Gelfand et al., 2006). Two meta-analyses showed that the risks of MI, stroke, and death caused by CVD, collectively termed as major cardiovascular events, is greatest among patients with psoriasis and appear to be greatest among those with severe or longer duration disease (Armstrong et al., 2013a; Samarasekera et al., 2013). Psoriasis, as an independent CV risk factor, was reported to strongly impact on the Framingham Risk Score for more than 60% of the patients (Mehta et al., 2012).

The epidemiology of the relationship between IBD and psoriasis is still unclear. Many studies reported that psoriasis may be associated with an increased incidence and prevalence of IBD (and *vice versa*), in particular Crohn's disease (Bernstein et al., 2005; Cohen et al., 2009). Psoriasis may be more strongly associated with Crohn's disease than ulcerative colitis withOn the other hand, the T-cell-derived ORs of 2.49 (95% CI 1.71–3.62) *versus* 1.64 (95% CI 1.15–2.23), respectively (Mehta et al., 2012). Patients with psoriasis and concomitant IBD have a higher rate of comorbidities (seronegative arthritis, thyroiditis, diabetes, and lymphoma) than patients with only psoriasis (Binus et al., 2012). Considering the potentially hepatotoxicity and nephrotoxicity of many psoriatic treatments, there is a great interest in the epidemiology of liver and renal disease in psoriatic patients. NAFLD is a common liver disease comprising mild forms of steatosis up to steato-hepatitis. Psoriasis is frequently associated to metabolic disorders that can favor liver steatosis. The prevalence of NAFLD among patients with psoriasis is greater compared with non-psoriatic patients, but the evidence of the association between psoriasis and hepatic diseases is based on seven low-to-moderate quality observational studies with pooled OR of 2.15 (95% CI 1.57–2.94) (Candia et al., 2015). Moderate-to-severe psoriasis may be an independent risk factor for chronic kidney disease (CKD) and end-stage renal disease. A cohort study found that severe psoriasis may be associated with CKD and end-stage renal disease with HRs of 1.93 (95% CI 1.79–2.08) and 4.15 (95% CI 1.70–10.11), respectively (Wan et al., 2013).

Several studies have reported association between psoriasis and other emerging comorbidities such as cancer, especially T-cell lymphoma, mood disorders, pneumopathies such as chronic pulmonary disease and obstructive sleep apnea, peptic ulcer disease, hyperuricaemia/gout, osteoporosis, and sexual dysfunction (Takeshita et al., 2017). Some of these need to be confirmed in larger studies.

PATHOGENESIS BEHIND THE COMORBIDITIES IN PSORIASIS

The pathogenesis behind psoriasis comorbidity remains partially unknown; however different factors may be involved, including common pattern of immune responses and inflammatory pathways, shared risk factors, and genetic predisposition (Takeshita et al., 2017) (**Figure 2**).

Patients with psoriasis are enriched for certain common genetic variants (HLA, FUT2, UBE2L3, SH2B3) that predispose to increased risk of dyslipidemia, hypertension, and CVD (Lu et al., 2013).

Most common inflammatory pathways between psoriasis and its comorbidities strictly depends on the expansion of circulating pathogenic T cells, instructed by DC activated at skin sites, and to the establishment of systemic inflammation. These pathways involve key cytokines and signal transducers, such as IL-23R, IL-12B, IL-21, IL-4, and IL-5, in psoriatic arthritis; IL-23R, IL-12B, IL-13, Rel, TYK2, and JAK2 in Crohn's disease (Ellinghaus et al., 2012; FitzGerald et al., 2015; Veale and Fearon, 2018). In addition to common cytokine hallmarks, psoriasis and cardiometabolic diseases may share other mutations, such as CDKAL1 and apolipoprotein E (Eiris et al., 2014).

A high number of studies have shown that psoriasis and cardiometabolic disorders have rather more commonly similar underlying immunologic mechanisms related to Th1 and Th17 cells activation (Lockshin et al., 2018). Inflammatory mediators released from psoriatic lesions, including TNF-α, IFN-α, IFN-γ, IL-1, IL-6, and IL-17, may have systemic effects contributing to atherogenesis. Consistently, recent studies conducted on human tissues showed that psoriasis and atherosclerosis exhibit significant overlap of their transcriptomes and in particular those dependent on TNF-α and IFN-γ, thus providing the linking between the two diseases (Mehta et al., 2017). By contrast, IL-17A and CCL20 genes were higher in psoriasis than in atherosclerosis tissue, whereas IL-17R was expressed at comparable levels. Because of the link between IL-17 and neutrophil infiltration in atherosclerotic plaques and its pathogenic role in psoriasis, it has been suggested that the IL-17/neutrophil axis could take part to atherogenesis associated with psoriatic disease (Sanda et al., 2017). Consistently, aortic vascular inflammation in psoriatic patients has been found to

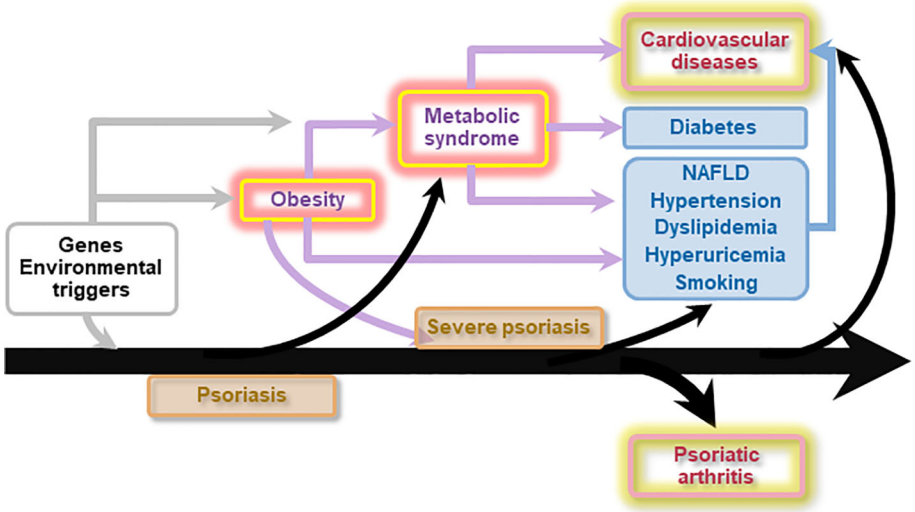

FIGURE 2 | Genetic and environmental factors predispose to psoriasis and obesity. Obesity is a risk factor for both psoriasis and metabolic syndrome. However, inflammation associated with moderate to severe psoriasis can in turn favor insulin resistance, dyslipidemia, obesity, and non-alcoholic fatty liver disease (NAFLD), hence directly and/or indirectly fuelling atherosclerosis, and configuring the so-called "psoriatic march". Ultimately, moderate to severe psoriasis directly and indirectly increases the risk of cardiovascular diseases and mortality. Psoriasis also precedes the development of psoriatic arthritis.

correlate with disease severity and high levels of S100A8/A9 neutrophil activation markers (Naik et al., 2015). In addition, the neutrophil extracellular traps (NET)osis, a defense mechanism operating in psoriasis and based on the formation of cytosolic granule proteins containing autoantigens, has been found to induce macrophage priming, Th17 activation, and immune cell recruitment in atherosclerosis similarly to psoriasis (Aldabbous et al., 2016; Delgado-Rizo et al., 2017; Doring et al., 2017). NET are also shown to directly induce endothelial dysfunction and plaque rupture in human carotid plaque sections (Quillard et al., 2015). Monocyte and neutrophil damage, increased oxidative stress, endothelial dysfunction, angiogenesis, and increased circulating micro particles are other common shared alterations (Quillard et al., 2015). Psoriasis and atherosclerosis patients also share dysfunctional peripheral T regulatory (Treg) cells, a subset of T lymphocytes highly releasing TGFβ, IL-10, and IL-35, with inhibitory function on T cell activation and proliferation, and anti-inflammatory roles through endothelial cell modulation (Sugiyama et al., 2005; Kagen et al., 2006; Takeshita et al., 2014; Meng et al., 2016). In psoriasis, this impairment may be dependent on high IL-6 levels, as demonstrated by blocking IL-6 in co-cultures of Treg cells and effector T cells from psoriatic patients (Goodman et al., 2009).

Systemic inflammation associated with psoriasis also promotes inflammation in the adipose tissue, which harbors cells and molecular components of the immunity system. Psoriatic adipose tissue contains immune cells that can influence cardiometabolic disease (Rose et al., 2014). Among them, T cells, DCs, neutrophils, mast cells, and adipose tissue macrophages contribute to obesity and insulin resistance, whereas eosinophils and Treg are protective. Obesity is also associated to systemic inflammation because of the release of adipokines, including chemerin, adiponectin, resistin, visfatin, C-reactive protein released by macrophages, and T cells infiltrating visceral adipose tissue. Adipokines can contribute to the pathogenesis of insulin resistance and fuel inflammation associated with psoriasis (Davidovici et al., 2010). Adipokines together with chemokines (i.e., CXCL8 and CCL2) produced by visceral adipose tissue can also contribute to progression of atherosclerosis and CDV disease development by influencing endothelial cell function and interaction with immune cells (Henrichot et al., 2005; Karastergiou et al., 2010; Britton and Fox, 2011). An association between obesity and PsA has been confirmed, and the presence of metabolic syndrome and related adipokines correlates with skin and joint disease activity (Eder et al., 2013). However, levels of adipokines have been found to not differ between PsA patients with clinical evident psoriasis and PsA sine psoriasis, reinforcing the concept that metabolic manifestations during psoriatic disease may be independent of severity of cutaneous and articular involvement and are potentially related to the subclinical chronic inflammation (Caso et al., 2019). As abdominal visceral fat, also epicardial adipose tissue has been shown to be increased in patients with psoriasis, and potentially contribute to increased CV risk (Torres et al., 2015). Additionally, epicardial adipose tissue has been referred as potentially responsible for a distinctive pattern of CV disorders seen in psoriasis (accelerated coronary atherosclerosis leading to myocardial infarction; atrial myopathy leading to atrial fibrillation and thromboembolic stroke, and ventricular myopathy leading to heart failure with a preserved ejection fraction) (Packer, 2019). Psoriasis-related inflammation could trigger the progression from normal liver to NAFLD.

Pro-inflammatory cytokines and adipokines, including TNF-α, play a pivotal role in pathogenesis of both psoriasis and NAFLD as well as in progression of NAFLD to NASH. Moreover, obesity induces a bio-mechanical stress that may be a possible trigger for PsA (Mantovani et al., 2016).

Finally, as psychosocial impact of psoriasis is relevant, this could favor unhealthy lifestyles, such as alcoholism and smoking habit that are well-known CV and metabolic risk factors. Anxiety, depression, and suicidal ideation and behavior (SIB) are prevalent in patient with psoriasis. There is growing evidence that inflammation is associated with pathophysiology of depression. Pro-inflammatory cytokines such as IL-1 and IL-6 are elevated both in psoriasis and depression. Cytokine-mediated systemic inflammation may be an underlying patho-mechanism of both psoriasis and mental health disorders, such as depression and SIB (Wu et al., 2018a).

SYSTEMIC TREATMENT OF PSORIASIS COULD AMELIORATE CARDIOVASCULAR COMORBIDITIES

New psoriasis treatment paradigms have gone beyond the belief of psoriasis as a disease limited to the skin. Borrowing the experience from the studies of other immune-mediated inflammatory diseases, such as Crohn's disease and rheumatoid arthritis, the goals of treating systemic inflammation in psoriasis are two: to prevent and even to reverse comorbidities (Takeshita et al., 2017; Korman, 2019).

Many surrogate laboratory and radiologic biomarkers of inflammation and endothelial dysfunction have been identified among patients with inflammatory conditions. These include C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), levels of glycoprotein acetylation, coronary flow reserve (CFR), flow-mediated dilatation, carotid intima media thickness, vascular inflammation measured through 18-fluorodeoxyglucose positron emission tomography-computed tomography (18F-FDG PET/TC) (Vadakayil et al., 2015; Puig, 2017). For example, in patients with moderate to severe psoriasis treated with systemic therapies studies have reported reduction in ESR and/or CRP levels (Popa et al., 2005). A prospective study found that improvement in Psoriasis Area Severity Index (PASI) score *via* TNF-α inhibitors was associated with reduced aortic vascular inflammation measured using 18F-FDG PET/TC (Bissonnette et al., 2013). A study on 37 patients treated with TNF-α inhibitors for an average of 6 months reported a significant increase in the value of CFR in the left anterior descending coronary (Piaserico et al., 2016). Recently, biologic therapy was shown to be associated with favorable modulation of coronary plaque indices by coronary computed tomography angiography in patients with severe psoriasis (Elnabawi et al., 2019). There are some evidences in favor of the hypothesis that treating psoriasis with systemic agents could prevent CVD, as a result of suppression of systemic inflammation. In a recent meta-analysis in patients with psoriasis and PsA, systemic therapy was found to significantly decrease the risk of all cardiovascular events with a RR of 0.75 (95% CI 0.63–0.91). With the exception of methotrexate, there are no studies formally evaluating the effect of any anti-psoriatic therapy as a treatment for coronary heart disease (Roubille et al., 2015). Two retrospective analysis of cardiovascular events rates in patients with psoriasis found that patients receiving rouTNF-α inhibitors had significant lower risks for MI compared with patients receiving topical therapies with OR 0.5 (95% CI 0.32–0.79) or phototherapy, HR 0.77 (95% CI 0.60–0.99) (Wu et al., 2012; Wu et al., 2018b). Few studies found no significant differences in overall MI risk between patients treated with systemic therapies and those who received ultraviolet B phototherapy. In a randomize double-blind clinical trial adalimumab reduced key markers of inflammation including glycoprotein acetylation compared with phototherapy, with no effect on glucose metabolism and vascular inflammation (Mehta et al., 2018). The protective cardiovascular effect could be not exclusive to TNF-α inhibitors. CARIMA study indicates that secukinumab might have a beneficial effect on cardiovascular risk by improving the endothelial function (von Stebut et al., 2019).

Obesity is accompanied by a dysregulation of adipocytokines and systemic inflammation and has a well-known effect on psoriasis severity and response to therapies. Current data suggest that weight loss improves psoriasis. Meta-analysis of three randomized control trials confirmed that weight loss following lifestyle interventions (diet or physical activity) improves psoriasis compared with reduction in the PASI score with a pooled mean difference of -2.49 (95% CI -3.90 to -1.08; $P = 0.004$) (Mahil et al., 2019). Long-term weight loss in patients with psoriasis has long-lasting positive effects on the severity of psoriasis (Gisondi et al., 2016). A possible role for biologic agents in reducing obesity in psoriasis has not been observed to date. Although TNF-α inhibitors can induce modest weight gain, they do not cause an increase in visceral adipose tissue and thee association between low-carbohydrates calorie-restricted diet and anti-TNF-alpha therapy seems to be able to improve the anthropometric profile of psoriasis patients (Campanati et al., 2017). No evidence of clinically weight gain has been observed in studies of ustekinumab or ixekizumab. Since IL-17A does not alter adipogenesis and/or insulin resistance mediated by an inflammatory environment and contributes only to the propagation of inflammation in human obese adipose tissues, anti-IL17A agents may play a beneficial effect in inflammatory pathologies, where obesity contributes to poorer response to biologic treatments (Pestel et al., 2019).

CONCLUSIONS

Psoriasis is increasingly being recognized as a systemic inflammatory disorder affecting not only skin and joints. The association with metabolic disorders, such as diabetes, dyslipidemia, and metabolic syndrome, and CVD deserves special attention. Common genetics and shared immuno-inflammatory pathways may partially explain this association. Cutaneous lesions produce a wide range of inflammatory

products that are released into systemic circulation and fuel the systemic inflammation. Other non-cutaneous sites, like adipose tissue, can contribute to the inflammatory state. Systemic therapies targeting psoriasis may prevent and even reverse cardio-metabolic comorbidities as a result of suppression of systemic inflammation. It is important for clinicians to recognize psoriasis comorbidity burden to ensure a comprehensive medical care for the patients. In the view of psoriasis as systemic inflammation disease new treatment paradigms may potentially reduce or prevent the comorbidities associated with systemic inflammation.

AUTHOR CONTRIBUTIONS

Each author has contributed in the ideation and writing of the manuscript, and each author has checked the final version of the paper.

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REVIEW
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Metabolic Checkpoints in Rheumatoid Arthritis

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Several studies have highlighted the interplay between metabolism, immunity and inflammation. Both tissue resident and infiltrating immune cells play a major role in the inflammatory process of rheumatoid arthritis (RA) via the production of cytokines, adipo-cytokines and metabolic intermediates. These functions are metabolically demanding and require the most efficient use of bioenergetic pathways. The synovial membrane is the primary site of inflammation in RA and exhibits distinctive histological patterns characterized by different metabolism, prognosis and response to treatment. In the RA synovium, the high energy demand by stromal and infiltrating immune cells, causes the accumulation of metabolites, and adipo-cytokines, which carry out signaling functions, as well as activating transcription factors which act as metabolic sensors. These events drive immune and joint-resident cells to acquire pro-inflammatory effector functions which in turn perpetuate chronic inflammation. Whether metabolic changes are a consequence of the disease or one of the causes of RA pathogenesis is still under investigation. This review covers our current knowledge of cell metabolism in RA. Understanding the intricate interactions between metabolic pathways and the inflammatory and immune responses will provide more awareness of the mechanisms underlying RA pathogenesis and will identify novel therapeutic options to treat this disease.

Keywords: rheumatoid arthritis, metabolism, immunity, mediators of inflammation, immunometabolism

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INTRODUCTION

Rheumatoid arthritis is an immune mediated inflammatory disease characterized by autoantibody production [including rheumatoid factor (RF) and anti–citrullinated protein antibody (ACPA), anti-carbamylated proteins antibodies (anti-CarP) etc.,] chronic synovial inflammation (synovitis) and hyperplasia, cartilage and bone destruction, as well as systemic complications such as cardiovascular, pulmonary, and neurological co-morbidity. Progressive disability and systemic complications are still a burden leading to socioeconomic costs and unmet needs. Indeed, current conventional and biologic disease modifying therapies produce good responses in only 60% of patients (Humby et al., 2019). Predictive biomarkers of prognosis, therapeutic response, and resistance to treatment, which currently include ACPA, RF, C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR), remain inadequate from a clinical decision making perspective (McInnes and Schett, 2011; Dennis et al., 2014).

The loss of immune tolerance that precedes the onset of inflammation in the joint is thought to represent a key process in RA pathogenesis (McInnes and Schett, 2011; Smolen et al., 2016) and is likely to occur at extra-articular sites (Tracy et al., 2017). Synovitis, the hallmark of established RA, is characterized by leukocyte infiltration, neo-angiogenesis and increased expression of adhesion molecules and chemokines which lead to increased leukocyte migration into the inflamed site. In addition, inadequate lymphangiogenesis, which limits cell egress, together with local fibroblast activation, promotes the establishment of synovial inflammation (Croft et al., 2019). Nutrient availability is also limited and immune and joint resident cells compete for available nutrients at a rate which exceeds their production thereby increasing the metabolic demand (**Figure 1**; Goetzl et al., 1971; Treuhaft and MCCarty, 1971; Patella et al., 2015; Biniecka et al., 2016; Tsokos, 2016; Yang et al., 2016; Zhou et al., 2016). All these events can, in the long-term, induce an alteration of immune responses and promote a continued breach of immune tolerance leading to inflammation and autoimmunity.

METABOLITES: A FOCUS ON LACTATE

The study of intermediates and end-products of metabolism in the context of immune cell functions is an emerging field that has been termed immunometabolism (Pearce et al., 2013). It is now clear that molecules such as succinate, lactate, acetyl-CoA, fumarate are more than intermediate by-products in metabolic pathways as they function as signaling molecules capable of linking metabolic reprograming with immune and inflammatory responses in immunity, inflammation and cancer (**Figure 1**; Haas et al., 2016). Whether metabolic perturbations are causal or the effect of the disease and how they can impact on the prognosis of RA is an area of significant current research.

Nuclear magnetic resonance (NMR) spectroscopy–based metabolomics on serum and urine samples from people with RA has identified a metabolic signature of patients with active established RA which differs from that of healthy controls (Young et al., 2013). Among the metabolites investigated 3-hydroxybutyrate and lactate were much higher in RA than in the control group. In addition choline, lactate and low-density lipoprotein (LDL) lipids strongly correlated with CRP a marker of disease activity (Young et al., 2013). This evidence suggests that NMR could be used as a tool to predict the development of atherosclerosis and other metabolic complications often associated with inflammatory disease. Similarly, a gas chromatography–mass spectrometry (GC–MS) study on serum samples, has shown a decrease in amino acid and glucose metabolism in combination with increased fatty acid metabolites such as palmitate, oleate and cholesterol (Zhou et al., 2016).

In the same vein, a correlation between serum metabolites and gene expression profiling in synovial tissue from patients with active RA was recently found (Narasimhan et al., 2018). The authors described an association of serine, glycine, and phenylalanine metabolism with a lymphoid cell gene expression signature in synovial tissue. In addition, amino acids (i.e., alanine, aspartate, glutamate) and choline-derived metabolites correlated with TNF-α synovial expression while circulating ketone bodies associated with synovial gene expression of metalloproteinases. These data pointed to a link between serum metabolite profiles and synovial biomarkers further suggesting that NMR may be a promising technique for mapping pathogenic pathways in RA (Narasimhan et al., 2018).

In vitro studies have further highlighted the role of metabolites as signaling molecules in mediating inflammatory responses. Studies on succinate have shown that lipopolysaccharides (LPS)-activated inflammatory (M1) macrophages accumulate this metabolite intracellularly as a consequence of an altered TCA cycle (Jha et al., 2015). Here succinate promotes the activation of hypoxia-inducible factor (HIF)-1α and increases pro-inflammatory interleukin (IL)-1β production. In addition, when activated by inflammatory stimuli, macrophages release succinate into the extracellular space and up-regulate G protein-coupled receptor (GPR)91, which functions as a sensor for extracellular succinate to enhance IL-1β production (Tannahill et al., 2013). Notably, GPR91-deficient mice display decreased macrophage activation and reduced IL-1β production during antigen-induced arthritis as well as decreased dendritic cell traffic and reduced differentiation of Th17 cells in the lymph nodes (Tannahill et al., 2013; Saraiva et al., 2018). High levels of succinate have been found in synovial fluid from RA patients, where it induces IL-1β release from macrophages in a GPR91-dependent manner. This evidence suggests that GPR91 antagonists may act as novel therapeutic molecules to treat RA (Littlewood-Evans et al., 2016). Interestingly, intracellular and extracellular succinate exhibit different functions. More specifically, intracellular succinate induces angiogenesis through HIF-1α, while extracellular succinate regulates GPR91 activation (Li et al., 2018). The abolition of succinate dehydrogenase (SDH) activity with dimethyl malonate limited succinate accumulation and prevented angiogenesis via blocking the HIF-1α/VEGF axis, revealing a new potential therapeutic strategy to attenuate neo-angiogenesis in arthritis (Li et al., 2018). If succinate

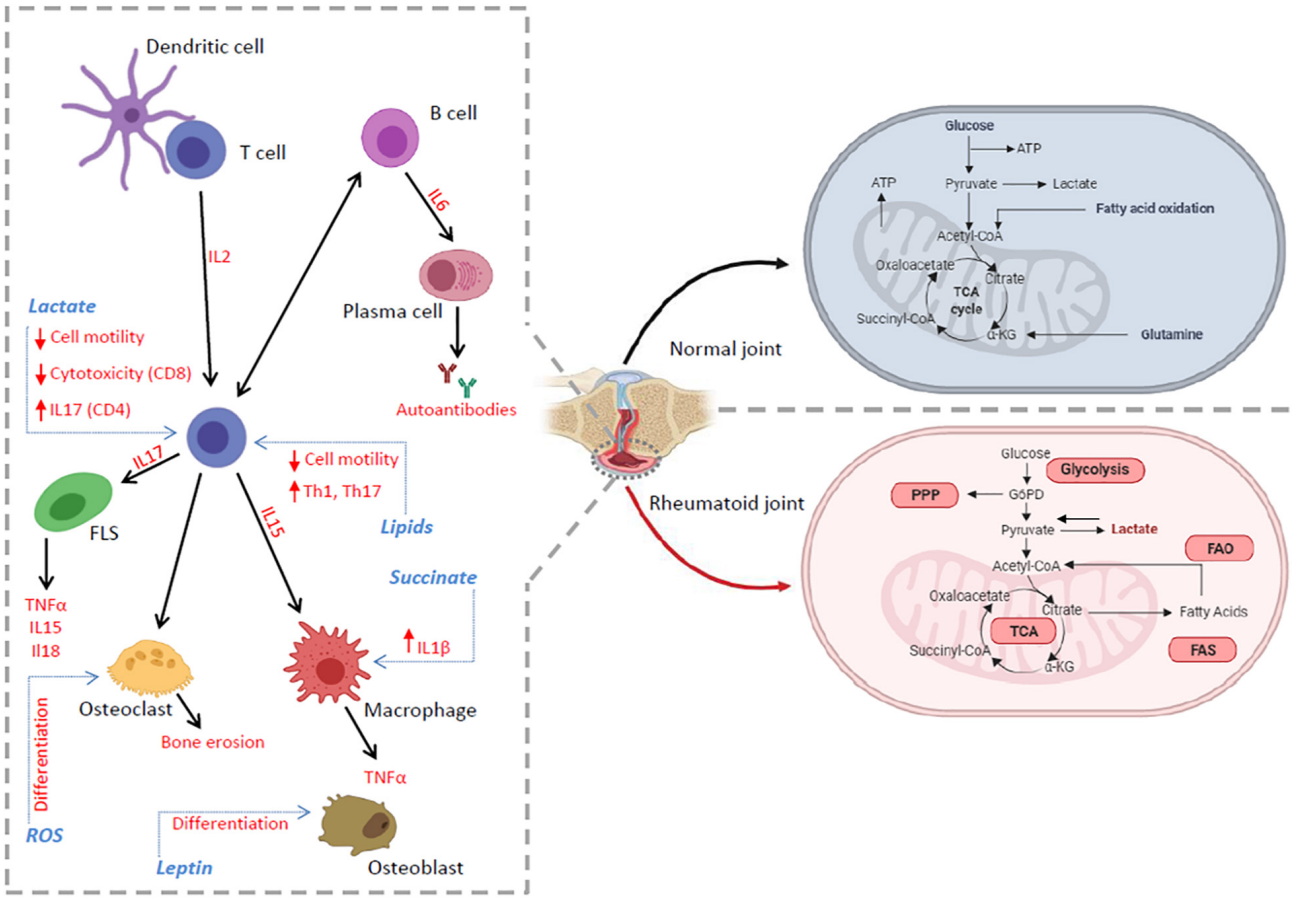

FIGURE 1 | The inflammatory environment in RA synovium. The RA tissue microenvironment is characterized by the accumulation of cytokines, adipo-cytokines and metabolic intermediates produced by the accelerated metabolism of infiltrating and tissue resident immune cells. These events can promote or be the consequence of a dysregulation in several metabolic pathways (red), including glycolysis, TCA cycle, PPP and lipid metabolism, which regulate many cell functions including activation, differentiation, proliferation, autoantibody and cytokine production leading to pro-inflammatory immune responses and the exacerbation of chronic inflammation.

exhibits pro-inflammatory activity, other metabolites such as fumarate and itaconate, have been observed to mediate anti-inflammatory effects (McGuire et al., 2016; Mills et al., 2018). With regard to fumarate, the methyl ester dimethyl fumarate (DMF) has been approved for the treatment of relapsing multiple sclerosis (MS) (Fox et al., 2012; Gold et al., 2012). Interestingly DMF has been reported to reduce osteoclastogenesis and bone destruction *via* increasing the expression of nuclear factor erythroid 2–related factor 2 (NRF2)-mediated antioxidant genes and decreasing reactive oxygen species (ROS) levels (Yamaguchi et al., 2018). The role of itaconate in RA is still debated. Despite evidence suggests an anti-inflammatory role (Mills et al., 2018) other studies have shown that reduced levels of itaconate correlate with a decreased pro-inflammatory (M1) signature in human macrophages isolated from healthy control subjects (Papathanassiu et al., 2017) and with a reduced arthritis severity *in vivo* (Michopoulos et al., 2016; Papathanassiu et al., 2017). It would be valuable to investigate how these observations in murine models translate into the human disease setting (i.e., OA vs. RA macrophages).

For more than 50 years, the inflamed joint has been recognized as a site with low levels of glucose and high amounts of lactate (Goetzl et al., 1971; Treuhaft and MCCarty, 1971), as a consequence of the intense cellular turnover in the synovium. Accumulation of lactate in RA synovial fluid is in part responsible for the acidic environment of RA synovitis. Indeed, it is well established that the PH of synovial fluidis significantly lower in inflamed arthritic joints than in healthy joints (Cummings and Nordby, 1966).

The rheumatoid synovial environment is paradigmatic of some of the lactate-induced features seen in T cells, including IL-17 secretion and loss of antigen responsiveness (Croia et al., 2013). In particular, lactate modulates specific T cell subsets via the interaction with lactate transporters. Sodium lactate selectively affects $CD4^+$ T cell functions via the solute carrier (SLC)5A12, while lactic acid was found to have an impact on $CD8^+$ T cell motility and cytolytic capability via its influx through SLC16A1 (MCT1) (Haas et al., 2015; Pucino et al., 2017).

Solute carrier 5A12 is highly expressed in RA synovial tissues and this expression significantly increases in association with the

inflammatory T cell score (Haas et al., 2015; Pucino et al., 2017). Notably we showed that SLC5A12 blockade promoted the egress of $CD4^+$ T cell from the inflamed tissue in an organ culture model and improved clinical scores of disease in an experimental model of arthritis (Pucino et al., 2019).

Another lactate transporter, the monocarboxylate transporter 4 (MCT4 or SLC16A3) was found to be up-regulated by RA synovial fibroblast (FLS) compared to osteoarthritis (OA) FLS (Fujii et al., 2015). Silencing MCT4, with MCT4-specific siRNA, inhibited the proliferation of RA FLS and was able to reduce the severity of arthritis in mice with collagen-induced arthritis (CIA) (Fujii et al., 2015).

These findings have established lactate signaling as integral feature of RA and open up the possibility of a new biomarker for disease progression and response to treatment as well as a novel target for therapeutic intervention. However, a better understanding of how the different synovial cell types co-ordinate their metabolism and the role of metabolites in cell-cell communication will be required to fully appreciate how the metabolic landscape in disease differs from that in health.

GLUCOSE METABOLISM

Proliferating cells mainly use aerobic glycolysis (Warburg effect) to generate energy. Indeed, in inflammatory conditions and tumors, aerobic glycolysis is preferred over oxidative phosphorylation for the production of ATP and for the stock of carbon sources necessary to build cell mass (Tsokos, 2016).

Both peripheral and tissue resident RA $CD4^+$ T cells have a unique metabolic signature (Weyand et al., 2017; Pucino et al., 2019). Indeed, RA $CD4^+$ T cells exhibit an impairment in engaging glycolysis. This is due to a deficiency of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3), a glycolysis regulator enzyme, resulting in delayed glycolysis and increased pentose phosphate pathway (PPP) via the up-regulation of glucose-6-phosphate dehydrogenase (G6PD). As a consequence, high levels of NADPH (reduced form nicotinamide adenine dinucleotide phosphate) and ROS consumption were observed. Moreover, altered activation of ataxia telangiectasia mutated (ATM), an enzyme involved in the cell cycle, was also reported. All these alterations result in increased cellular proliferation, a switch toward pro-inflammatory $CD4^+$ T cell subsets (Th1 and Th17) and chronic inflammation (Yang et al., 2013, 2016). Interestingly the replenishment of ROS was able to reverse these phenomena (Yang et al., 2013, 2016). Similarly, $CD4^+$ T cell from naive-to-treatment RA synovial tissues display a reduced expression of glycolytic genes coupled with increased PPP and Kreb cycle genes (Pucino et al., 2019). These findings correlate with increased Th17 cell tissue infiltration and the formation of ectopic lymphoid structure (ELS) (Pucino et al., 2019).

6-phosphofructo-2-kinase/fructose-2 deficiency also limits the ability of RA T cells to engage autophagy with increased susceptibility to apoptosis (Yang et al., 2013). This is linked with the recent discovery that RA T cells lack N-myristoyltransferase (NMT)-induced AMP-activated protein kinase (AMPK) activation which is a positive regulator of autophagy by suppressing the mammalian target of rapamycin (mTOR) activity (Kim et al., 2013; Cassano et al., 2014; Wen et al., 2019). Further studies are needed to better comprehend the intracellular mechanisms linking metabolism, apoptosis, and autophagy in RA to understand potential therapeutic implications.

In contrast to T cells, RA FLS display increased glycolytic metabolism under metabolic stress (Falconer et al., 2018). Indeed, glucose deprivation or glycolytic inhibitors [i.e., 2-deoxy-D-glucose (2-DG)], reduced FLS cytokine secretion, proliferation, and migration as well as disease severity in a mouse model of arthritis (Garcia-Carbonell et al., 2016). In this context, RA FLS show an higher expression of the inducible isoform of hexokinase (HK)2, which catalyze the phosphorylation of glucose to glucose 6 phosphate (G6P), in comparison to OA FLS. Interestingly, HK2 silencing reduced RA FLS tissue invasiveness; by contrast, the overexpression of HK2 increased the levels of MMP, IL6, and IL8 along their migratory rate (Bustamante et al., 2018). These data were further confirmed *in vivo*, in a mouse model of arthritis, where the HK2 deletion in murine FLS ameliorated disease severity of arthritis (Bustamante et al., 2018). Similarly, the HK2 inhibitor, 3-bromopyruvate (BrPA), was found to modulate the Th17/Treg ratio and suppress dendritic cells (DC) activation and cytokine expression (Okano et al., 2017). In addition to its canonical role in glucose metabolism, HK2 translocates to mitochondria where it triggers an autophagic and anti-apoptotic responses through its interaction with the voltage-dependent anion channel (VDAC) (Tan and Miyamoto, 2015). Intriguingly we found that lactate, which is abundant in the RA synovium, modulates HK2 mitochondrial translocation suggesting a potential role of this enzyme in promoting T cell survival. This provides an important link between metabolism and apoptosis resistance in the RA synovium that needs to be further explored (Pucino et al., 2019).

Abnormal metabolism by RA FLS may be a consequence of the hypoxic microenvironment found in inflamed sites. Indeed, hypoxia by itself is able to induce a downregulation of mitochondrial respiration and an increase of glycolysis in RA fibroblasts, leading to synovial invasiveness, angiogenesis and synovial hyperplasia (Biniecka et al., 2014, 2016). Moreover stimulation *in vitro* of RA FLS with platelet derived growth factor (PDGF) or TNF increased glucose metabolism (Garcia-Carbonell et al., 2016).

Enhanced glycolysis is also observed in synovial monocytes and macrophages in RA. RA macrophages express high levels of the glycolytic enzyme α-enolase, which induces secretion of pro-inflammatory cytokines through autoantibody recognition (Bae et al., 2012). High concentrations of glucose have also been shown to increase IL-1β secretion from RA monocytes through an NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3)/inflammasome-dependent mechanism (Ruscitti et al., 2015) and the glycolytic enzyme HK1 is known to drive cleavage and activation of pro–IL-1β in macrophages (Moon et al., 2015). Following these results, a clinical trial (NCT02236481) has recently been published showing the efficacy of IL-1 inhibition, in terms of RA disease activity and glycated

hemoglobin percentage (HbA1c%), as a targeted treatment in patients with RA and type 2 diabetes (T2D). Notably patients treated with TNF inhibitors did not achieve the same results (HbA1c reduction) in this trial suggesting a different pathogenic mechanism linking inflammation, T2D and RA. Further studies are needed to dissect the implication of NLRP3 and the risk of developing T2D in patients with RA (Ruscitti et al., 2019) and to highlight the potential application of NLRP3-targeted therapies for these diseases. Driven by surrounding environmental conditions, glycolytic enzymes can translocate to the nucleus ("moonlighting"), where they regulate the expression of their target mRNAs and modulate immune responses (De Rosa et al., 2015; Boukouris et al., 2016). For instance, the glycolytic enzyme pyruvate kinase M2 (PKM2) plays a crucial role in the regulation of transcription factors and cytokine production in both coronary artery disease (CAD) and RA macrophages (Shirai et al., 2016; Weyand et al., 2017). Specifically, increased ROS production during inflammation, promotes PKM2 dimerization enabling its nuclear translocation and transcription factor STAT3 phosphorylation, thereby enhancing IL-6 and IL-1β production (Shirai et al., 2016; Weyand et al., 2017). Reducing glycolysis, limiting superoxide production and promoting PKM2 tetramerization, repaired the pro-inflammatory phenotype of CAD macrophages (Shirai et al., 2016). Similarly we found that lactate induces the nuclear translocation of PKM2 in activated $CD4^+$ T cells, boosting IL-17 production in a STAT3 dependent manner (Pucino et al., 2019).

Emerging evidence suggests that hypoxia and HIFs play a pivotal role in the regulation of several pathophysiological features of RA including synovitis, angiogenesis, and cartilage destruction (Hua and Dias, 2016). In particular, HIF-1α, a master regulator of glycolysis, is highly expressed by macrophages in the RA synovium, compared to macrophages in OA and healthy control synovium (Hollander et al., 2001) suggesting HIF-1α as novel potential therapeutic target. It will be interesting to determine whether these observations reflect up or down regulation of HIF-1α in the same macrophage subset in RA and OA or alternatively is a reflection of different subsets of macrophages in RA and OA (Croft et al., 2019).

Vascular endothelial growth factor-dependent HIF-1α pathways play a key role in endothelial cell (EC) metabolism. Indeed, in response to growth factor stimulation, such as by vascular endothelial growth factor (VEGF), EC become highly activated, proliferative, and acquire migratory capability (Potente et al., 2011; Varricchi et al., 2018) with increased glycolysis (Yeh et al., 2008; Parra-Bonilla et al., 2010; De Bock et al., 2013). Blockade of the glycolytic enzyme, PFKFB3, inhibited angiogenic tube formation *in vivo* and reduced the secretion of pro-inflammatory/angiogenic mediators in RA FLS and EC suggesting a key role of this glycolytic enzyme in promoting angiogenesis and inflammation (Biniecka et al., 2016). G6PI was also found to be important in the regulation of VEGF secretion from RA FLS (Lu et al., 2017). Indeed, in hypoxic conditions, both G6PI and HIF-1α were increased. This phenomenon was accompanied by enhanced proliferation of RA FLS and angiogenic tube formation of human dermal microvascular endothelial cells (HDMECs) *in vivo*. These events were reversed in G6PI loss-of-function experiments, thus confirming the requirement for G6PI in promoting angiogenesis in RA (Lu et al., 2017).

MITOCHONDRIAL METABOLISM

Mitochondrial functions in RA are still under investigation. Mitochondrial DNA (mtDNA) mutations and ROS production were found to be higher in RA compared to OA FLS (Da Sylva et al., 2005). In addition, they correlated with elevated MMP expression and a more invasive phenotype of FLS (Harty et al., 2012). Another study showed that mitochondria in macrophages isolated from the RA synovium, produced more ATP, consumed more oxygen and developed inter-organelle connections with the endoplasmic reticulum, forming mitochondria-associated membranes (MAM). MAMs promote mitochondrial hyperactivity and calcium transport, and induce the inactivation of glycogen synthase kinase 3b (GSK3b). In turn, the inactivation of GSK3b increases the production of the collagenase cathepsin K, a macrophage effector molecule, whose levels correlates with RA clinical disease activity (Zeisbrich et al., 2018). Lipopolysaccharide (LPS) stimulated macrophages (M1 macrophages) display a decreased TCA cycle. Moreover the mitochondrial oxidative phosphorylation pathway is coupled to the up-regulation of glucose transporter 1 (Glut1) to facilitate efficient uptake of glucose (Corcoran and O'Neill, 2016). ROS production is increased, partly as a consequence of reversed electron transport in mitochondria, and the accumulation of TCA cycle intermediates such as succinate, as previously described. These events promote the expression of the pro-inflammatory cytokine IL-1β by inhibiting prolyl hydroxylases and activating the transcription factor HIF-1α. Succinate has also been linked to changes in DNA methylation and associated histone proteins which in turn modulate gene expression (Mills and O'Neill, 2014). In animal models of arthritis, succinate has been shown to induce synovial angiogenesis through VEGF-dependent HIF-1α pathways (Li et al., 2018).

In RA, ROS are thought to directly contribute to destructive and proliferative synovitis (Datta et al., 2014). High levels of ROS accumulate in the synovial fluid and peripheral blood of RA patients where they can modify (e.g., via oxidation) major components of cartilage and bone, such as collagen and hyaluronic acid, inducing bone and cartilage destruction (Ishibashi, 2013; Chimenti et al., 2015). Moreover ROS levels positively correlate with disease activity (Datta et al., 2014) and contribute to osteoclast differentiation via RANK signaling (Lee et al., 2005).

LIPID METABOLISM

Recent discoveries have highlighted the role of lipid metabolism in the regulation of immune cells functions (Cucchi et al., 2019) and targeting lipid mediators is becoming an attractive field in autoimmune and allergic disorders (Marone et al., 2019).

It has been recently reported that the short chain fatty acids (FAs) such as acetate, propionate and butyrate are able

to orchestrate several $CD4^+$ T cell functions by modulating the activity of histone deacetylases (HDAC) (Park et al., 2015) and via the peroxisome proliferator-activated receptor (PPAR) signaling (Berger and Moller, 2002; Klotz et al., 2009; Cipolletta et al., 2012). Lipid metabolism is also crucial for T cell activation and proliferation. Indeed, T cell activation is accompanied by the upregulation of sterol regulatory element binding protein (SREBP). Lack of SREBP by genetic inactivation is detrimental to T cells undergoing clonal expansion after activation (Kidani et al., 2013).

T cells from patients with RA display increased fatty acid synthesis (FAS) leading to their increased tissue invasiveness. More specifically, reduced glycolytic flux due to PFKFB3 deficiency, promotes a shunt toward anabolic glucose utilization (increased PPP and FAS) and the up-regulation of the podosome scaffold adapter protein TKS5 (SH3PXD2A), which is involved in the formation of cell membrane protrusions (Yang et al., 2013; Shen et al., 2017). In addition, enhanced FAS causes the accumulation of cytoplasmic lipid droplets, which are necessary for T cell functions including cell growth, proliferation and for naïve to memory T cell conversion. Interestingly, restoring pyruvate level was able to replenish T cell locomotion and limit tissue-invasiveness and inflammation in non-obese diabetic (NOD) SCID mice (NSG mice) engrafted with human synovial tissue. In addition, inhibition of FAS efficiently reduced tissue inflammation, decreased the number of $RANKL^+$ and $IFN\text{-}\gamma^+$ T cells and diminished the total number of T cell infiltrating the synovial tissue (Shen et al., 2017). *De novo* FAS regulate Th17 differentiation (Berod et al., 2014). Indeed the inhibition of acetyl-CoA carboxylase (ACC) *in vitro*, using the specific inhibitor Sorafen A, leads to an impaired differentiation of Th17, favoring instead the differentiation of $Foxp3^+$ Treg cells (Berod et al., 2014). Consistent with these results, we have recently shown that in the presence of lactate, at concentrations comparable to those measured in the synovial tissue, $CD4^+$ T cells upregulate the *de novo* FAS, leading to increased IL-17 and reduced cell motility (Pucino et al., 2019). Interestingly, these events were restored after treating $CD4^+$ T cells with a range of FAS inhibitors and reducing the lactate-induced NADPH levels (Pucino et al., 2019).

While *de novo* FAS has been shown to play an important role in effector $CD4^+$ T cell functions, cholesterol metabolism is involved in the regulation of the anti-inflammatory response in human $CD4^+$ T cells (Perucha et al., 2019). Inhibition of the cholesterol biosynthesis pathway with atorvastatin or 25-hydroxycholesterol during switching from $IFN\gamma^+$ to $IL\text{-}10^+$ showed a specific block in immune resolution, defined as a significant decrease in c-Maf/IL-10 expression (Perucha et al., 2019).

Metabolomics profiling has shown alterations in the lipid metabolism in RA versus OA FLS. In line with this evidence, choline and choline like transporter (CTL)1 (high-affinity) and CTL2 (low affinity), were found to be highly expressed by synovial RA FLS (Ahn et al., 2016; Volchenkov et al., 2017) and their functional inhibition promoted FLS cell death (Seki et al., 2017). Supporting these findings, positron emission tomography (PET) scanning with ^{11}C-choline showed increased uptake in inflamed arthritic joints (Seki et al., 2017). Further studies are needed to understand the mechanisms linking lipid metabolism to FLS effector functions and subset differentiation in RA.

TANSCRIPTION FACTORS AS METABOLIC SENSORS

Catabolic and anabolic pathways are regulated by specific transcription factors which act as metabolic sensors. In this context, 5′ AMPK is a redox sensor, being activated by increased AMP:ATP ratios (Shirwany and Zou, 2014). AMPK modulates several metabolic functions, including glucose uptake, mitochondrial biogenesis and lipid metabolism, as well as cellular functions (i.e., transcriptional activity and cell cycle). Therapeutic AMPK activation was reported to suppress experimental arthritis. Moreover, methotrexate-induced activation of AMPK-dependent pathway has been shown to protect the vasculature against inflammation (Kang et al., 2013; Yan et al., 2015; Thornton et al., 2016). AMPK activation is myristoylation dependent. Notably, RA T cells display a defect in N-myristoyltransferase (NMT) function, which prevents AMPK activation and enables mTORC1 signaling activation, resulting in pro-inflammatory Th1 and Th17 differentiation. NMT1 loss of function experiments induced an inflammatory response both *in vitro* and *in vivo*; by contrast, NMT1 overexpression restored AMPK activation and suppressed synovial inflammation (Wen et al., 2019).

Finally, metformin, an anti-diabetic drug, which indirectly activates AMPK, has been shown to mitigate disease in mouse models of arthritis (Son et al., 2014) *via* the inhibition of mTOR pathway, the suppression of NF-κB-mediated inflammatory cytokine production as well as enhanced autophagic flux (Yan et al., 2015).

Together with AMPK, mTOR is a central integrator of environmental signals and nutrient availability with cellular functions (Delgoffe and Powell, 2015; Pollizzi and Powell, 2015; Pucino et al., 2016). Indeed, aberrant mTOR activation is associated with cellular senescence, and rapamacyin, the mTOR complex 1 inhibitor, has been investigated as a therapeutic agent to treat degenerative, autoimmune and hyperproliferative diseases (Perl, 2016). The ability of mTOR to integrate nutrient supply, bioenergetics and T cell functions, makes it a promising target for therapeutic intervention to suppress abnormal T cell differentiation during the early stages of RA (Perl, 2016).

ADIPO-CYTOKINES

A link between the neuroendocrine and immune systems has been shown to contribute to the pathogenesis of several immune mediated inflammatory disorders (Cassano et al., 2014; Procaccini et al., 2014). In this context adipo-cytokines, such as leptin and adiponectin, hormones secreted mainly by the adipose tissue, have been shown to play a role in RA pathogenesis (Hamaguchi et al., 2012; Ruscitti et al., 2018). For instance, it has been shown that *ob/ob* mice develop resistance to experimental antigen-induced arthritis compared to wild-type mice (Busso et al., 2002). In addition a decrease in serum leptin concentration

following fasting, limited $CD4^+$ activation, promoted a shift toward Th2-type cytokine secretion, and improved clinical disease in RA patients (Fraser et al., 1999). Leptin can also directly modulate chondrocyte biology. Indeed, leptin induces, in combination with IFN-γ and IL-1, nitric oxide synthases (NOS) type II activation in cultured chondrocytes (Otero et al., 2003). These events promote pro-inflammatory cytokine production in joint cartilage, causing chondrocyte apoptosis, metalloproteases activation and consequently inflammation (Otero et al., 2005). However there is conflicting evidence regarding the role of leptin in RA (Tian et al., 2014). Some studies have found elevated leptin in serum from RA patients (Bokarewa et al., 2003; Xibille-Friedmann et al., 2010; Yoshino et al., 2011) in particular in patients with erosive RA (Targonska-Stepniak et al., 2010; Olama et al., 2012). Conversely other reports have showed no difference in serum leptin levels between RA patients and healthy controls (Harle et al., 2006; Hizmetli et al., 2007; Oner et al., 2015). Leptin has also been detected in RA synovial fluid and tissue. A study by Seven et al. (2009) reported that serum and synovial fluid leptin levels were higher in RA patients when compared to controls, with positive correlation with disease activity. Another study showed instead a negative correlation between leptin synovial fluid levels and bone erosions. In addition, leptin levels were higher in the serum than in the synovial fluid suggesting that leptin may be consumed in the joints and have a protective role against erosions (Bokarewa et al., 2003).

Similar to leptin, adiponectin has also been suggested to play a role in the pathogenesis of RA, though again results are inconsistent. Adiponectin is a 28–30 kDa collagen-like protein predominantly secreted by adipocytes. In some studies, increased levels of adiponectin were found in synovial fluid and serum of patients with RA (Schaffler et al., 2003; Otero et al., 2006) and were associated with the production of pro-inflammatory mediators and arthritis (Ehling et al., 2006). In other studies, serum adiponectin showed no association or a negative correlation with disease activity in RA (Senolt et al., 2006; Rho et al., 2009; Yoshino et al., 2011). In the DBA/1 mouse model of collagen-induced arthritis, adiponectin treatment significantly alleviated the severity of arthritis together with a decrease in the expression of pro-inflammatory cytokines such as TNF-α and IL-1, and the reduction of metalloproteinase (MMP)-3 in synovial tissues (Lee et al., 2008). These latter findings suggest that in RA the role of adiponectin is anti-inflammatory rather than pro-inflammatory.

TREATMENTS AFFECTING METABOLIC SIGNALING PATHWAYS IN RA

Several drugs currently in use to treat RA affect metabolic signaling pathways. Glucocorticoids for example, inhibit the glycolytic enzyme fructose 2,6-bisphosphate in rat tymocytes and regulate respiratory rate in peripheral blood mononuclear cells from patients with rheumatic diseases (Moreno-Aurioles and Sobrino, 1991; Kuhnke et al., 2003). Methotrexate's anti-inflammatory effects depend on the modulation of purine or pyrimidine nucleotide metabolism (Cronstein and Aune, 2020). Similarly, biologic disease modifying anti rheumatic drugs (DMARDs) can modulate specific metabolic pathways. For example, anti-TNF-α and JAK inhibitor (i.e. tofacitinib) treatments decrease glycolysis in RA synovium (Biniecka et al., 2016; McGarry et al., 2018).

In the context of tofacitinib, it significantly increased oxidative phosphorylation, mitochondrial respiration in RA FLS, coupled with a decrease in glycolysis and several key glycolytic enzymes such as HK2, glycogen synthase kinase 3α (GSK-3α), lactate dehydrogenase A, and HIF-1α both in RA FLS and synovial explants (McGarry et al., 2018). It would be interesting to evaluate if these events are associated with reduced lactate levels and impaired lactate/STAT3 signaling as we have recently shown (Pucino et al., 2019).

The anti IL-6 receptor antibody tocilizumab decreases oxidative stress in RA leucocytes (Ruiz-Limon et al., 2017). Over-expression of HK2 has been associated with resistance to rituximab (anti-CD20) in aggressive lymphoma, whilst the impact of rituximab on immune cell metabolism in RA patients is still unknown (Gu et al., 2018).

CONCLUSION AND FURTHER PERSPECTIVES

The tissue microenvironment plays a pivotal role in the pathology of inflammatory diseases such as RA. A lack of nutrients, low oxygen concentrations, accumulation of metabolic intermediates as well as unbalanced metabolic pathways drive the local immune response in such a way as to exacerbate chronic inflammation (**Figure 1**).

TABLE 1 | Potential metabolic therapeutic targets in RA.

Cells	Defective metabolic Pathway	Potential therapeutic targets
T cell	Glycolysis (–) PPP (+) Lipid (+)	PFKFB3 G6PD FASN Lactate/SLC5A12 AMPK/mTOR
Monocytes/macrophages	Glycolysis (+) TCA (+) AMPK (–)	PKM2 HIF Succinate/GPR91 Lactate/MCT1 and 4
Fibroblasts	Glycolysis (+) Lipid (+)	GLUT1 HK2 PFKFB3 Choline/Chokα
Dendritic cells	Glycolysis (+)	HK2 iNOS AKT/mTOR

AMPK, adenosine monophosphate-activated protein kinase; Chokα, choline kinase alpha; FAS, fatty acid synthase; G6PD, glucose-6-phosphate dehydrogenase; G6PI, glucose 6 phosphate isomerase; HK2, hexokinase 2; iNOS, inducible nitric oxide synthase; HIF-1α, hypoxia-inducible factor 1-alpha; MCT, monocarboxylate transporter; mTOR, mammalian target of rapamycin; PFKFB3, 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase; PKM2, pyruvate kinase muscle isozyme 2; PPP, pentose phosphate pathway; SLC5A12, solute carrier 5A12; (–) decreased, (+) enhanced.

Immunometabolism studies have recently highlighted the possibilty of targeting metabolic pathways, metabolites, transcription factors and enzymes that are altered in RA (**Figure 1** and **Table 1**). Several drugs currently in use to treat RA affect metabolic signaling pathways. However, we are now in a position from which we can consider developing therapies to specifically target pathogenetically relevant metabolic pathways. For example, targeting specific metabolic pathways has been demonstrated to reduce inflammation both *in vitro* and *in vivo* models of arthritis (Yan et al., 2015; Biniecka et al., 2016; Okano et al., 2017; Shen et al., 2017; Bustamante et al., 2018). In addition, targeting metabolic intermediates such as lactate (Pucino et al., 2018; Certo et al., 2019) or succinate (Littlewood-Evans et al., 2016), is also becoming an attractive possibility. Animal models remain a crucial tool for preclinical screening of new therapeutics in pharmaceutical development. However, potential therapeutics, which have been shown to be safe and effective in animal studies, have in certain cases failed when tested in humans. Further knowledge on human immunology and additional development of animal models that bear more resemblance to the human condition are needed (Hegen et al., 2008; Bevaart et al., 2010). Another important area of investigation is the impact of sex and gender on RA immunometabolism. Prevalence of RA is higher in women than in men (van Vollenhoven, 2009). This is partly ascribed to the effect of sex hormones on the immune system and their interaction with environmental and genetic factors (Alpizar-Rodriguez et al., 2017). Estrogenic control of mitochondrial function and glycolysis metabolism has been studied (Cai et al., 2013; Klinge, 2020), however what are the sex-based differences in RA cell immunometabolism is still unknown and needs further investigation.

Correlation studies between serum metabolites and synovial and blood biomarkers suggests that NMR and mass-spectrometry may be promising tools for predicting specific pathogenic pathways altered in RA (Young et al., 2013; Zhou et al., 2016; Narasimhan et al., 2018). In addition they may be useful in the future to to identify which RA patients are at higher risk to develop artheroslerosis. Metabolomics profiles in serum, plasma, or urine do not necessarily correlate with joint metabolism as well as synovial fluid metabolites may not identify metabolic pathway alterations in the synovial tissues.

Further studies are needed to better determine whether specific metabolic signatures can be used to stratify patients with RA in terms of outcome, disease stage and response to therapy. Single cell RNA-seq techniques will be of help to shed light on metabolic pathways used by specific immune cells (i.e., macrophages, lymphocytes, fibroblast) in the context of the RA inflammatory environment.

Advanced RNA-seq techniques are also developing. In this context, the droplet-based single-cell RNA-seq has recently been shown to be a promising tool for cellular profiling allowing the analysis of thousands of individual cells simultaneously by encapsulating them in tiny droplets (Salomon et al., 2019). Similarly single cell metabolomic analysis will facilitate the identification of new biomarkers and the development of novel therapeutic molecules targeting abnormal metabolic signaling pathways at single cell level without dampening homeostatic immune responses.

AUTHOR CONTRIBUTIONS

VP, CM, KR, and CB contributed to the conceptualization. VP, MC, CM, KR, and CB contributed to the preparation of the original draft. VP, MC, GV, GM, FU, FR, AD, CM, KR, and CB contributed to the final editing and revision.

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REVIEW
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Pathophysiological Role of Synovitis in Hemophilic Arthropathy Development: A Two-Hit Hypothesis

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Despite an increasing access to prophylaxis with clotting factor concentrates, arthropathy still represents the main chronic complication of hemophilia. Whereas previous studies described hemophilic arthropathy (HA) as a degenerative arthropathy, somehow resembling osteoarthritis (OA), most recent evidence suggests that complex inflammatory and immunologic mechanisms are also involved in the pathophysiology of HA. In the present review, we described available data on major mechanisms leading to arthropathic changes in patients with hemophilia, with a specific focus on the role of synovium. The presence of hemosiderin in the joint space induces synovium proliferation, thus leading to formation of several lytic enzymes determining chondrocytes apoptosis and proteoglycans levels reduction. This leads to a direct joint "chemical" damage representing early damages in the pathogenesis of HA (first hit). In parallel, synovial membrane and synovial endothelial cells become a dynamic reservoir of inflammatory cells and mediators, and propagate the inflammatory response (second hit), switching the process from a chemical damage to an inflammatory damage. Overall, consistent data pointed out synovitis as the keystone in HA pathophysiology. This opens novel potential therapeutic targets in this clinical setting.

Keywords: hemophilic arthropathy, cytokines, inflammation, synovitis, pathophisiology

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INTRODUCTION

Hemophilia is a genetic X-linked coagulative disorder caused by the deficiency of coagulation factor VIII (hemophilia A) or coagulation factor IX (hemophilia B). Incidence is 1/5000 for hemophilia A and 1/30000 for hemophilia B (Acharya, 2012). Affected individuals report an increased bleeding risk, with joints being the anatomical site most often involved (Di Minno et al., 2016). All joints can be potentially involved, but hemarthrosis usually occurs in large synovial joints (knee, ankles, and elbows), thus progressively leading to a severe and disabling arthropathy (Arnold and Hilgartner, 1977).

Although a more severe bleeding phenotype has been recognized in patients with severe hemophilia A (<1% FVIII activity), some data showed that we can observe a significant incidence of HA also in patients with moderate hemophilia (2–5% FVIII activity) (Di Minno et al., 2013).

While an effective prophylactic factor replacement therapy considerably reduced joint bleeding episodes, some signs of hemophilic arthropathy (HA) are still reported by 25–30%

of patients, even in highly developed countries (Arnold and Hilgartner, 1977; Manco-Johnson et al., 2007; Wojdasiewicz et al., 2018). Thus, arthropathy still represents the main chronic complication of hemophilia.

Several previous studies described HA as a degenerative arthropathy, somehow resembling osteoarthritis (OA) (Pulles et al., 2017). In contrast, most recent evidence suggests that complex inflammatory and immunologic mechanisms are also involved in the pathophysiology of HA. The aim of the present review is to describe available data on major mechanisms leading to arthropathic changes in patients with hemophilia, focusing on the role of synovial tissue.

SYNOVIAL TISSUE

In physiologic conditions, the synovial tissue is involved in the production of synovial fluid that fills articular cavity and lubricates bony structures to ensure a correct articular excursion. On the other hand, synovial tissue has a pivotal role in pathogenesis of HA (Arnold and Hilgartner, 1977).

Indeed, the synovial membrane, a specialized connective tissue, consists of two layers, the intima and the sub-intima, with a small amount of hyaluronic acid between layers. The intima is relatively acellular and consists of two types of synoviocytes: type A (monocyte-macrophage cell-like) and type B (fibroblast-like). The sub-intima is composed of lymphatic vessels and is highly vascularized (Smith, 2011). Although the presence of numerous capillaries in the synovial tissue is of great importance for physiologic functions, unfortunately they are also the source of joint bleeds (Jansen et al., 2008).

IRON CHEMICAL DAMAGE IN SYNOVITIS (FIGURE 1)

When a hemarthrosis occurs, blood-derived iron (hemosiderin) deposition determines a chemical damage to the synovial tissue leading to activation of inflammatory and anti-apoptotic patterns. In a study conducted on murine models of hemarthrosis, an iron-induced chemical damage was demonstrated, also emphasizing the pathogenic role of iron-derived metabolites [Ferroportin (an iron cell exporter); Hepcidin (regulator of FPN); Hemoglobin scavenger receptor (CD163); Heme carrier protein 1 (heme cell importer); Feline leukemia virus subgroup C (heme cell exporter)] (Nieuwenhuizen et al., 2013). These data have been confirmed in a study comparing synovial histological sections of patients affected by rheumatoid arthritis (RA), OA, and HA. Nuclear and cytoplasm expression of iron-derived metabolites was much more abundant in synovial tissue of hemophilic patients as compared to OA and RA, thus suggesting a crucial role in pathophysiology of HA (Nieuwenhuizen et al., 2013). In particular, hemosiderin deposition within synoviocytes and the presence of iron metabolites are associated with the production of reactive oxygen species (ROS) via the Haber–Weiss/Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^-$) (Manco-Johnson et al., 2007; Valentino, 2010; Blobel et al., 2015). In turn, the thin synovial membrane becomes a hypertrophic and villous membrane, via induction of DNA-synthesis and cell proliferation. In fact, hemosiderin inhibits synovial cell apoptosis by stimulating the amplification of myelocytomatosis viral oncogene (c-MYC) (a proto-oncogene associated with cell proliferation) and of mouse double minute 2 (MDM2) homolog (a protein that targets the tumor suppressor gene p53) (Hakobyan et al., 2004; Pulles et al., 2017).

These findings are consistently confirmed by results of the study by Wen et al. (2002) showing that iron is involved in the modulation of the expression of c-MYC and MDM2 homolog, leading to proliferation of the synovium.

Hypertrophic synovium produces several lytic enzymes that, by means of a "chemical damage," induce chondrocytes apoptosis and proteoglycans level reduction. According with these pathophysiological mechanisms, a recent study conducted on hemophilia murine models in which hemarthroses were artificially induced showed that HA-related signs of degenerative manifestations quickly appear after exposition to blood products. In fact, histological section evaluation highlighted that synovitis was developed within 24 h, whereas cartilage and bone damage became manifest within 48–96 h. This could suggest a direct influence of blood on these processes besides indirect effect of inflammation (Christensen et al., 2019).

Overall, early damages secondary to iron-mediated chemical injury could represent the first step in the pathogenesis of HA (*FIRST HIT*) (Roosendaal et al., 1999).

In parallel, iron plays a crucial role in the induction of the expression of several pro-inflammatory cytokines, including interleukin 1 beta (IL-1β), tumor-necrosis factor alpha (TNFα), and interleukin 6 (IL-6) (Melchiorre et al., 2017).

In detail, type A synoviocytes, after incorporating iron, produce inflammatory cytokines (IL-1β, IL-6, TNFα), in turn inducing migration of polymorphonuclear cells and, later, of monocytes and lymphocytes. This leads to a self-maintaining cycle further increasing inflammatory response and inducing an enhanced angiogenesis (Lafeber et al., 2008; Agapidou et al., 2016). Indeed, the inflamed and hypertrophic synovium has an enhanced oxygen demand, stimulating both locally and systemically the release of growth factors like vascular-derived endothelial growth factor (VEGF), thus promoting neo-angiogenesis (Pulles et al., 2017).

These phenomena involving synovial tissue can induce a chronic inflammatory process mediated by cytokines and pro-angiogenic molecules, switching the process from a chemical damage to an inflammatory damage characterized by progressive synovial pannus growth and articular cartilage damage worsening (Valentino, 2010; Pulles et al., 2017).

Thus, synovial membrane and synovial endothelial cells become an active reservoir of inflammatory cells and mediators, and propagate the inflammatory response (*SECOND HIT*).

Currently, IL-1β and TNFα are the most widely studied inflammatory cytokines involved in the pathogenesis of HA (Pulles et al., 2017; Wojdasiewicz et al., 2018).

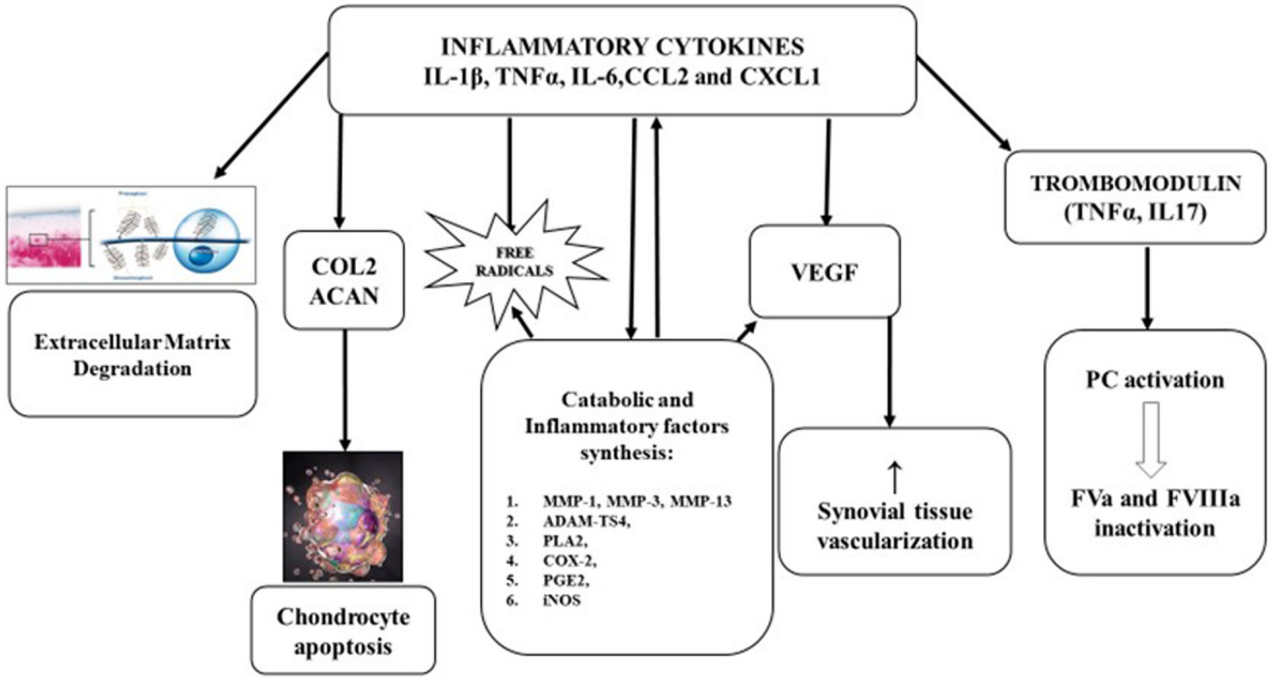

FIGURE 1 | Pathophysiology of hemophilic arthropathy. Type A synoviocytes, after incorporating iron, produce and relapse inflammatory cytokines (IL-1β, IL-6, TNFα) and chemokines (CCL2, CXCL1), leading to migration of polymorphonuclear cells and later, of monocytes and lymphocytes. The consequent inflammatory response promotes:

- Extracellular matrix degradation.
- Inhibition of proteoglycan and collagen type II (COL2) synthesis by chondrocytes and induce apoptosis.
- Expression of metalloprotease (MMP-I, MMP-3, MMP-13, andADAMTS4) that have a pivotal role in catabolic joint processes.
- Expression of cyclooxygenase 2 (COX-2) and prostaglandin E2 (PGE2) involved in development and maintenance of inflammatory process.
- Neo-angiogenesis, stimulating, both locally and systemically, the release of growth factors like vascular-derived endothelial growth factor (VEGF).
- Liberation of trombomodulin (TM) by inflammatory cells, TM binds, then activates protein C (PC) inducing factor V (FVa) and FVIIIa degradation.

ROLE OF INFLAMMATORY CYTOKINES (FIGURE 1)

IL-1β

IL-1β is one of the main regulators of inflammatory response. IL-1β induces catabolic processes in synovial joint both directly, acting on cell, and amplifying inflammatory processes through activation of transduction signal pathways.

The "inflammasome" is a crucial factor regulating the maturation and secretion of pro-inflammatory IL-1 (Dutra et al., 2014; Srivastava, 2015). After interacting with its receptor, IL-1 leads to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcriptive factor and other transcriptive factors such as c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinases (p38MAPK). As a result, there is an increased expression of various genes responsible for the synthesis of enzymes, adhesion molecules, or inflammatory mediators including cytokines and chemokines (Wojdasiewicz et al., 2018). This is in line with observations of the role of NFkB in synovitis development and cartilage degeneration in OA and RA (Melchiorre et al., 2012; Pulles et al., 2017).

Consistently confirming the involvement of IL-1β in the pathophysiology of HA, some authors (Tagariello and di Giovine, 1996; Valentino, 2010; Dutra et al., 2014; Srivastava, 2015) documented markedly elevated IL-1β levels in histological section of the synovial membranes collected during synovectomy or joint replacement from HA patients as compared to patients without hemophilia (Roosendaal et al., 1999).

On the other hand, IL-1β can also increase transferrin-bound iron uptake into type B synoviocytes which leads to deposition of hemosiderin and IL-1β autocrine secretion and, consequently, to development of chronic synovitis (Telfer and Brock, 2004).

TNFα

TNFα is a member of tumor necrosis factor superfamily and plays a crucial role in HA pathophysiology. TNFα also induces catabolic processes in synovial joint and directly regulates intra-articular levels of FVIIIa modulating expression of thrombomodulin (TM) (Aggarwal et al., 2012; Wojdasiewicz et al., 2018).

In particular, TNFα inhibits proteoglycan and collagen type II (COL2) synthesis by chondrocytes. It can induce the expression of metalloprotease (MMP-1, MMP-3, MMP-13, and ADAMTS4) that have a pivotal role in catabolic joint processes (Wojdasiewicz et al., 2018).

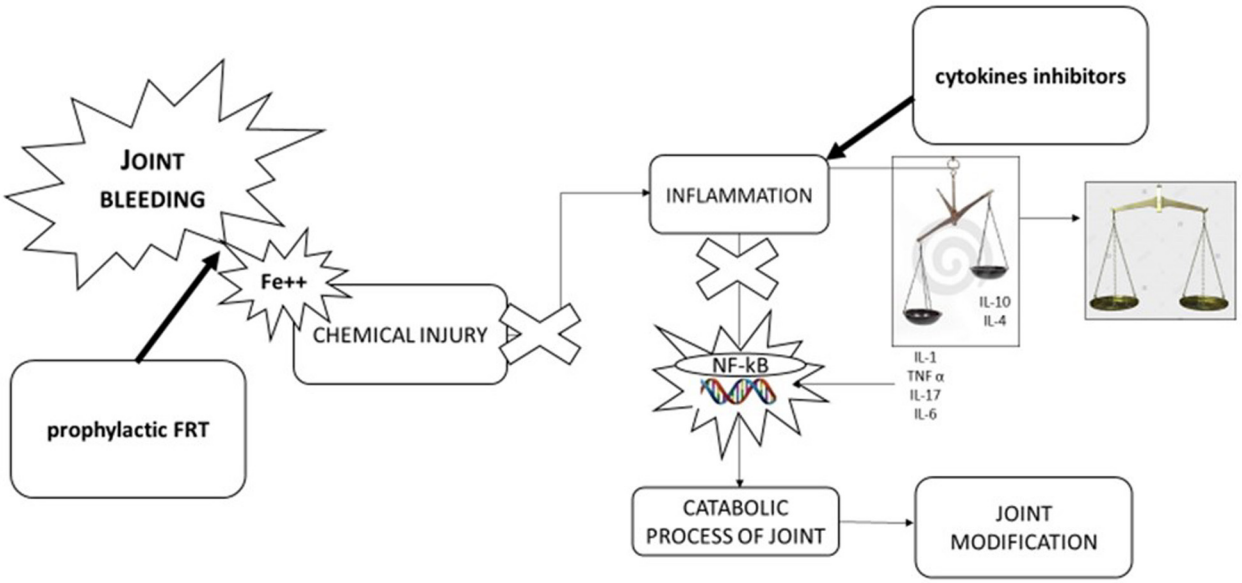

FIGURE 2 | Pathophysiology of hemophilic arthropathy and potential therapeutic approach. Joint bleeding and consequent chemical injury due to iron exposition is the "*primwn movens*" in development of hemophilic arthropathy leading to inflammation and alteration of natural balance between inflammatory cytokines (IL-1β, IL-6, TNFα, IL-17) and anti-inflammatory cytokines (IL-10, IL-4). Tins leads activation of nuclear transcription factor [nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)] inducing catabolic process of joint and in turn joint modification. Therapeutic approaches can be direct to stop key point of pathological process: first factor replacement to stop joint bleeding, using anti-inflammatory drugs [cyclooxygenase 2 (COX-2) inhibitors, monoclonal antibodies anti-TNFα, and anti-IL-1β] to balance cytokines pathway.

On the other hand, TNFα has a direct role in increasing the risk of bleeding recurrence. TNFα is associated with a substantial reduction of TM synthesis by synoviocytes, due to a huge liberation of TM into the synovial fluid induced by an intensive action of neutrophils and cytokines on synovial cells. Additionally, a recent study shows that synovial fluid TM levels were more elevated in patients with HA (56 ± 25 ng/mL) as compared to healthy controls (39 ± 21 ng/mL). In physiologic conditions, TM binds thrombin in a 1:1 stoichiometric ratio, then activates protein C (PC) (Dargaud et al., 2012). PC is a zymogen which belongs to a group of proteins which inhibits coagulation by inducing factor V (FVa) and FVIIIa degradation (Anastasiou et al., 2012; Wojdasiewicz et al., 2018). This interaction between inflammatory mediators and hemostasis components might explain why the hemorrhagic process can sometimes be sustained, despite the FVIII replacement therapies.

Thus, IL-1β and TNFα, triggering and amplifying inflammatory damage and its consequences on joint, represent the cornerstone in pathophysiology of HA. Moreover, TNFα has an important and documented role in the regulation of hemostatic balance of joint in patients with HA.

Moreover, recent evidence showed an increased synovial tissue expression of the TNFα/TNF receptor (TNF-R) system. The activation of this system could represent a crucial mediator of synovial proliferation and a potential novel target for therapy (Manetti et al., 2019).

Furthermore, a recent study showed that similarly to OA and RA, patients with HA exhibit increased levels of progranulin (PGRN), a molecule known for its protective role toward TNFα catabolic effects (Kotela et al., 2018). This evidence could open future hypotheses on its potential role as a serum-maker for monitoring disease activity.

CHALLENGES IN THE TREATMENT OF HA (FIGURE 2)

Clotting factor replacement therapy represents the stronghold in hemophilia treatment but new knowledge about the pathophysiology of HA leads to new issues concerning potential therapeutic targets. An alternative potential approach is represented by the reduction of intra-articular iron deposition by means of iron chelators (deferoxamine, deferasirox) to stop the process at very early stages.

A further option is represented by anti-inflammatory therapy using cyclooxygenase 2 (COX-2) inhibitors, monoclonal antibodies anti-TNFα and anti-IL-1β with the aim to avoid the self-maintaining inflammatory cycle. Evidence showed that COX-2 inhibitors (celecoxib and rofecoxib) are safe and effective in treating chronic synovitis and joint pain, and currently represent a potential choice to treat pain in hemophilia patients (Rattray et al., 2006; Tagliaferri et al., 2018; Santoro et al., 2020). In 2013, Melchiorre et al. reported data about a drastic reduction of joint bleeding in three patients treated with an anti-TNFα monoclonal antibody. These interesting findings are potentially due to the cross-talk between inflammation and hemostasis mediated by TM inhibition (Melchiorre et al., 2014). On the other

hand, in another study on human chondrocyte cells cultures exposed to human blood cells (as a model of joint bleeding), the addition of monoclonal anti-TNFα antibodies did not reduce chondrocytes apoptosis and did not improve proteoglycans synthesis. On the contrary, the addition of monoclonal antibodies against IL-1β reduced chondrocytes apoptosis and enhanced proteoglycans synthesis. These findings suggest that TNFα inhibition, although able to reduce joint bleeding, could not have a direct positive effect on joint deterioration, whereas promising effects on cartilage could be expected using anti IL-1β monoclonal antibodies (van Vulpen et al., 2015).

Furthermore, a recent study conducted on murine models showed that the inhibition of iRhom2/ADAM17/TNFα pathway by TNFα inhibition is able to prevent synovitis and bone degenerative damage development (Haxaire et al., 2018).

Overall, despite higher costs, monoclonal antibodies could provide further beneficial effects beyond the pure inflammatory effect and, therefore, could be considered as a valuable therapy instead of COX-2 inhibitors. However, further studies are needed to address this issue.

CHALLENGE IN HA MONITORING

To identify early arthropathic changes for prevention of joint degeneration due to progression of HA is advised a periodic follow up of the joint status (Di Minno et al., 2017). The gold standard for evaluation of HA to date is MRI (Di Minno et al., 2016). Although MRI can be reputed highly sensitive to detect signs of disease activity and effective to perform a full evaluation of the joint surfaces, this exam presents some important limitation of execution in daily clinical practice. In fact, it is not possible to evaluate more than a joint for each exam, the time of exam performing is at least 30 min, and it is not comfortable for the patient. Furthermore, execution of MRI might require sedation for children and it is a high-cost technique (Di Minno et al., 2017).

In view of these limitations, rising interest has been reported in ultrasound (US) as a useful tool to evaluate joint status and to observe disease progression in hemophilic patients (Di Minno et al., 2016). The first practice to assess joint disease in hemophilic patients with US was performed by Wilson et al. (1987) in 38 patients with acute hemarthroses.

Ultrasound exam is able to detect and quantify most important biomarkers of disease activity such as joint effusion and synovial hypertrophy. Furthermore, one can find degenerative damages such as osteo-chondral changes through application of scoring scales (Martinoli et al., 2016a; Di Minno et al., 2017). In recent years, six scoring systems based on US have been proposed to quantify joint abnormalities in patients with hemophilia. Interestingly, all the US scores emphasize the role of synovitis detection as a maker of disease activity (Hermans et al., 2015; Martinoli et al., 2016b). Based on these, some recent UK guidelines defined the concept of "at risk joint" as a joint with synovitis (Hanley et al., 2017). On this hand, US examination by easily identifying synovitis could help guide physicians in the decision-making process of the optimal treatment for hemophilia patients.

Furthermore, identification of serum markers of disease activity (i.e., plasma levels of IL-1, TNFα, PGRN) could be a useful clinical tool to evaluate the severity of the joint disease and to guide the decision-making process for the treatment of each patient. Future studies should be designed to address this issue.

CONCLUSION

At variance with previous evidence suggesting a purely degenerative nature of HA, several and consistent data clarified more complex underlying mechanisms, involving both degenerative alterations and inflammatory response, and pointing out synovitis as the keystone in HA pathophysiology. This opens novel potential therapeutic targets for HA and suggests a role of US for monitoring synovitis and guiding treatment tailoring in patients with hemophilia.

AUTHOR CONTRIBUTIONS

IC contributed to literature evaluation and manuscript drafting. GI contributed to literature evaluation and supporting in manuscript drafting. FD contributed to literature evaluation and supporting in manuscript drafting. MD coordinated and supervised manuscript drafting.

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REVIEW
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Potential Bidirectional Relationship Between Periodontitis and Alzheimer's Disease

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Alzheimer's disease (AD) is the most prevalent form of dementia in the elderly population, representing a global public health priority. Despite a large improvement in understanding the pathogenesis of AD, the etiology of this disorder remains still unclear, and no current treatment is able to prevent, slow, or stop its progression. Thus, there is a keen interest in the identification and modification of the risk factors and novel molecular mechanisms associated with the development and progression of AD. In this context, it is worth noting that several findings support the existence of a direct link between neuronal and non-neuronal inflammation/infection and AD progression. Importantly, recent studies are now supporting the existence of a direct relationship between periodontitis, a chronic inflammatory oral disease, and AD. The mechanisms underlying the association remain to be fully elucidated, however, it is generally accepted, although not confirmed, that oral pathogens can penetrate the bloodstream, inducing a low-grade systemic inflammation that negatively affects brain function. Indeed, a recent report demonstrated that oral pathogens and their toxic proteins infect the brain of AD patients. For instance, when AD progresses from the early to the more advanced stages, patients could no longer be able to adequately adhere to proper oral hygiene practices, thus leading to oral dysbiosis that, in turn, fuels infection, such as periodontitis. Therefore, in this review, we will provide an update on the emerging (preclinical and clinical) evidence that supports the relationship existing between periodontitis and AD. More in detail, we will discuss data attesting that periodontitis and AD share common risk factors and a similar hyper-inflammatory phenotype.

Keywords: Alzheimer's disease, periodontitis, dysbiosis, neurodegeneration, dementia

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder affecting millions of people worldwide, with a frequency that is rapidly rising as the life expectancy increases and the world population becomes older (Brookmeyer et al., 2002, 2007; Sosa-Ortiz et al., 2012; Alzheimer's Association., 2016). Importantly, AD is characterized by neuronal loss with a slow and progressive decline in memory, language, and other cognitive skills, leading to the final stage of the disease, which is ultimately fatal (Alzheimer's Association 2016).

Despite decades of intense investigation, how degenerative neurodisorders, such as AD, develop remains unclear. Aggregates (plaques) of the amyloid-β peptide (AβP), as well as neurofibrillary tangles of the hyperphosphorylated protein, tau, are among the most sought-after therapeutic targets for AD (Braak and Braak, 1995; Long and Holtzman, 2019). However, many clinical trials investigating the effects of anti-amyloid drugs failed to demonstrate improvement in patients' cognitive performance and in countering the primary adverse events (Pinheiro and Faustino, 2019). Hence, there is an increasing interest in identifying new strategies to prevent and/or treat AD. For instance, several modifiable risk factors have been considered so far, such as physical inactivity, mood disorders, hypertension, diabetes mellitus, and obesity (Mayer et al., 2018). Moreover, many reports are now supporting the role of inflammation as a significant pathological driver of AD development and cognitive decline, with evidence that communication between the brain and peripheral immune systems also exists (Goldeck et al., 2016; Cao and Zheng, 2018; Alexandraki et al., 2019; Long and Holtzman, 2019; Tejera et al., 2019). In this sense, multiple studies have raised that an infectious hypothesis might underlie the pathogenesis of AD (Long and Holtzman, 2019). For instance, several studies have demonstrated the presence of herpesvirus (HSV) within the amyloid plaques and in the brains of AD patients (Jamieson et al., 1991; Jamieson et al., 1992; Wozniak et al., 2009; Carbone et al., 2014). In line with these data, HSV-1 particles can directly induce the fibrillization of Aβ42 *in vitro* (Ezzat et al., 2019). Moreover, two retrospective cohort studies demonstrated that HSV infection significantly increased the risk of developing all-cause dementia. Of note is that this risk was almost eliminated in patients treated with antiherpetics (Chen et al., 2018; Tzeng et al., 2018; Long and Holtzman, 2019).

Further to these viral effects on AD development, the research in the field has focused its attention on periodontitis, a chronic oral inflammatory condition, and its potential bidirectional link with AD (Kim and Amar, 2006; Kamer et al., 2008; Chen et al., 2017; Marchini et al., 2019; Long and Holtzman, 2019). Importantly, people with periodontitis have an increased risk of developing AD (Chen et al., 2017), and those with AD or dementia have impaired oral health, as a result of cognitive decline, and are more prone to develop chronic oral diseases, such as periodontitis, tooth loss, and mucosal lesions (Tada et al., 2006; Gonsalves et al., 2008; Noble J.M. et al., 2013; Maldonado et al., 2018). Mechanistically, periodontal pathogens not only invade the oral cavity but can also percolate through the epithelium of the periodontal pocket. From here, they can enter the bloodstream, where they can induce the release of several endotoxins and exotoxins, thus fueling infection in different compartments, including the brain (Nazir, 2017; Sudhakara et al., 2018; Bui et al., 2019; Dominy et al., 2019; Liccardo et al., 2019).

Thus, this review aimed to provide the readers with an update on the most recent findings that support the existence of a relationship between periodontitis and AD, with particular emphasis on the common risk factors, phenotype, and bidirectionality.

PATHOPHYSIOLOGY OF ALZHEIMER'S DISEASE: β-AMYLOID, TAU, AND APOE

AD is generally classified into two forms: the inherited and the sporadic one (Bekris et al., 2010; Dorszewska et al., 2016). Although there are differences in terms of the triggering factors and the proportion of the affected population, the underlying neuropathology of both conditions remains similar: with patients progressing from normal to mild cognitive impairment (MCI), followed by increasing dementia severity, eventually leading to the final stage of the disease that is ultimately fatal (Donev et al., 2009; Sperling et al., 2011; Scheltens et al., 2016; Davis et al., 2018). At a molecular level, both the sporadic and the inherited forms are characterized by the same diagnostic hallmarks such as AβP plaques and neurofibrillary tangles (Braak and Braak, 1995; Long and Holtzman, 2019).

AβP Plaques

In 1907, Alois Alzheimer, a German neurologist, reported the presence of a not well-identified substance in the cortex associated with a progressive behavioral and cognitive disorder (Alzheimer et al., 1995; O'Brien and Wong, 2011). Almost 80 years later, Glenner and Wong (1984) demonstrated that this substance was constituted by a ∼4 kDa peptide called AβP. AβP is a fragment derived from the proteolytic cleavage of the amyloid precursor protein (APP). APP is a transmembrane protein with a large ectodomain, a C-terminal (CT) membrane-bound domain and short intracellular domain (AICD) (Passer et al., 2000; Serpell, 2000; Gu et al., 2001; Weidemann et al., 2002; Kakuda et al., 2006; Walsh and Selkoe, 2007). Importantly, two main proteolytic pathways have been described for APP: the nonamyloidogenic and the amyloidogenic (Andrew et al., 2016; **Figure 1**). In the non-amyloidogenic pathway, α-secretase ADAM10 cleaves APP within the Aβ domain, generating a soluble proteolytic fragment, termed sAPPα, and a membrane-bound CT fragment (CTFα). Importantly, CTFα is subsequently processed by another proteolytic process that involved γ-secretases to generate p3 and the AICD. Conversely, in the amyloidogenic pathway, β-secretase 1, also known as β-site APP cleaving enzyme 1 (BACE1), and presenilin-containing γ-secretase (PS/γ-secretase) multi-subunit complex are involved in the generation of AβP (De Strooper et al., 1998; Struhl and Greenwald, 1999; Wolfe et al., 1999; Gu et al., 2001; De Strooper et al., 2012; Ben Halima et al., 2016; Andrew et al., 2016). More in detail, BACE1 cleaves APP, liberating a sAPPβ fragment and a

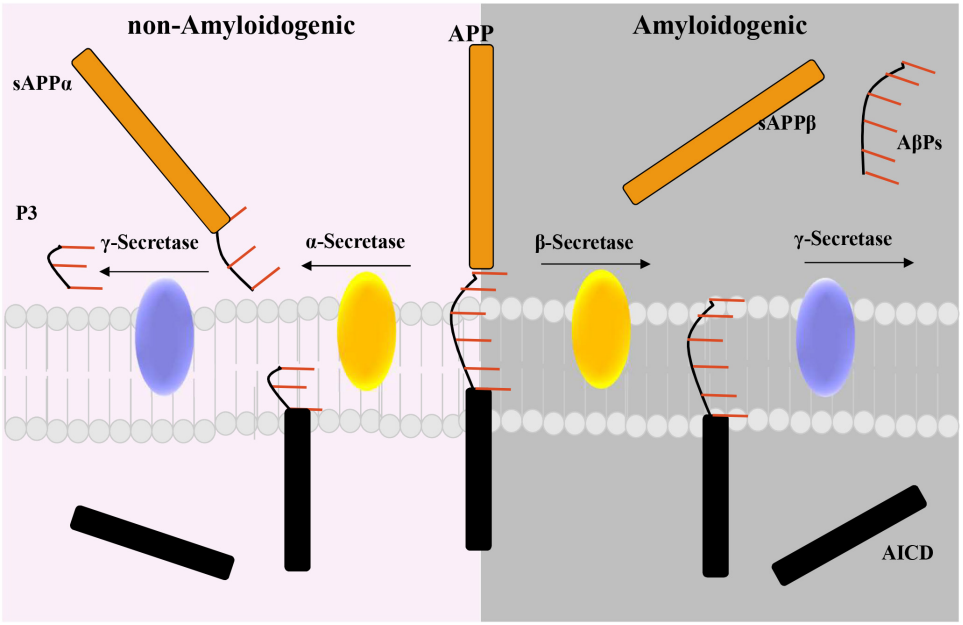

FIGURE 1 | Diagram of the non-amyloidogenic and the amyloidogenic proteolytic pathway for the amyloid precursor protein (APP). Non-amyloidogenic pathway: α-secretase cleaves the transmembrane protein APP to release the soluble APP fragment, sAPPα. The APP C-terminal fragment is then processed by γ-secretase to release an intracellular domain (AICD) and the P3 fragment. Amyloidogenic pathway: β-secretase processes APP to generate the soluble fragment, sAPP-β, then cleaved γ-secretase Aβ peptides (AβPs), and the AICD.

99-amino acid remaining CTF (CTFβ) (De Strooper et al., 2012; Andrew et al., 2016; Ben Halima et al., 2016). Then, the CTFβ is processed at the ε-site by PS/γ-secretase, thereby releasing the AICD (Andrew et al., 2016; Ben Halima et al., 2016). The AICD, either produced by α- or β-secretase, translocates into the nuclei of neurons. Here, it acts as a regulator of gene expression, including that of the Aβ-degrading neprilysin, or is degraded into the cytosol (Belyaev et al., 2010; Grimm et al., 2015; Multhaup et al., 2015). Importantly, different Aβ forms are generated by PS/γ-secretase cleavages at the ζ and γ sites that trim the transmembrane domain of CTFβ to liberate several forms of AβPs of variable lengths [from 38 (Aβ38) to 42 (Aβ42) amino acids] (De Strooper et al., 2012). In this regard, Aβ40 is the major product generated, along with minor amounts of Aβ38 and Aβ42 (De Strooper et al., 2012). However, besides these Aβ forms, it has been reported that, in this process, tiny amounts of Aβ37 and Aβ43 are also generated (De Strooper et al., 2012).

Importantly, although AβP's function is still debated and uncertain, these products are generated throughout life and appear to be normally stimulated by synaptic activity (Pearson and Peers, 2006). Conversely, dysregulation of the trimming process of APP can lead to a substantial increase in the levels of the insoluble Aβ42 isoform. This isoform is more prone to form oligomers, which correlate with synaptic dysfunction (Hayden and Teplow, 2013), protofibrils, or fibrils. Importantly, Aβ42 oligomers represent the most soluble and potent toxic conformers of AD (Haass and Selkoe, 2007), and their presence correlates with the severity of the disease (McLean et al., 1999). However, as recently suggested by Gulisano et al. (2018), Aβ42 oligomers are crucial both in physiological and pathological conditions. Indeed, only when present in excessive concentrations or for a prolonged time do these Aβ isoforms can negatively affect long-term potentiation (LTP) and memory. Conversely, low-dose administration positively affects synaptic plasticity and memory.

Thus, all the described processes, in a multitude, generate amyloid plaques resulting toxic to neurons and participating in synaptic destruction during the early stages of AD (Andrew et al., 2016). However, it is worth stressing that several studies are now supporting the idea that amyloid plaques are not the major toxic AβP entity, and amyloid plaques are not a direct indicator of AβP-induced brain damage in AD. For instance, the Arctic APP mutation (E693G) (Nilsberth et al., 2001) leads to enhanced Aβ protofibril formation and AD dementia. Still, no amyloid is visible on positron emission tomography (PET) imaging through the ^{11}C-labeled Pittsburgh Compound B (PiB) ligand (Schöll et al., 2012). Similarly, the Osaka mutation (E693Δ) in APP causes the aggregation of AβP with little amyloid accumulation on PiB-PET (Shimada et al., 2011). Similarly, transgenic mice carrying the Osaka mutation do not show, by immunohistochemistry, amyloid deposits (Tomiyama et al., 2010). Thus, several therapeutic strategies targeting Aβ have been tested in the last decades, such as secretase inhibitors, AβP aggregation inhibitors, and Aβ immunotherapy (Pinheiro and Faustino, 2019). However, almost all of these strategies have been discontinued, either because of side effects or the lack of sizable therapeutic effects. Nevertheless, the failure of past clinical trials targeting Aβ does not mean that Aβ is a wrong target. Indeed,

the current common concern is that AD patients must be treated at an earlier stage, i.e., right when the pathological "amyloid" cascade likely begins.

Tau Protein

The protein tau has been identified and purified in 1970 (Weingarten et al., 1975; Cleveland et al., 1977) as a microtubule-interacting protein that stabilizes the neuronal cytoskeleton. The tau protein structure is composed of four main regions: an acidic N-terminal (NT); a proline-rich region responsible for the binding to microtubules; four repeat domains (R1–4), also called microtubule-binding domains (MBDs) (Drewes et al., 1995; Sengupta et al., 1998; Gendron and Petrucelli, 2009); and a C-terminal (CT) region. Importantly, tau activity can be modulated by a wealth of posttranslational modifications (PTMs), such as acetylation, glycosylation, glycation, methylation, truncation, nitration, ubiquitination, and phosphorylation (Almansoub et al., 2019). However, phosphorylation is the most commonly described and investigated since it is centrally involved in the formation of pathologic aggregates. Indeed, the aggregation of tau has been correlated to a broad spectrum of neurological diseases, including AD, known as "tauopathies" (Congdon and Sigurdsson, 2018; Almansoub et al., 2019). This PTM is physiologically regulated by the balance between tau kinases and phosphatase activities (Martin et al., 2013). Importantly, among 85 phosphorylation sites, about 45 of these are phosphorylated in AD brains (Noble W. et al., 2013). More in detail, the early phosphorylation events, at specific serine residues such as Ser199, Ser202/205, and Ser262, can disrupt the association of tau with microtubules. This event, in turn, can lead to alterations in tau-dependent cellular functions with dysregulated axonal growth and vesicle and organelle transport (Gendron and Petrucelli, 2009; LaPointe et al., 2009; Congdon and Sigurdsson, 2018). Otherwise, phosphorylation at other serine residues, such as Ser396, has been suggested as a prominent subsequent event that correlates with the progression of AD (Congdon and Sigurdsson, 2018).

Like phosphorylation, tau acetylation may arise from multiple mechanisms, and the dysregulation of this process can chiefly contribute to neurodegeneration. For instance, acetylation appears to prevent the binding of ubiquitin and then tau turnover (Min et al., 2010). This event can prompt a rise in cytosolic tau levels that makes the protein prone to aggregation (Congdon and Sigurdsson, 2018). Finally, unlike other tau posttranslational modifications, *O*-GlcNAcylation seems to be protective against tau-induced pathology. Indeed, in the AD brain, the levels of *O*-GlcNAcylated tau are reduced when compared to those in healthy subjects (Liu et al., 2009). Thus, targeting these posttranslational modifications may offer new avenues to prevent tau aggregation, restoring the normal function of the protein.

Apolipoprotein E

Apolipoprotein E (ApoE) is a primary cholesterol carrier highly expressed in astrocytes and, to a lesser extent, in the microglia which mediates both the transport and delivery of lipids from a cell type to another (Mahley and Rall, 2000). In humans, three different alleles (ε2, ε3, and ε4) give rise to three different isoforms of ApoE, which differ in amino acids in positions 112 and 158: ApoE2 (Cys112 and Cys158), ApoE3 (Cys112 and Arg158), and ApoE4 (Arg112 and Arg158) (Mahley and Rall, 2000; Liu et al., 2013). Importantly, the single amino acid difference in the ApoE protein influences its ability to bind lipids, receptors, and also Aβ(REFF). Indeed, several studies have demonstrated that ApoE has a crucial role in Aβ aggregation and clearance influencing senile plaque formation and AD development (Ellis et al., 1996; Liu et al., 2013). In this context, several reports, including clinical, epidemiological, and genetic studies, have demonstrated an association between ApoE genotypes and AD. For instance, genome-wide association studies (GWAS) have confirmed that the ε4 allele of ApoE is one of the strongest genetic risk factors for AD (REFF). Indeed, the ε4 allele is significantly enriched in AD patients (Corder et al., 1993) and is associated with an increased Aβ plaque load in the brain (Schmechel et al., 1993), a higher brain atrophy (Agosta et al., 2009), and an earlier onset and accelerated progression of the disease (Shi et al., 2017).

Moreover, it has been shown that Aβ deposition and aggregation to form senile plaques are a phenomena predominantly observed in ApoE ε4 allele carriers compared with non-carriers (Schmechel et al., 1993; Polvikoski et al., 1995; Kok et al., 2009). In addition, ApoE ε4 carriers present lower Aβ42 levels in cerebrospinal fluids (CSFs) and higher PiB-positive imaging (Prince et al., 2004; Head et al., 2012).

NEUROINFLAMMATION IN THE PATHOGENESIS OF ALZHEIMER'S DISEASE

In addition to the two classic diagnostic hallmarks of AD, Aβ plaques and neurofibrillary tangles, the brain of patients with AD exhibits evidence of a sustained inflammatory response (Mandrekar and Landreth, 2010; Femminella et al., 2018, 2019). In the acute phase, inflammation in the brain represents an established defense against infections, toxins, and injury. However, a disruption in the equilibrium between the pro- and anti-inflammatory mediators results in a chronic inflammatory condition of the brain which is identified as a neuroinflammation (Mandrekar and Landreth, 2010). Importantly, this process is currently attributed to the accumulation of reactive microglia and astrocytes that, in AD, appears to be localized to amyloid deposits (Alzheimer et al., 1995; Bornemann et al., 2001; Stalder et al., 2001; Mandrekar and Landreth, 2010). Microglia are the resident phagocytes of the central nervous system that are activated in response to AβP accumulation, change their morphology to ameboid cells, migrate to the plaques, and release inflammatory mediators, starting the phagocytosis of the plaques (Kettenmann et al., 2011; Du et al., 2017; Wolf et al., 2017). However, while in the acute phase, the activation of microglia is neuroprotective; in chronic phase, it exacerbates neuroinflammation with consequent neurodegeneration (Solito and Sastre, 2012; Heppner et al., 2015; Zuroff et al., 2017; Kinney et al., 2018). Emerging evidences have demonstrated that astrocyte-mediated neuroinflammation is also involved in the pathogenesis of neurodegenerative diseases, including

AD (Verkhratsky et al., 2010; Colombo and Farina, 2016). Astrocytes are specialized glial cells involved in the production of neurotrophic factors and in the maintenance of the blood brain–barrier (BBB), which protect the central nervous system (CNS) from harmful molecules and cells (including pathogens) (Sofroniew and Vinters, 2010). In response to brain insults, these cells become activated, a process known as reactive astrogliosis, and they release reactive oxygen species (ROS), nitric oxide (NO), and pro-inflammatory molecules, including interleukins (ILs) and tumor necrosis factor (TNF) (Phillips et al., 2014; Neal and Richardson, 2018). Although initially this process is aimed at removing noxious stimuli, prolonged astrocyte activation causes detrimental effects, leading to neuronal dysfunction and cell loss (Steardo et al., 2015). Reactive astrogliosis is a hallmark of AD and is responsible for the exacerbation of AβP-induced neurotoxicity and increased tau phosphorylation (Garwood et al., 2011; Osborn et al., 2016).

The involvement of neuroinflammation in the pathogenesis of AD has been supported by observational and epidemiological studies demonstrating that chronic use of nonsteroidal anti-inflammatory drugs (NSAIDs) can exert beneficial roles in reducing the risk of AD (Ali et al., 2019). Moreover, mutations in the genes encoding for immune receptors, including triggering receptor expressed on myeloid cells 2 (TREM2) and myeloid cell surface antigen CD33, have been associated with an elevated risk of developing AD (Griciuc et al., 2013; Gratuze et al., 2018). TREM2 is a transmembrane immune receptor expressed on the surface microglia, and in AD, it is involved in the clearance of Aβ plaques (Boche et al., 2013; Jevtic et al., 2017; Lagarde et al., 2018). For this reason, an alteration in TREM2 function is reported as harmful and correlates with AD development. For instance, Wang and coworkers have demonstrated that TREM2 deficiency resulted in an increased AβP accumulation in the brain with reduced clustering of the microglia around the plaques (Wang et al., 2015). Importantly, the most common TREM2 mutation is the arginine 47 histidine (R47H) variant, which appears to be associated with a reduced microglial uptake of Aβ and an increased risk of AD development (Guerreiro et al., 2013; Jonsson et al., 2013; Tanzi, 2015; Hansen et al., 2018). In this regard, Cheng-Hathaway et al. (2018) demonstrated that AD mice heterozygous for the TREM2 R47H presented reduced immune cells and enhanced neuritic dystrophy around Aβ plaques. Importantly, other TREM2 variants have also been studied for their association with the risk of AD, including R62H (Huang et al., 2004; Guerreiro et al., 2013; Jonsson et al., 2013; Jin S.C. et al., 2014; Roussos et al., 2015; Ghani et al., 2016; Song et al., 2017; Sims et al., 2017). More in detail, Kleinberger et al. demonstrated, in human macrophages *in vitro*, that the TREM2 R62H variant led to an impairment of the phagocytic functions of TREM2 with a reduced uptake of Aβ-LDL complexes compared to wild-type control cells (Kleinberger et al., 2014; Yeh et al., 2016). Of note is that numerous recent findings suggest a link between tau protein aggregation and TREM2 dysfunction. For instance, in the CSF of AD patients, the levels of soluble TREM2 correlate with the amount of total and phosphorylated tau, but not with those of Aβ42 (Piccio et al., 2016). Importantly, either soluble TREM2 or the phosphorylated tau levels in the CSF are related to the cognitive decline and clinical progression of AD (de Leon et al., 2004; Buerger et al., 2006; Andersson et al., 2008; Ewers et al., 2019). Contrary to the protective role of TREM2, CD33 induces a negative response in AD because this receptor inhibits phagocytosis, thus reducing microglial uptake and clearance of Aβ (Griciuc et al., 2013). There is also evidence for the existence of a potential crosstalk between CD33 and TREM2. More in detail, Griciuc and coworkers have demonstrated, in a murine model of AD, that loss of CD33 resulted in a decreased Aβ pathology and improved cognition (Griciuc et al., 2019). However, these effects were significantly abrogated by additional TREM2 knockout (Griciuc et al., 2019). Conversely, TREM2 knockout mice presented increased Aβ pathology and exacerbated neurodegeneration, which was not rescued by additional knockout of CD33. Thus, the authors concluded that TREM2 acts downstream of CD33.

Importantly, an association between TREM2 and ApoE has also been discussed. For instance, Jendresen et al. (2017) have demonstrated that human ApoE protein contains a binding site for TREM2 (amino acids 130–149), and this binding is isoform-dependent. In line with this report, Atagi et al. (2015) showed that ApoE can increase the phagocytosis of apoptotic neurons *via* TREM2 binding.

Importantly, ApoE activity has also been associated with microglia function. Indeed, LaDu and colleagues have demonstrated that glial cells cultured from ApoE knockout (KO) mice show an increased production of pro-inflammatory markers in response to treatment with Aβ (LaDu et al., 2001). In line with these data, in 2003, Lynch et al. (2003) have demonstrated that intravenous administration of lipopolysaccharide (LPS) in animals expressing the ε4 allele resulted in a more significant systemic and brain inflammation compared with their ε3 allele counterparts. Analogously, in a tauopathy murine model, ApoE knockdown markedly reduced the activation of microglia and astrocytes (Shi et al., 2017). This evidence supports the role of ApoE in neurodegenerative disorders. In the same vein, in one report, Rodriguez and colleagues demonstrated a direct relationship between ApoE, neuroinflammation, and AD (Rodriguez et al., 2014). Indeed, in the cortex of transgenic mice expressing five familial AD mutations (FAD), these authors found that the ApoE genotype can influence both Aβ deposition and Aβ-induced glial activation. Consistent with this notion, NSAIDs have been shown to reduce AD risk only in ε4 allele carriers, further supporting the role of the *ApoE* genotype in AD progression and development (Szekely et al., 2008).

Finally, in addition to these mechanisms, chronic complement activation has been linked to neuroinflammation and AD (Fischer et al., 1995; Fischer and Popa-Wagner, 1996). In particular, recent pieces of evidence from GWAS have identified complement component receptor (CR1), which binds complement proteins C3b and C4b, as a risk factor for AD (Lambert et al., 2009). In line with these data, Brouwers and coworkers found four single-nucleotide polymorphisms (SNPs) in the CR1 locus that were associated

with elevated levels of Aβ in the CSF of patients with AD (Brouwers et al., 2012). Furthermore, intragenic duplication of low copy repeats (LCR) within the CR1 gene appears to be associated with an increased risk of late-onset AD (Kucukkilic et al., 2018).

ORAL DYSBIOSIS, INFLAMMATION, AND PERIODONTITIS

The microbiome plays a crucial role in human physiology influencing nutrition, immunity, organ development, and function (Sudhakara et al., 2018). In the last decades, the observation that several chronic diseases of the gastrointestinal tract and mouth are associated with the perturbation of microbiome (dysbiosis) has achieved growing attention from scientists. Thus, several studies have been designed to evaluate the potential association between dysbiosis and systemic diseases, including cardiovascular and neurological disorders (Beck and Offenbacher, 2005; Poole et al., 2013). Periodontitis is a chronic inflammatory disease caused by the abnormal growth and aggregation of different microorganisms (Kassebaum et al., 2014; Poole et al., 2015). In periodontitis, of the about 800 microorganisms identified so far, it appears that the vast majority of germs are Gram-positive (early colonizers), followed by Gram-negative bacteria (late colonizers). The latter are on the tooth surface, where they contribute to form the dental plaque (Belstrøm et al., 2014; Liccardo et al., 2019). These species include *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Treponema denticola*, *Prevotella intermedia*, *Campylobacter rectus*, *Tannerella forsythia*, *Fusobacterium nucleatum*, *Selenomonas* spp., *Parvimonas micra*, and *Eubacterium timidum* (Socransky et al., 1998; Belstrøm et al., 2014; Liccardo et al., 2019). Interestingly, poor oral hygiene results in the increase of the anaerobic environment in the dental plaque, promoting the proliferation of these pathogens and the release of their toxic factors. Moreover, defects in host immunoregulation enable pathogen proliferation and increase local inflammation (Barth et al., 2013). Paradoxically, also neutrophils, i.e., the most efficient phagocytes and primary cellular defense recruited to the periodontal pocket, participate in the pathogenesis of periodontitis (Hajishengallis, 2015). Indeed, these immune cell types release several molecules (antimicrobial peptides, enzymes, and reactive oxygen species) that cannot discriminate between pathogens and host tissue. Moreover, certain agents, such as *P. gingivalis*, may subvert neutrophil function, inhibiting the phagocytosis, thus expanding the inflammatory response (Sochalska and Potempa, 2017). Because of this process, additional mediators and cytokines are produced, and more neutrophils, T cells, and monocytes are recruited to the periodontium, leading to chronic local and systemic inflammation (Cekici et al., 2014; Hajishengallis, 2014; Hajishengallis, 2015). Importantly, T cells promote the release of several cytokines and inflammatory mediators, including tumor necrosis factor alpha (TNF-α), interleukin (IL)-1, IL-4, IL-10, and transforming growth factor β (TGF-β) (Graves, 2008). In addition to these inflammatory mediators, in response to pathogen infection, the gingival epithelial cells and fibroblasts release other cytokines and mediators [i.e., IL-1, IL-8, TNF-α, and prostaglandin E2 (PGE2)] that, in turn, recruit more macrophages and neutrophils. Moreover, these cells promote the expression of matrix metalloproteinases (MMPs), tissue-derived enzymes that participate in the extracellular matrix remodeling. Altogether, these processes result in the stimulation of osteoclasts with subsequent alveolar bone reabsorption (Neely et al., 2005; Jin J. et al., 2014). Periodontitis leads to systemic inflammation due to the direct infiltration of bacteria and their virulence factors into the bloodstream (Poole et al., 2013). For this reason, periodontitis has been linked to the onset and progression of disorders systemically, such as cancer, diabetes, and cardiovascular and neurological diseases (Hajishengallis, 2015; Liccardo et al., 2019). Importantly, virulence factors expressed by periodontal pathogens are important pathogenic determinants in the initiation, progression, and severity of the disease, and they are responsible for the local and systemic inflammatory response observed in patients with periodontitis. For instance, *P. gingivalis*, long considered as one of the most important members of the periodontopathic microbiota, presents a specific LPS (LPS-Pg), which is recognized by immune cells *via* Toll-like receptors 2 and 4 (TLR2/4), and toxic proteases called gingipains (gps) and other surface components such as carbohydrates and fimbriae (Potempa et al., 1995, 1997; Holt et al., 1999; Imamura, 2003; Hasegawa et al., 2008; Yilmaz, 2008; Guo et al., 2010). gps are cysteine proteases that comprise lysine-gp (Kgp) and arginine-gp A (RgpA) and B (RgpB) are released and transported to the outer bacterial membrane surfaces (Guo et al., 2010). In synergy with other virulence factors (Guo et al., 2010; Dominy et al., 2019) these proteases are crucially involved in *P. gingivalis* survival and pathogenicity, allowing the colonization and invasion of gingival/periodontal tissues as well as other tissues, systemically. Importantly, the initial colonization of cells, including fibroblasts, epithelial cells, and other bacteria, is mostly mediated by the coordination between gps and the fimbrial and non-fimbrial components. Moreover, gps play a critical role in iron and nutrient acquisition (*P. gingivalis* agglutinates erythrocytes and lyses them to release hemoglobin), tissue destruction (Guo et al., 2010), and in the inactivation of host defenses escaping phagocytosis from immune cells (i.e., neutrophils) (Maekawa et al., 2014). Analogously, *A. actinomycetemcomitans* produces numerous factors that have been well characterized, including adherence proteins, LPS, and toxins like the cytolethal distending toxin (CDT) and leukotoxin (LtxA) (Kolodrubetz et al., 1989; Lally et al., 1989; Shenker et al., 2005). Of note is that these toxins are involved in immune evasion mechanisms (Kachlany, 2010). Finally, *T. forsythia* expresses several proteases that contribute to bacterial virulence in multiple manners. For example, proteases participate in degrading the host periodontal tissue, modifying host cell proteins, thus allowing bacterial colonization. Moreover, all the above-mentioned factors are able to activate host degradative enzymes that process components involved in innate and adaptive immunity, thus blocking the host immune response (Sharma, 2010).

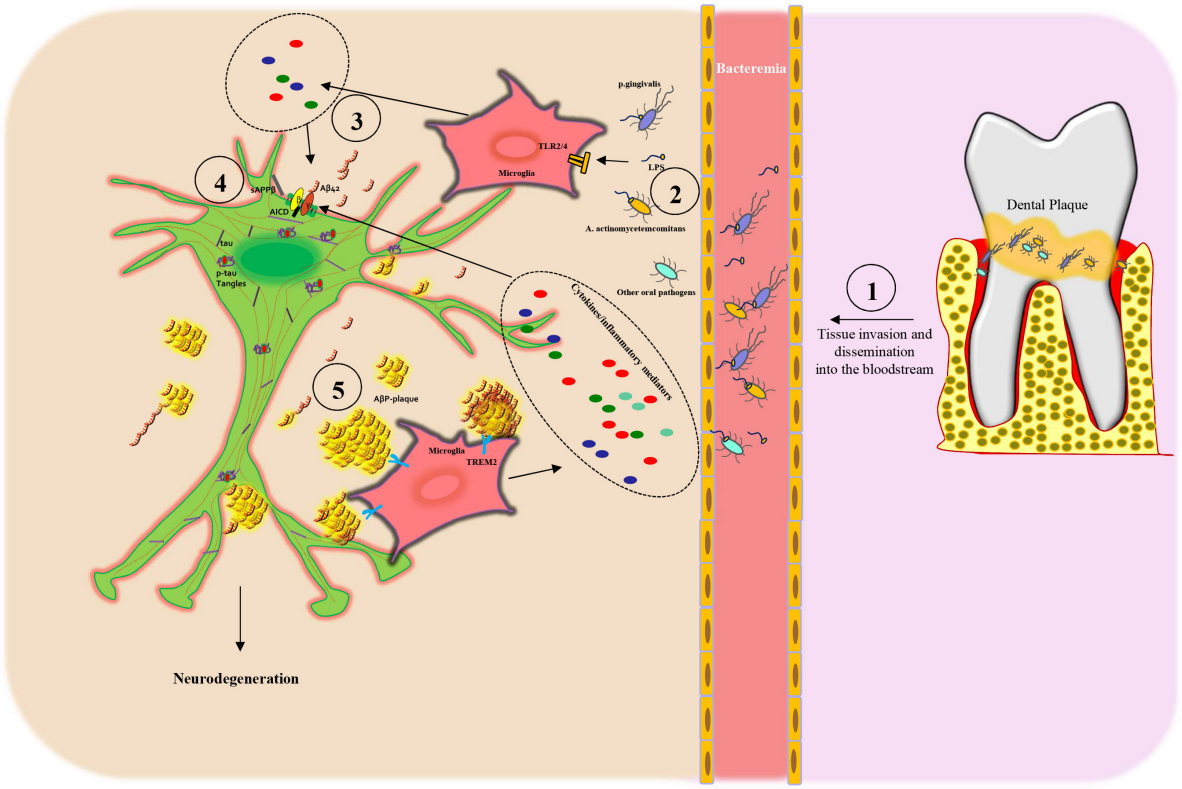

FIGURE 2 | Scheme of the proposed mechanism linking periodontitis to Alzheimer's disease: (1) Oral dysbiosis of the dental plaque leads to proliferation, tissue invasion, and then dissemination into the bloodstream of oral pathogens. (2) Next, the oral pathogens and their toxic molecules, such as lipopolysaccharide (LPS), bind to microglia *via* Toll-like receptors 2/4 (TLR2/4), inducing the release of cytokines (3) and inflammatory mediators that, in turn, lead to APP processing from neuronal cells. (3–4) Subsequently, the activation of β- and γ-secretase leads to an increased secretion of Aβ peptides (AβPs), in particular Aβ42 monomers and sAPPβ, outside of the cells and AICD intracellularly. (4) AβPs form oligomers, protofibrils, or fibrils and then amyloid plaques that are recognized by TREM2 receptors on the microglia plasma membrane, thus triggering an inflammatory response, which again stimulates AβP production. Dysfunctional neurons present also increased tau phosphorylation (p-tau) with the formation of neurofibrillary tangles (p-tau tangles). All these processes induce neuronal degeneration.

RELATIONSHIP BETWEEN PERIODONTITIS AND ALZHEIMER'S DISEASE

Although the brain is considered an immune-isolated environment, several shreds of evidence have indicated that systemic inflammation contributes to neurodegeneration through the microglial activation and release of pro-inflammatory molecules, thus driving AD progression (Perry et al., 2007; Holmes, 2013). For instance, Capsoni and colleagues, in 2012, have demonstrated that pathogen-free conditions can delay the onset of neurodegeneration in a murine model of nerve growth factor (NGF) deprivation (Capsoni et al., 2012). Furthermore, LPS, the main component of the membrane of Gram-negative bacteria, can be found in large amounts in the brain of AD patients compared to healthy controls (Zhan et al., 2016). In this context, several studies have found that LPS co-localized with AβPs (Aβ40/42) in the amyloid plaques and around vessels of the brain of AD patients (Zhan et al., 2018). And peripheral injection of LPS in mice can activate microglia, inducing the release of pro-inflammatory cytokines, such as interleukins and TNF-α (Godbout et al., 2005). In line with these data, Sheng et al. (2003) demonstrated that mice infused with LPS presented an increased neuroinflammation associated with the enhanced expression and processing of APP and Aβ40/42 levels inside neurons. Lastly, Lee and colleagues showed that in rTg4510 mice expressing a mutated tau protein (TauP301L) that develop tauopathy between 3 and 5 months of age, LPS infusion increases microglial activation and tangle formations (Lee et al., 2010). Thus, these reports indicate that bacteria can induce local inflammatory damage, which, in chronic condition, is a trigger of neuroinflammation, constituting a significant contributor of neurodegeneration and AD. For this reason, periodontal pathogens have been investigated for their involvement in AD development and progression. For instance, Chen and colleagues have demonstrated, in a retrospective study, that periodontitis exposure is associated with an about 1.7-fold increase in the risk of developing AD (Chen et al., 2017). Analogously, a recent study analyzing the National Health and Nutrition Examination Survey (NHANES) database demonstrated that subjects with mild to severe periodontitis presented a decreased cognitive function compared

with the healthy group (Sung et al., 2019). Mechanistically, this association has been demonstrated in a different number of studies. Kamer et al. (2009) have observed that elevated serum levels of TNF-α and serum antibodies to *P. gingivalis*, *A. actinomycetemcomitans*, and *T. forsythia* were present in AD patients compared to the controls. In line with these data, Sparks Stein and coworkers demonstrated that antibody levels to *F. nucleatum* and *P. intermedia*, at baseline, resulted significantly increased compared to the controls and correlated with a declined cognitive function in AD patients (Sparks et al., 2012). Furthermore, in a preclinical study from Ilievski and coworkers, it has been shown that in wild-type mice, *P. gingivalis* infection resulted in the neurodegeneration and formation of extracellular Aβ42 (Ilievski et al., 2018). Analogously, Díaz-Zúñiga et al. (2019) demonstrated that *in vitro* LPS (from *A. actinomycetemcomitans*) increased neuroinflammation *via* the activation of microglia and the subsequent increase in pro-inflammatory cytokines and chemokines coupled to the accumulation of Aβ42. Importantly, LPS from *P. gingivalis* (LPS-PG) binds to glial cells (Poole et al., 2013), and in the AD brain, it is co-localized with Aβ plaques (Zhan et al., 2016; Zhao et al., 2017). Of note is that a direct connection between oral dysbiosis and AD has been suggested by Poole et al. (2013), who reported the presence of periodontal pathogen components in AD subjects. Subsequently and in line with these data, Dominy et al. (2019) demonstrated that *P. gingivalis* and their virulence factors, gingipains, were exclusively detected in the brain of AD patients compared to the controls. Moreover, in this study, the authors demonstrated that in mice, this oral pathogen migrates from the mouth to the brain, increasing the production of Aβ42, exerting significant neurotoxic effects. Conversely, these processes were abolished following treatment with gingipain inhibitors. In this context, a phase II/III clinical trial has been designed and initiated in order to test the effects of the gingipain inhibitor COR388 in patients with a diagnosis of mild to moderate AD (NCT03823404).

Importantly, as discussed above, the ApoE genotype appears to be crucially involved in neuroinflammation, and as previously demonstrated, it can also contribute to enhancing *P. gingivalis* brain colonization. For example, in 2015, Poole and coworkers observed the presence of *P. gingivalis* DNA (Poole et al., 2015) in the brain of $ApoE^{null}$ mice infected at gingival levels with this Gram-positive pathogen. Interestingly, as demonstrated by Singhrao et al., in these mice, gingival infection with *P. gingivalis* also resulted in the early appearance of age-related granules (Singhrao et al., 2015). These data, in line with the results obtained in another study by Hafezi-Moghadam et al., suggest that the lack of functional ApoE protein and the increased systemic inflammation, observed in periodontitis, induce an impairment of the BBB (Hafezi-Moghadam et al., 2007; Singhrao et al., 2017; Ranjan et al., 2018).

Importantly, a dysfunctional BBB allows periodontal pathogens to access the systemic circulation (bacteremia) and invade the brain (Hafezi-Moghadam et al., 2007; Singhrao et al., 2017; Ranjan et al., 2018) and represents an early feature of AD and cognitive decline (Van de Haar et al., 2016; Carter, 2017). In aggregate, these data strengthen the potential relationship between periodontitis and AD development and progression (**Figure 2**).

CONCLUSION

In summary, periodontitis and AD often coexist. However, the current debate focuses on one main question: *what comes first*? Some studies have demonstrated that people with periodontitis present a major risk of developing AD (Chen et al., 2017); however, other reports suggest that those with AD or dementia suffer from inadequate oral health, stemming from cognitive decline, and are, therefore, more likely to develop periodontitis (Tada et al., 2006; Gonsalves et al., 2008; Maldonado et al., 2018). Thus, further studies are urgently needed to establish the *raison d'être* for the mutual association between periodontitis and AD. Along this line of reasoning, the trial (NCT03823404) discussed above shall give us the proof-of-concept of the beneficial role of oral pathogen blockade in human AD. Yet, while waiting for the publication of the trial outcome, we can ascertain, with no additional hesitation, that a more careful dental treatment effectively improves the quality of life/cognitive impairment of patients with mild AD (Rolim et al., 2014). Likewise, in decreasing the incidence of dementia in patients treated for dementia or periodontitis (Lee et al., 2017; Yoo et al., 2019). Therefore, oral hygiene care strategies should be included in the routine health care of patients with dementia and cognitive impairment and become a dominant part of adult oral health programs to avoid any extra-neuronal source of inflammation as well as to prevent the onset of neurodegeneration. Thus, these findings highlight the necessity to prevent the progression of periodontitis and encourage healthcare service at the national level.

AUTHOR CONTRIBUTIONS

DL wrote, edited, and revised the manuscript. FM, FC, MG, GF, LB, JA, AA, and IM contributed to the writing and editing of the manuscript. AV, CR, NF, and GR revised the manuscript. AC supervised the project, revised the manuscript, and generated the figures.

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BRIEF RESEARCH REPORT
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Persistence of Mast Cell-Positive Synovitis in Early Rheumatoid Arthritis Following Treatment With Conventional Synthetic Disease Modifying Anti-Rheumatic Drugs

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Mast cells (MCs) are immune cells infiltrating the synovial membrane and implicated in the pathogenesis of Rheumatoid Arthritis (RA). Their infiltration in the synovia of early RA patients has been shown to be associated with systemic inflammation, disease activity and autoantibody positivity. Here, we analyzed their presence in matched synovial samples obtained by ultrasound-guided synovial biopsies pre- and post-treatment with conventional synthetic Disease Modifying Anti-Rheumatic Drugs (csDMARDs) (n=20). Upon IHC staining, patients were classified as $MC^{+ve/-ve}$ based on the presence/absence of CD117+ synovial MCs. At baseline, MC^{+ve} patients had significantly higher synovial inflammation, inflammatory markers, disease activity and a higher prevalence of lympho-myeloid aggregates. Synovial biopsies after 6 months of treatment with csDMARDs showed a significant reduction of synovitis scores, but only a partial reduction of MC numbers. Accordingly, 45% of patients (9/20) were MC^{+ve} after treatment, in association with significantly higher degree of synovitis and higher proportion lympho-myeloid aggregates. Finally, significantly lower patients with MC^{+ve} synovitis at 6 months reached Low Disease Activity (LDA), while the association of MCs with disease activity was independent from lymphoid aggregates, after adjustment for BMI and age. Overall, this study confirms the relevance of MCs as part of the inflammatory infiltrate in the synovia of RA patients, warranting further investigations in larger cohorts to clarify their role in disease progression and response to treatment and their relevance as prognostic markers and potential therapeutic targets.

Keywords: mast cells, synovitis, synovial membrane, rheumatoid arthritis, inflammation, treatment response

INTRODUCTION

Mast cells (MCs) are tissue-resident cells of the innate immunity, involved in a number of physiological and pathological processes, including infections, cancer, and chronic inflammatory diseases (Voehringer, 2013; Krystel-Whittemore et al., 2016; Varricchi et al., 2017; Varricchi et al., 2018). Mast cells are present in the synovial membrane (SM) in physiological conditions (De Paulis et al., 1996) and have been implicated in various rheumatic diseases (Suurmond et al., 2016), including rheumatoid arthritis (RA) (Rivellese et al., 2017). In fact, synovial MC numbers are significantly increased in inflammatory conditions such as RA (Crisp et al., 1984; Gotis-Graham and McNeil, 1997; Gotis-Graham et al., 1998). However, recent evidences suggest that MC contribution to autoimmune diseases can be complex and multifaceted (Brown and Hatfield, 2012). In the context of RA, for example, human MCs have been shown to induce immunomodulatory effects *in vitro* (Rivellese et al., 2015). Similarly, *in vivo* findings in animal models yielded contrasting results on the contribution of mast cells to the development of arthritis (Lee et al., 2002; Zhou et al., 2007; Pitman et al., 2011). Recent data suggest that their contribution to RA may be different in various disease stages, i.e. essential during the early phases, but dispensable during the late effector phases (Schubert et al., 2015; van der Velden et al., 2016). Thus, despite a substantial amount of data produced over the last years, the role of MCs in RA remains to be clarified (Rivellese et al., 2019b). When considering the well-known heterogeneity of RA (Pitzalis et al., 2013; Smolen et al., 2016; Firestein and McInnes, 2017) and the multifaceted functions of MCs, it is possible to hypothesize that the presence and functions of MCs in synovia may be different in various disease subsets. Recently, the analysis of MCs in the synovia of a large cohort of conventional synthetic disease-modifying anti-rheumatic drugs (csDMARDs)-naïve early RA patients has substantiated such hypothesis, as it showed a strong association of MCs with the infiltration of lymphoid and myeloid cells, which are defining a specific histological subset of patients with a so-called lympho-myeloid pathotype (Pitzalis et al., 2013; Rivellese et al., 2018). Since the lympho-myeloid synovial pathotype has been associated with disease outcomes (Humby et al., 2019), here we aimed to analyze the presence/absence of MCs in synovial biopsies at baseline and 6 months after treatment with csDMARSDs.

MATERIALS AND METHODS

Patient Samples and Ultrasound-Guided Synovial Biopsy

Synovial tissue was obtained by ultrasound-guided synovial biopsy from DMARD-naïve patients with early (<12 months) RA (n=20), enrolled in the Pathobiology of Early Arthritis Cohort (PEAC) cohort of the Centre for Experimental Medicine and Rheumatology of Queen Mary University (London) at Barts Health NHS trust (Kelly et al., 2015). At the baseline visit, following written informed consent, patients underwent synovial biopsy of the most inflamed joint (Synovial thickening >=2). Afterwards, patients started treatment with csDMARDs with a treat-to-target approach, according to a standardized protocol in line with local guidelines [NICE (National Institute for Health and Care Excellence), 2018]. All patients were started on methotrexate—unless contraindicated—in combination of hydroxychloroquine or sulfasalazine. At 6 months, patient had a repeated ultrasound-guided synovial biopsy of the same joint (n=20). An overview of csDMARDs use up to 6 months is presented in **Table 2**. More specifically, two patients were not treated with csDMARDs, two were in monotherapy with Methotrexate, one with hydroxychloroquine, four in combination therapy with methotrexate and hydroxychloroquine, 10 with methotrexate and sulfasalazine, and one with methotrexate, hydroxychloroquine, and sulfasalazine. All patients fulfilled the 2010 EULAR criteria for RA (Aletaha et al., 2010). All procedures were performed following written informed consent and were approved by the hospital's ethics committee (REC 05/Q0703/198).

Histological Analyses of Synovial Samples

Synovial sections underwent standard H&E staining and semi-quantitative (SQ) assessment of synovitis according to a previously validated score (Krenn) (Krenn et al., 2006). Sequentially cut sections underwent Immunohistochemical (IHC) staining and upon SQ scoring (0-4), sections were stratified into synovial pathotypes according to the degree of immune cell infiltration: i) Lymphoid- grade 2/3 B cell aggregates, CD20≥ 2 and/or CD138>2, ii) Myeloid- CD68 SL≥ 2, CD20 ≤ 1 and/or CD3≥1, CD138 ≤ 2, and iii) Fibroid- CD68 SL<2 and CD3, CD20, CD138<1) (Humby et al., 2009; Rivellese et al., 2019a). Following IHC staining for CD117, patients were classified as MC+/MC-, based on the presence/absence of synovial mast cells and MC density (n of cells/mm^2) was calculated by automated cell counting (cellSens, Olympus).

Statistical Analyses

Measures of central tendency and dispersions and statistical analyses are indicated in each figure legend. P values of <0.05 were considered statistically significant. For the regression analyses, the glm function from package stats v3.6.2 was used, using DAS28 at 6 months as predicted value and BMI, age, lymphoid aggregates (binary), and MCs (binary) as predictors. The performance of the models without and with MCs were compared by ANOVA. Data have been analysed using R Studio Version 1.2.5033.

RESULTS

Mast Cells Are Associated With Defined Histological Features of Synovitis and Severe Disease Activity in Early RA

First, we explored whether the presence of MCs in synovia could identify early RA patients with a severe phenotype. To this aim, we classified patients into MC^{+ve} and MC^{-ve} based on the

presence/absence of synovial MCs. Representative images of this classification are shown in **Figure 1A**. MC^{+ve} patients (9/20, 45%) had significantly higher levels of inflammatory markers (ESR and CRP) and disease activity (DAS28) compared to MC^{-ve} (**Figure 1B** and **Table 1**). Overall, this indicates that the presence of MCs in synovia identifies patients with higher levels of systemic inflammation and severe disease. Accordingly, MC^{+ve} patients had significantly higher synovitis scores and a higher prevalence of the lympho-myeloid pathotype (**Table 1** and **Figure 1C**). However, there were no significant differences in DAS28, in the delta change of DAS28 from baseline and the prevalence of low disease activity in patients stratified as MC- or MC+ at baseline (**Table 1**).

Treatment With csDMARDs Induced a Significant Reduction of Synovial Inflammation and a Partial Reduction of Mast Cell Numbers

Having demonstrated that the presence of MCs in synovia identifies early RA patients with severe disease, we next looked at the effect of csDMARDs treatment on synovial inflammation, by analyzing repeated synovial biopsies at 6 months. We observed a significant reduction of the synovitis score (Krenn score, **Figure 2A**) and only a partial non-significant reduction of MC numbers (**Figure 2B**). Accordingly, synovial MCs were present in 45% of patients (MC^{+ve} 9/20) at 6 months, in association with higher synovitis scores and a higher prevalence of lympho-myeloid aggregates (**Figure 2C** and **Table 2**). This shows that treatment with csDMARDs has an impact on synovial inflammation, as it induces a significant reduction of synovitis. Nonetheless, almost half of the patients have non-resolving synovial inflammation, with MC infiltration accompanied by lympho-myeloid aggregates.

Persistence of Mast Cells at 6 Months Was Associated With a Higher Disease Activity and a Lower Remission Rate

In parallel to non-resolving synovial inflammation, the presence of MCs after 6 months of treatment with synthetic DMARDs was associated with numerically higher DAS28 values (p=0.07) and significantly higher tender joint count (p=0.03) and significantly lower rates of Low Disease Activity (LDA), as defined by DAS28 <3.2 (**Table 2** and **Figure 2D**). As shown in **Table 2**, most of patients were treated with a combination of two csDMARDs, without statistically significant differences between MC+ ad MC- groups.

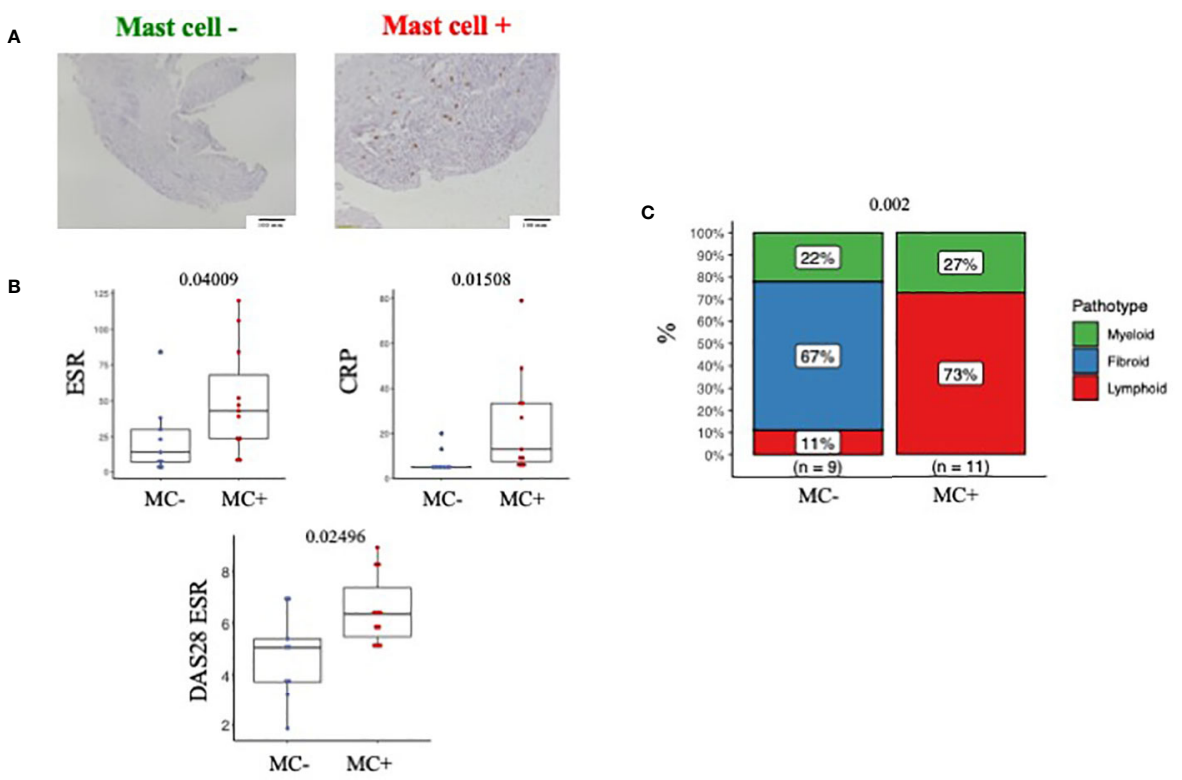

FIGURE 1 | (A) Representative example of IHC staining for CD117 (c-kit) and classification of patients into MC^{-ve} (left) and MC^{+ve} (right). Line at 100um. **(B)** ESR, CRP and DAS28 in MC^{-ve} and MC^{+ve} patients. n=20 patients, pre-treatment synovial biopsy. ESR, Erythrocyte Sedimentation Rate; CRP, C Reactive Protein; DAS28, Disease activity score 28 joints. **(C)** Pathotype distribution in MC^{-ve} and MC^{+ve} patients.Mann Whitney test in B, Fisher in C, p value is displayed when significant (<0.05), ns, non-significant (>0.05).

TABLE 1 | Baseline features in patients stratified according to Mast cell presence.

	Overall (N=20)	MC negative (N=9)	MC positive (N=11)	P value
Age				0.3215
Mean (SD)	52.9 (18.7)	47.0 (20.9)	57.3 (16.5)	
Median [Min, Max]	53.0 [23.0, 81.0]	45.5 [23.0, 79.0]	60.0 [27.0, 81.0]	
Disease onset				1
Mean (SD)	4.61 (2.48)	4.29 (1.70)	4.82 (2.93)	
Median [Min, Max]	4.00 [2.00, 12.0]	5.00 [2.00, 7.00]	4.00 [2.00, 12.0]	
ESR				0.0400
Mean (SD)	38.3 (34.9)	23.4 (25.8)	50.5 (37.7)	
Median [Min, Max]	27.5 [2.00, 120]	14.0 [2.00, 84.0]	43.0 [8.00, 120]	
CRP				0.0150
Mean (SD)	16.9 (19.3)	7.56 (5.36)	24.5 (23.3)	
Median [Min, Max]	7.50 [5.00, 79.0]	5.00 [5.00, 20.0]	13.0 [5.00, 79.0]	
ACPA positive, %	60.00%	55.60%	63.60%	0.6499
RF positive, %	50.00%	44.40%	54.50%	0.6562
DAS28 ESR				0.0249
Mean (SD)	5.69 (1.75)	4.67 (1.69)	6.52 (1.36)	
Median [Min, Max]	5.59 [1.88, 8.92]	5.05 [1.88, 7.00]	6.34 [5.09, 8.92]	
Tender joint count				0.1184
Mean (SD)	12.1 (8.91)	8.67 (8.43)	14.9 (8.64)	
Median [Min, Max]	11.5 [1.00, 28.0]	5.00 [2.00, 27.0]	12.0 [1.00, 28.0]	
Swollen Joint Count				0.1809
Mean (SD)	8.00 (7.09)	5.67 (4.47)	9.91 (8.40)	
Median [Min, Max]	6.50 [1.00, 26.0]	4.00 [1.00, 16.0]	8.00 [2.00, 26.0]	
VAS global health				0.0200
Mean (SD)	67.3 (28.5)	50.9 (31.4)	80.6 (17.7)	
Median [Min, Max]	77.5 [2.00, 100]	48.0 [2.00, 100]	79.0 [48.0, 100]	
Joint biopsied				n.a.
Knee	1 (5.0%)	0 (0%)	1 (9.1%)	
MCP	1 (5.0%)	1 (11.1%)	0 (0%)	
PIP	1 (5.0%)	0 (0%)	1 (9.1%)	
Wrist	17 (85.0%)	8 (88.9%)	9 (81.8%)	
Synovitis scores				0.0311
No synovitis (0-2)	8 (40.0%)	6 (66.7%)	2 (18.2%)	
Low synovitis (3-5)	4 (20.0%)	2 (22.2%)	2 (18.2%)	
High Synovitis (6-9)	8 (40.0%)	1 (11.1%)	7 (63.6%)	
Pathotypes				
Fibroid	6 (30.0%)	6 (66.7%)	0 (0%)	0.0019
Lymphoid	9 (45.0%)	1 (11.1%)	8 (72.7%)	
Myeloid	5 (25.0%)	2 (22.2%)	3 (27.3%)	

ES, RErythrocyte Sedimentation Rate; CRP, C Reactive Protein; ACPA, Anti Citrullinated Protein Antibodies, RF, Rheumatoid Factor; DAS28, Disease Activity Score 28 joints; VAS, Visuoanalog scale.

When patients were stratified according to the presence/absence of lympho-myeloid aggregates at 6 months, we found no significant differences in the prevalence of low disease activity (**Figure 2E**) nor in any other clinical parameter at 6 months (data not shown).

To further confirm the association of MCs with disease activity at 6 months, we used multiple linear regression to predict 6 months DAS28, using the presence/absence of MCs and lymphoid aggregates as predictors, after correcting for age and BMI. Interestingly, the presence of lymphoid aggregates per se did not improve the prediction model, while the presence of MCs significantly improved its performance (ANOVA p value 0.00657 when comparing the full model including MCs, lymphoid aggregates, age and BMI to the model with lymphoid aggregates, age and BMI). The results of the full regression model with age, BMI, lymphoid aggregates and MCs are shown in **Table 3**.

Although the small numbers should be taken into account when interpreting these results, these analyses suggests that the presence of MCs at 6 months biopsy is associated with disease severity, independently of BMI, age and lymphoid aggregates.

DISCUSSION

We here report the analysis of mast cells in the synovia of patients with early untreated Rheumatoid Arthritis undergoing ultrasound-guided synovial biopsies before and after treatment with csDMARDs. Our results indicate that synovial MCs are associated with disease severity at baseline and non-resolving MC^{+ve} synovitis after treatment with csDMARDs is associated with a lower response to csDMARDs.

The results at baseline are in agreement with previous publications (Gotis-Graham and McNeil, 1997; Gotis-Graham

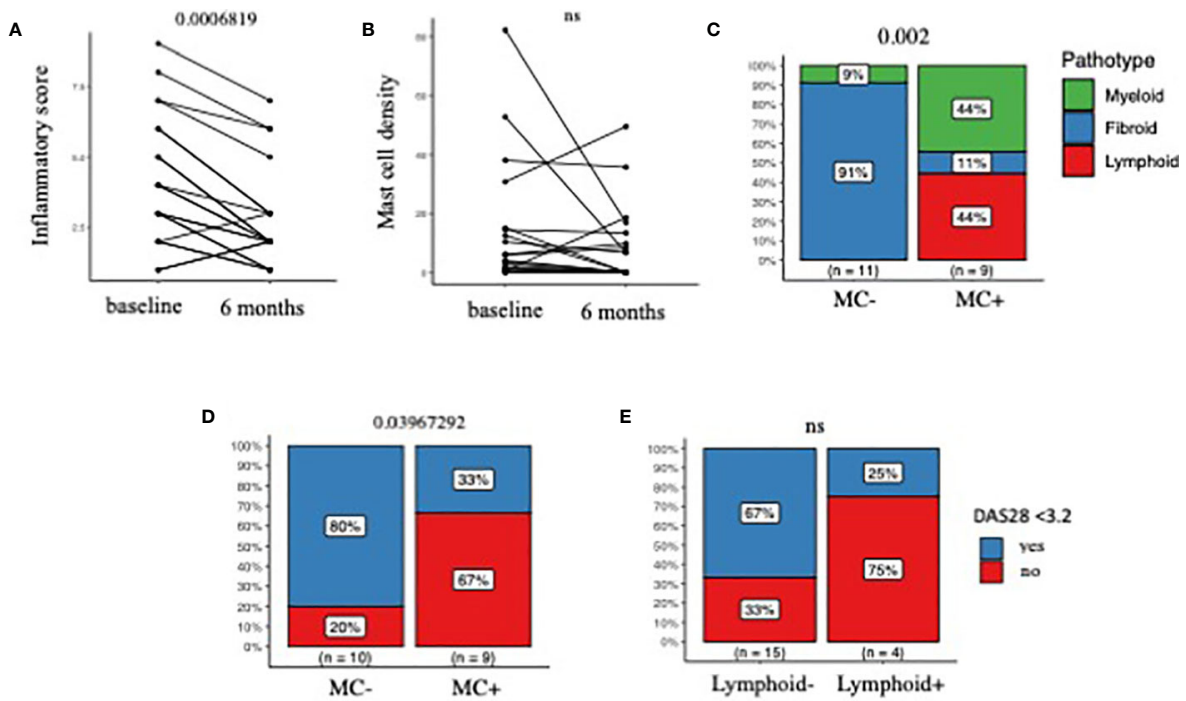

FIGURE 2 | (A, B) Synovitis score (Krenn score) in A and MC density (number/mm2) in B, in matched ultrasound-guided biopsies at baseline and 6 months after treatment with csDMARDs. **(C)** Distribution of pathotypes at 6 months in MC^{-ve} and MC^{+ve} patients. **(D, E)** Prevalence of low disease activity (LDA), defined as DAS28<3.2, at 6 months in in MC^{-ve} and MC^{+ve} patients (D) and lymphoid-ve and lymphoid+ve patients (E); N=20 patients, pre- and post-treatment biopsy in A-B, post-treatment in C-E. MC, Mast cells; DAS28, Disease activity score 28 joints; csDMARDs, conventional synthetic Disease Modifying Anti-Rheumatic Drugs; paired samples Wilcoxon test in A and B, Fisher exact test in C-D-E. P values are displayed when significant (<0.05), ns, non-significant (>0.05).

et al., 1998), including our recent observations showing that stratification of patients according to the abundancy of synovial MCs in early untreated RA identifies patients with severe disease (Rivellese et al., 2018).

Here, we classified patients based on the simple presence/absence of synovial MCs, rather than into groups based on the relative abundancy of mast cells. Thus, our results indicate that the simple presence of MCs in the synovia of patients with early untreated RA, independently from the degree of infiltration, is able to identify patients with higher disease severity at baseline.

Additionally, we assessed MCs in matched ultrasound guided synovial biopsies following treatment with csDMARDs, showing a significant reduction of the synovitis score and a partial reduction of MC density. Accordingly, 45% of the patients were classified as MC^{+ve} in the repeated post-treatment biopsy, in association with a lower prevalence of low disease activity. In other words, non-resolving MC^{+ve} synovitis is associated with a reduced response to csDMARDs, as only 33% of MC+ patients reach a low disease activity, in comparison to 80% of MC negative.

To our knowledge, one previous work reported the analysis of MCs in post-treatment synovial biopsies, that failed to identify a clear pattern in the effects of treatment on synovial MC infiltration, most likely because of the lack of treatment standardization and the very low number of patients (n=6) (Gotis-Graham et al., 1998). In our manuscript, we analysed matched post-treatment synovial biopsies from an observational cohort, thus in the absence of randomization, however patients were treated according to a standardized protocol in line with treat-to-target local guidelines and tight follow-up, with visits at 1 month, 3 months, and 6 months [NICE (National Institute for Health and Care Excellence), 2018]. Accordingly, most patients were treated with a combination of two csDMARDs, with no differences in MC+ and MC- groups, suggesting that the lack of response in MC^{+ve} patients is not associated with sub-optimal treatment.

This observation is in line with a recent publication describing significantly higher numbers of synovial mast cells and B cells in patients who did not maintain remission after 1 year, which suggest that synovial MCs could be used as predictors of disease flare (Ramírez et al., 2016).

Importantly, since the presence of MCs in synovia is strongly associated with lympho-myeloid cells, it could be argued that the association of MCs with treatment outcomes is indirect. Indeed, we have previously shown that high MC in synovia are associated with the infiltration of lympho-myeloid cells, both *ex vivo* in early untreated RA patients and *in vivo* in animal models of antigen induced arthritis, and MCs were able to activate B cells inducing the production of ACPA autoantibodies (Rivellese et al., 2018). Thus, it could be hypothesized that lympho-myeloid cells are the real culprit in defining response/non-response to treatment. However, when patients in our cohort

TABLE 2 | Six months outcomes in patients stratified according to MC presence at baseline and 6 months.

	BASELINE BIOPSY			
6 months outcomes	**Overall (N=20)**	**MC negative (N=9)**	**MC positive (N=11)**	
DAS28ESR				0.1518
Mean (SD)	2.41 (1.03)	3.80 (2.01)	3.21 (1.78)	
Median [Min, Max]	2.08 [1.36, 3.83]	3.50 [1.46, 7.56]	3.03 [1.36, 7.56]	
Delta DAS28ESR				0.4326
Mean (SD)	2.51 (1.32)	2.21 (1.66)	2.73 (1.03)	
Median [Min, Max]	2.22 [0.160, 5.21]	2.07 [0.160, 5.21]	2.22 [1.36, 4.89]	
DAS28 <3.2	55.00%	66.70%	45.50%	0.1977
Methotrexate, %	85.00%	66.70%	100%	0.1637
csDMARDs numbers				0.2669
0	2 (10.0%)	2 (22.2%)	0 (0%)	
1	3 (15.0%)	1 (11.1%)	2 (18.2%)	
2	14 (70.0%)	5 (55.6%)	9 (81.8%)	
3	1 (5.0%)	1 (11.1%)	0 (0%)	
Steroids, %	12 (60.0%)	5 (55.6%)	7 (63.6%)	1
	6 MONTHS BIOPSY			
	Overall (N=20)	**MC negative (N=11)**	**MC positive (N=9)**	
ESR				0.3627
Mean (SD)	15.7 (22.5)	8.20 (5.77)	24.0 (30.8)	
Median [Min, Max]	8.00 [2.00, 95.0]	7.50 [2.00, 19.0]	8.00 [2.00, 95.0]	
CRP				0.5383
Mean (SD)	6.06 (2.67)	6.00 (3.16)	6.13 (2.10)	
Median [Min, Max]	5.00 [5.00, 15.0]	5.00 [5.00, 15.0]	5.00 [5.00, 10.0]	
DAS28ESR				0.0788
Mean (SD)	3.21 (1.78)	2.40 (0.919)	4.11 (2.10)	
Median [Min, Max]	3.03 [1.36, 7.56]	2.09 [1.36, 3.83]	3.64 [1.46, 7.56]	
Tender Joint Count				0.0325
Mean (SD)	5.20 (7.34)	2.27 (4.15)	8.78 (8.96)	
Median [Min, Max]	2.00 [0, 22.0]	0 [0, 14.0]	6.00 [0, 22.0]	
Swollen Joint Count				0.09357
Mean (SD)	2.40 (2.93)	1.27 (1.74)	3.78 (3.56)	
Median [Min, Max]	1.00 [0, 10.0]	1.00 [0, 5.00]	3.00 [0, 10.0]	
VAS globa health				0.2234
Mean (SD)	29.7 (29.1)	21.5 (22.9)	39.7 (34.0)	
Median [Min, Max]	19.5 [0, 89.0]	12.0 [0, 70.0]	34.0 [0, 89.0]	
Delta DAS28ESR				1
Mean (SD)	2.51 (1.32)	2.45 (1.56)	2.57 (1.08)	
Median [Min, Max]	2.22 [0.160, 5.21]	2.78 [0.160, 5.21]	2.14 [1.36, 4.89]	
DAS28V3 <3.2	55.00%	72.70%	33.30%	0.0396
Methotrexate, %	85.00%	72.70%	100%	0.4736
csDMARDs number				0.4687
0	2 (10.0%)	2 (18.2%)	0 (0%)	
1	3 (15.0%)	2 (18.2%)	1 (11.1%)	
2	14 (70.0%)	6 (54.5%)	8 (88.9%)	
3	1 (5.0%)	1 (9.1%)	0 (0%)	
Steroids, %	12 (60.0%)	5 (45.5%)	7 (77.8%)	0.1968
Synovitis scores				0.0045
No synovitis (0-2)	12 (60.0%)	10 (90.9%)	2 (22.2%)	
Low synovitis (3-5)	4 (20.0%)	1 (9.1%)	3 (33.3%)	
High Synovitis (6-9)	4 (20.0%)	0 (0%)	4 (44.4%)	
Pathotypes				0.0009
Fibroid	11 (55.0%)	10 (90.9%)	1 (11.1%)	
Myeloid	5 (25.0%)	1 (9.1%)	4 (44.4%)	
Lymphoid	4 (20.0%)	0 (0%)	4 (44.4%)	

DAS28, Disease Activity Score 28 joints; csDMARDs, conventional synthetic Disease Modifying Anti-Rheumatic Drugs; ESR, Erythrocyte Sedimentation Rate; CRP, C Reactive Protein; ACPA, Anti Citrullinated Protein Antibodies, RF, Rheumatoid FactorVAS, Visuoanalog scale.
p, Fisher exact test or Mann-Whitney, as appropriate.

TABLE 3 | Multiple linear regression for 6 months DAS28.

Term	Estimate	Std error	Statistic	p.value	95% CI	
(Intercept)	-1.74	2.91	-0.59	0.56	-7.46	3.97
Mast cell presence	3.04	1.12	2.71	0.02	0.84	5.24
Lymphoid aggregates	-2.26	1.24	-1.81	0.10	-4.71	0.18
Age	0.03	0.02	1.53	0.15	-0.01	0.08
Body Mass Index	0.0	0.08	0.94	0.37	-0.08	0.23

were classified as lympho-myeloid positive/negative, based on the presence/absence of lympho-myeloid aggregates at 6 months, we did not observe significant differences in the number of patients reaching a low disease activity. Because of the small numbers, we can't exclude a type 2 error, thus we cannot exclude the association of lymphoid aggregates with treatment response. However, regression analyses suggested MC presence at 6 months is an independent predictor of disease activity, independently of lymphoid aggregates, after correction for BMI and age.

Overall, these observations are in line with our recent manuscript, showing that histologically defined lympho-myeloid patients are associated with worse disease activity at baseline and higher levels of radiographic progression at 12 months, but pathotypes at baseline do not associate with response to csDMARDs. On the contrary, reduction of lymphoid related genes assessed by molecular analysis of pre and post-treatment biopsies was associated with treatment response (Humby et al., 2019). However, also when considering the relatively low numbers of patients included in our current study, the relevance of other immune cells in addition to MCs can't be excluded, since it is possible that with a larger cohort other significant differences will emerge. At the same time, the identification of significant differences in such a small cohort points to the relevance of MCs, particularly when considering that their simple presence in synovia, independently from the degree of infiltration, is able to identify patients with higher disease severity. Nonetheless, additional work in larger cohorts is essential to confirm our observations and dissect the diverse contribution of MCs and other immune cells as markers of disease severity, progression and response to treatment. In fact, although a few study have looked at synovial membrane factors as predictors of response to csDMARDs (Vieira-Sousa, 2011) and at the effect of treatment on synovitis (Haringman, 2005), none to our knowledge include the analysis of MCs. In recent years, the attention has switched to biologic treatment, and a number of publications described both histological and molecular signatures that can predict treatment response to biologics (Dennis et al., 2014; Aterido et al., 2019). However, to date, the relevance of synovial MCs in relation to response to conventional or biologic DMARDs has not been explored.

In conclusion, in a small observational cohort of early RA patients with matched pre and post treatment synovial biopsies, we describe higher disease activity in association with the presence of synovial MCs at baseline and lower response to csDMARDs in patients with persistence of MC infiltration. Although additional studies in larger cohorts are needed to dissect the diverse contribution of mast cells and other immune cells, these results suggest that the analysis of synovial MCs contributes to the definition of the synovial inflammatory landscape.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Queen Mary University – REC 05/Q0703/198. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FR: study design, experiments, data acquisition, data analysis, manuscript preparation and revision. FWR: data analysis, manuscript preparation and revision. GG: data analysis, manuscript revision. FN: data analysis, manuscript revision. AP: interpretation of experimental results, manuscript revision. CP: study design, interpretation of experimental results, manuscript revision. FR wrote the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Physiology

BRIEF RESEARCH REPORT
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Relationship Between Plasma Osteopontin and Arginine Pathway Metabolites in Patients With Overt Coronary Artery Disease

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Introduction: Osteopontin (OPN) is involved in ectopic calcification. Its circulating form is upregulated in coronary artery disease (CAD) patients. Circulating OPN levels positively correlate with oxidative stress, one of the major triggers of endothelial dysfunction. Endothelial dysfunction is, in turn, associated with reduced nitric oxide (NO) bioavailability due to the impaired arginine pathway. The aim of this study was to better understand the correlations between OPN, oxidative stress markers, and the arginine pathway metabolites.

Methods and Results: ELISA and mass spectrometry techniques have been used to evaluate circulating OPN and arginine pathway/oxidative stress metabolites, respectively, in twenty-five control subjects and thirty-three patients with overt atherosclerosis. OPN positively correlates with 2,3-dinor-8isoPGF2a levels ($p = 0.02$), ornithine ($p = 0.01$), ADMA ($p = 0.001$), SDMA ($p = 0.03$), and citrulline ($p = 0.008$) levels only in CAD patients. In addition, citrulline positively correlated with ADMA ($p = 0.02$) levels, possibly as result of other sources of citrulline biosynthetic pathways.

Conclusion: The association between OPN and impaired arginine/NO pathway could play a role in the inhibition of endothelial NO synthase (eNOS) and/or in the arginase activation in the context of CAD patients. However, further studies are needed to verify the cause-effect relationship between OPN, oxidative stress, and arginine/NO pathway dysregulation.

Keywords: atherosclerosis, endothelial dysfunction, OPN, nitric oxide, citrulline

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INTRODUCTION

Osteopontin (OPN) is a phosphoglycoprotein secreted by different cellular types (monocytes, macrophages, cardiac fibroblasts, vascular smooth muscle cells, and endothelial cells), implicated in many molecular and cellular pathophysiological processes, including ectopic calcification (Cho and Kim, 2009). It has been shown that OPN plays an important role in the atherosclerotic plaque formation as well as in coronary artery diseases (CAD) (Wolak, 2014). In particular, several studies showed that circulating OPN levels are elevated in coronary artery disease (CAD) patients (Abdel-Azeez and Al-Zaky, 2010; Tousoulis et al., 2013; Wolak, 2014; Maniatis et al., 2019) and correlated with the disease extent and severity (Ohmori et al., 2003; Momiyama et al., 2010; Wolak, 2014). Indeed, circulating OPN has been proposed as a predictor of major cardiac events, such as acute myocardial infarction and ischemic heart disease (Georgiadou et al., 2010; Okyay et al., 2011). These observations, taken together with large literature evidences, corroborate the direct link between OPN and CAD development/progression (Wolak, 2014).

In addition, the upregulation of OPN transcription is also driven by oxidative stress (Branchetti et al., 2013) that represents one of the main initial atherosclerotic triggers, leading also to endothelial dysfunction (Incalza et al., 2018). Indeed, circulating OPN positively correlates with malondialdehyde levels, a recognized biomarker of oxidative stress (Cavalca et al., 2001; Georgiadou et al., 2008).

It has also been shown that increased levels of reactive oxygen species, in patients with CAD, lead to a progressive endothelial dysfunction (Incalza et al., 2018). Furthermore, it has been shown that endothelial vascular function impairment is associated with high OPN levels (Shemyakin et al., 2012; Schreier et al., 2016; Batko et al., 2019).

The endothelial dysfunction, among other causes, is associated with the impairment of the nitric oxide (NO) pathway, where the NO synthase (NOS) plays a pivotal role (Yang and Ming, 2013). NOS, using arginine as substrate, produces NO equimolarly to citrulline (Morris, 2007). Then, NO diffuses locally and mediates endothelium-dependent vasodilatation, acting on adhesion molecules and avoiding the infiltration of inflammatory cells and subsequent detrimental effects (Tousoulis et al., 2012). Undeniably, the reduction of NO bioavailability have a crucial importance in cardiovascular diseases (Cavalca et al., 2013; Eligini et al., 2013). Thus, in this study, we investigated the link between circulating OPN, oxidative stress, and endothelial dysfunction. We, therefore, performed an association study to explore the dysregulation of the arginine pathway and different oxidative stress markers in patients with overt CAD requiring surgical myocardial revascularization.

MATERIALS AND METHODS

Study Population

Thirty-three patients that underwent coronary artery bypass grafting (CABG) and twenty-five control subjects were enrolled in the study between January and June 2011 at Centro Cardiologico Monzino IRCCS. Pre-operative inclusion criteria were isolated surgical myocardial revascularization, elective surgery, age more than 18 years old, ejection fraction >30% and normal sinus rhythm. Exclusion criteria were prior cardiac surgery, rheumatic heart disease, endocarditis, active malignancy, chronic liver, and kidney diseases, calcium regulation disorders (hyperparathyroidism, hyperthyroidism and hypothyroidism) and chronic or acute inflammatory states (sepsis, autoimmune disease and inflammatory bowel disease). The Institutional Review Board and Ethical Committee of Centro Cardiologico Monzino (IRCCS) approved the study. Written informed consent to participate in this prospective observational study was obtained from all enrolled patients. The study protocol was conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Blood and Urine Sampling

Whole blood: 6 mL of peripheral blood sample was drawn from patients while fasting, into tubes containing EDTA (9.3 mM, Vacutainer Systems, Becton Dickinson, Franklin Lakes NJ, United States) kept on ice. 250 μL of whole blood was immediately precipitated with 250 μL of 10% trichloroacetic acid (Sigma-Aldrich, Darmstadt, Germany) plus 1 mM EDTA solution. Samples were stored at −80°C until analysis.

Plasma EDTA: anti-coagulated EDTA blood was centrifuged at 1700 g for 10 min at 4°C within 30 min after being drawn. Plasma was separated and aliquots were stored at −80°C until analysis.

Urine: urine collection was carried out the night before surgery or the night before the visit and samples stored at −80°C until analysis.

Osteopontin Evaluation

Plasma levels of soluble OPN were measured with an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine, R&D) following manufacturer instructions.

Oxidative Stress Markers Measurement

Reduced (GSH) and oxidized glutathione (GSSG) forms were determined in whole blood by liquid chromatography-tandem mass spectrometry (LC-MS/MS) method (Squellerio et al., 2012; Valerio et al., 2019). The separation of analytes was conducted on a Luna PFP analytical column (100 mm × 2.0 mm, 3 m, Phenomenex) maintained at 35°C under isocratic conditions (flow rate of 250 μL/min, mobile phase:1% methanol in 0.75 mM ammonium formate adjusted to pH 3.5 with formic acid). LC-MS/MS analysis was performed using an Accela HPLC (high performance liquid chromatography) system coupled with a triple quadrupole mass spectrometer TSQ Quantum Access (Thermo Fisher Scientific, Waltham, MA, United States) equipped with an electrospray ionization (ESI) source working in multiple reaction monitoring (MRM) and in positive ionization mode.

Data were obtained after comparison with calibration curves using GSH and GSSG pure standard solutions (Sigma-Aldrich, Darmstadt, Germany). The intra- and inter-CVs (%) obtained

with standard samples were <5% for both the analytes. The limits of detection were 0.031 μmol/L for GSH and 0.008 μmol/L for GSSG. Levels of GSH and GSSG were corrected for haemoglobin (Hb) and expressed as μ mol/g Hb.

Urinary 2,3-dinor-8isoPGF2a was detected by LC-MS/MS method according to Cavalca et al. (2010). The urinary concentration was calculated from the area ratio of the ion peaks of the 2,3-dinor-8isoPGF2a over the deuterated standard (8-iso-PGF2a-d4). The estimated values were corrected for the urinary creatinine levels and expressed as pg/mg of creatinine.

Arginine Pathway Analytes Measurement

The assessment of arginine, ornithine, citrulline, asymmetric dimethylarginines (ADMA), and symmetric dimethylarginine (SDMA) was performed by LC-MS/MS using a target metabolomic approach (Squellerio et al., 2011). Briefly, the chromatographic analysis was conducted on a Luna HILIC (hydrophilic interaction liquid chromatography) analytical column (50 mm × 2.0 mm, 3 μm, Phenomenex, Torrance, CA, United States). The mobile phases consisted of aqueous 1.5 mM ammonium formate (pH 3.2) (A) and 1.5 mM ammonium formate in acetonitrile/methanol (95.5:0.5, v/v) (pH 3.2) (B) at a flow rate of 250 μL/min. The mobile phase gradient ran from 10% A to 70% A over 7 min, from 70% A to 94.5% A over 2 min and was held at 94.5% A for 5 min, returning to 10% A over 2 min and held at 10% A for re-equilibration. The sample injection volume was 10 μL and the column temperature was set at 30°C. Total run time per sample, including column cleaning and re-equilibration, was 25 min. The mass spectrometric analysis was performed using a TSQ Quantum Access (Thermo Fisher Scientific, Waltham, MA, United States) triple quadrupole mass spectrometer equipped with ES) interface operating in MRM and positive ionization mode. The LOQ value is 0.25 M for all compounds, making this method suitable for the analysis of samples containing relatively low concentrations of the analytes, with a satisfactory precision as documented by the intra- and inter-day CVs of less than 10%. The method is linear in a wide range of concentrations (between 0 and 20 μM), with correlation coefficients greater than 0.99 and limit of detection (LOD) around 3–10 nm for all compounds. Global arginine bioavailability (GABR) was calculated as the ratio of arginine levels and the total amount of ornithine plus citrulline levels. GABR is an index of circulating arginine bioavailability associated with markers of endothelial dysfunction and increased risk of cardiovascular mortality (Morris et al., 2005; Sourij et al., 2011).

Statistical Analyses

Continuous variables were analyzed using Student's *T*-test and summarized as mean ± SD, while categorical ones were analyzed using Chi-square test and summarized as frequency (n) and percentage (%). Circulating biomarkers were analyzed by the Pearson product-moment correlation coefficient (r_p) and plotted using Graphpad Prism v7.0. A value of $p \leq 0.05$ was deemed statistically significant.

RESULTS

Demographic and clinical characteristics, as well as pharmacological therapies of the study population are listed in **Supplementary Table S1**. As previously reported by other authors (Tousoulis et al., 2013; Wolak, 2014; Maniatis et al., 2019), circulating OPN levels were lower in controls compared to the CAD patients (57.76 ± 9.8 vs 68.37 ± 24.2 pg/ml, respectively, $p = 0.04$, **Supplementary Figure S1**).

Osteopontin and Oxidative Stress

We assess the possible relationship between OPN levels and oxidative stress status, represented by 2,3-dinor-8isoPGF2a and the ratio between the reduced (GSH) and the oxidized (GSSG) forms of glutathione, in patients before the surgical intervention.

Linear regression analysis reported that there was no significant association between OPN levels and GSH/GSSG ratio, in controls ($r_p = 0.002$, $p = 0.99$, **Supplementary Figure S2A**), as well as in CAD patients ($r_p = -0.15$, $p = 0.42$, **Figure 1A**). The same analysis showed that there was no association between OPN levels and 2,3-dinor-8isoPGF2a urine levels in control group ($r_p = -0.38$, $p = 0.08$, **Supplementary Figure S2B**). However, OPN levels were directly correlated with 2,3-dinor-8isoPGF2a urine levels in CAD patients ($r_p = 0.42$, $p = 0.02$, **Figure 1B**).

Osteopontin and Arginine Pathway Metabolites

Since arginine is the substrate of NOS, we evaluated the metabolites involved in arginine pathway as representative molecules of NO production (Morris, 2007, 2016). In the control group, there were no correlations between the considered metabolites and circulating OPN levels (arginine, $r_p = -0.33$, $p = 0.11$, ornithine, $r_p = -0.04$, $p = 0.85$, citrulline, $r_p = 0.07$, $p = 0.75$, ADMA, $r_p = -0.30$, $p = 0.14$, SDMA, $r_p = -0.23$, $p = 0.28$, GABR, $r_p = 0.30$, $p = 0.14$, **Supplementary Figure S3**). In CAD patients, the linear regressions showed that OPN levels were not associated with arginine levels ($r_p = 0.20$, $p = 0.27$, **Figure 2A**) and the global arginine bioavailability (GABR, $r_p = -0.29$, $p = 0.11$, **Figure 2F**). However, OPN levels were positively correlated with ornithine ($r_p = 0.44$, $p = 0.01$, **Figure 2B**), ADMA ($r_p = 0.54$, $p = 0.001$, **Figure 2D**), and SDMA ($r_p = 0.37$, $p = 0.03$, **Figure 2E**) levels.

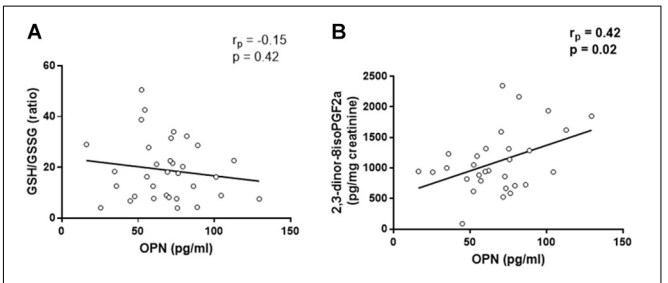

FIGURE 1 | Linear regression analyses between osteopontin (OPN) and GSH/GSSG ratio **(A)** and 2,3-dinor-8isoPGF2a **(B)**.

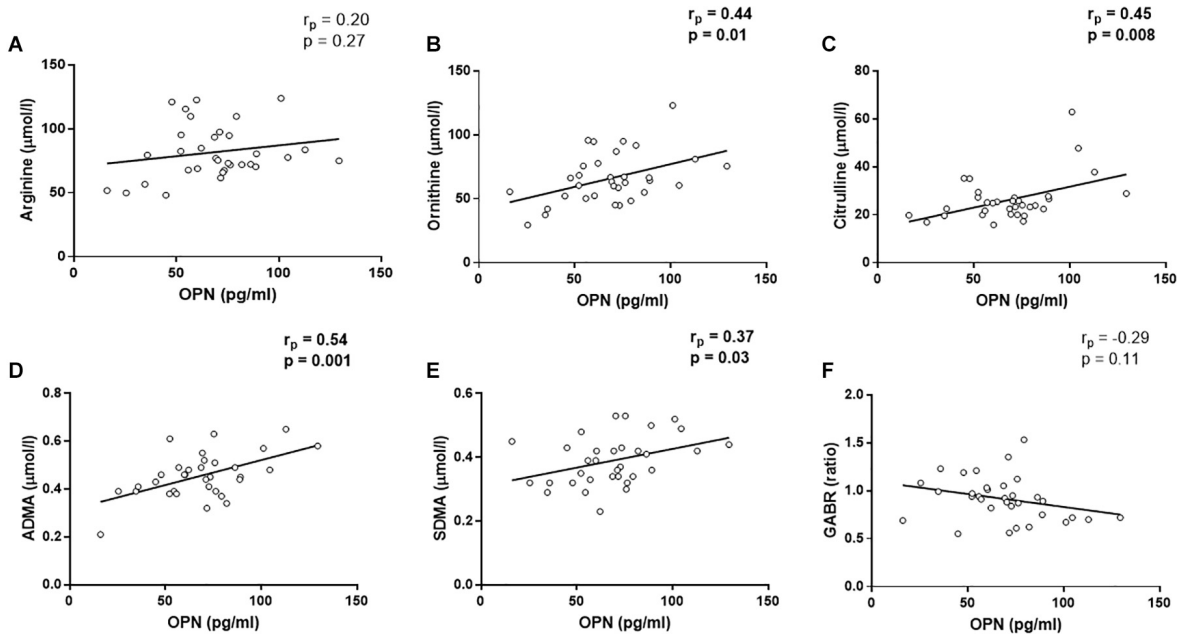

FIGURE 2 | Linear regression analyses between osteopontin (OPN) and arginine **(A)**, ornithine **(B)**, Citrulline **(C)**, asymmetric dimetilarginine [ADMA, **(D)**], symmetric dimethilarginine [SDMA, **(E)**], and global arginine bioavailability [GABR, **(F)**].

In addition, a positive correlation was found between OPN and citrulline (r_p = 0.45, p = 0.008, **Figure 2C**). Citrulline is known to be produced by (i) NOS from arginine, equimolarly with NO, (ii) ornithinetranscarbamilase (OTC) form ornithine, and (iii) dimethylarginine dimethylaminohydrolase (DDAH) from ADMA. In this regard, citrulline was not associated with ornithine (r_p = 0.18, p = 0.33, **Figure 3A**), although, we found that citrulline levels were associated with ADMA (r_p = 0.40, p = 0.02, **Figure 3B**) levels.

DISCUSSION

To our knowledge, we show for the first time that OPN could be linked to the pathological dysregulation of the arginine pathway in CAD patients.

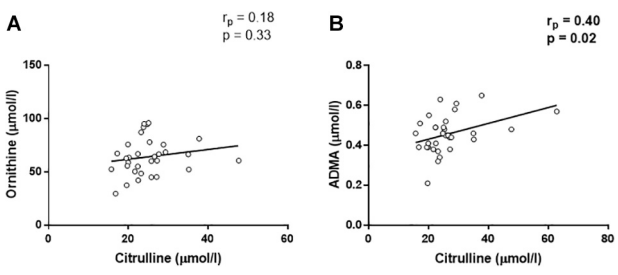

FIGURE 3 | Linear regression analyses between citrulline and ornithine **(A)** and asymmetric dimetilarginine [ADMA, **(B)**].

As largely reported before also CAD patients in our cohort showed high levels of circulating OPN. Nonetheless, CAD patients were characterized by an increased oxidative stress status and impaired endothelial function associated with a NO bioavailability reduction (Abdel-Azeez and Al-Zaky, 2010; Yang and Ming, 2013; Incalza et al., 2018). Recently some authors reported that high OPN levels could in some way interfere with vessel endothelial function (Shemyakin et al., 2012; Batko et al., 2019; Maniatis et al., 2019). – In this study, we investigated the relationship between OPN, oxidative stress, and endothelial dysfunction taking into account the arginine metabolism. In our cohort, plasma OPN levels correlated with urinary 2,3-dinor-8isoPGF2a, in agreement with literature evidences on the intensified production of OPN caused by an increased systemic oxidative stress status. However, we did not see any significant association between plasma OPN and GSH/GSSG ratio. These data suggest that lipid peroxidation may be the main process induced by the oxidative stress in the context of CAD, instead of protein oxidation. The link between OPN and lipid peroxidation is corroborated by the lack of any association between OPN and 2,3-dinor-8isoPGF2a in the control group.

It has been shown that OPN could interfere with the arginine pathway by inhibiting the inducible form of the NOS (iNOS) enzyme (Singh et al., 1995; Rabenstein et al., 2016). Thus, it is likely that the same mechanisms could cause an inhibition of endothelial NOS (eNOS) enzyme as a result of increased OPN levels. In this scenario, the reduction of NO synthesis, in combination with increased oxidative stress status, would favor the atherosclerotic milieu (Mahdi et al., 2019). For this purpose, we analyzed the metabolites of the arginine pathway both in controls and CAD patients. We found no correlation

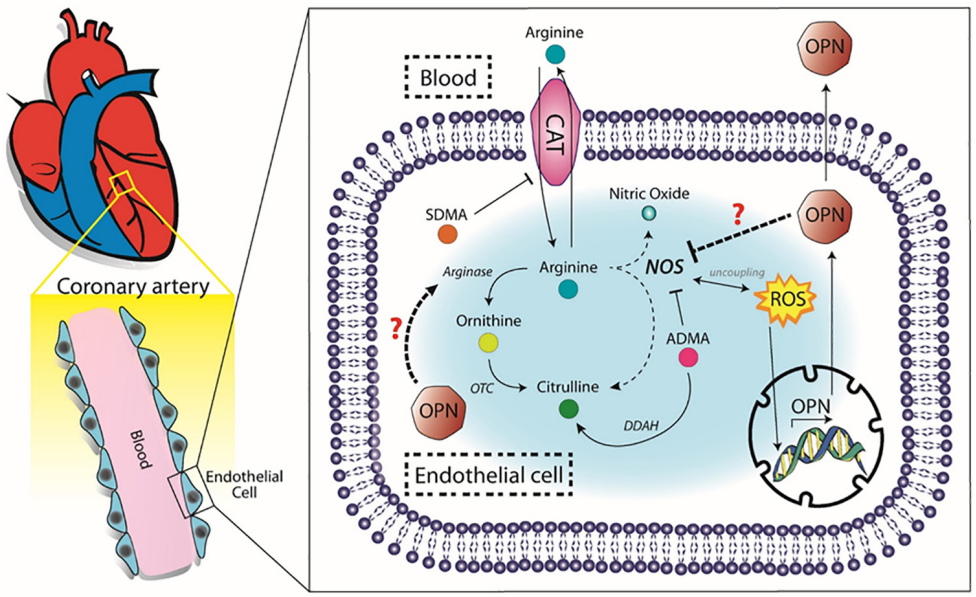

FIGURE 4 | Proposed molecular pathway. OPN, osteopontin, SDMA, symmetric dimethilarginine, ADMA, asymmetric dimetilarginine, CAT, cationic aminoacid transporter, OTC, ornithinetranscarbamilase, DDAH, dimethylarginine dimethylaminohydrolase, ROS, reactive oxygen species.

between OPN and any metabolite in control group, while in the in CAD patients, we found that OPN directly correlated with several metabolites belonging to the arginine pathway. Indeed, we found positive correlations between OPN, ADMA, SDMA, and ornithine. SDMA is not only an inhibitor of the arginine transporter CAT (Closs et al., 1997), but also a pro-inflammatory molecule (Chen et al., 2012), like OPN (Icer and Gezmen-Karadag, 2018). In the context of CAD, SDMA could play both roles acting in synergy with OPN in the development of the inflammation. However, we also observed positive correlations between citrulline and OPN. To explain this last correlation, we have to take into account that citrulline is normally produced equimolarly to NO from arginine by eNOS, but other sources of its production are known (Morris, 2007). In particular, citrulline could derive from ADMA by DDAH activity. Indeed, in our cohort we found a positive correlation between citrulline and ADMA, indicating that high levels of citrulline could be due to the activity of DDAH enzyme.

In 2012, Shemyakin et al. (2012) showed, in CAD patients, an improved endothelial functionality probably due to the inhibition of arginase. This evidence suggests that arginase activation reduces arginine bioavailability, thus NOS-mediated NO production, fundamental to maintain the endothelial function. Of notice, it has been reported a possible interaction between OPN and arginase (Partridge et al., 2008). Thus, it is likely that OPN could stimulate arginase activity in the CAD context.

We strongly believe that OPN could be directly or indirectly implicated in the decreased activity of eNOS in atherosclerosis, contributing to the endothelial dysfunction typically observed in CAD patients. We therefore propose a schematic view of the possible components that could link OPN to the arginine metabolism (**Figure 4**).

In summary, our results showed a correlation between OPN levels, oxidative stress status, and endothelial dysfunction markers in CAD patients. Nonetheless, further studies are required to determine if OPN really drives the endothelial dysfunction by direct or indirect eNOS inhibition in CAD patients. Endothelial cells from coronary artery, genetically modified to silence or overexpress OPN, could be the appropriate *in vitro* model to determine the functionality of the enzymes involved in the NO/arginine pathway. While OPN knockout mice would represent the best *in vivo* model to evaluate the relationship between the NO/arginine pathway and the OPN (Pedersen et al., 2013).

Limitations

This study has different limitations. First, we could not investigate the influence of each pharmacological treatment on the analyzed metabolites due to our small cohort. Second, we could not measure eNOS, arginase, and DDAH levels and activity. Third, although flow mediated dilation (FMD) is a recognized technique to assess endothelial dysfunction, we could not evaluate it given the status of our patients before surgery, as well as high number of drugs taken as per the 2019 European Society of Cardiology (ESC) guidelines for FMD evaluation (Thijssen et al., 2019). Lastly, we have not measured other common oxidative stress markers, such as malondialdehyde, since we wanted to investigate the glutathione system and the lipid peroxidation. Our study showed an association between OPN and endothelial dysfunction, however, further studies are necessary to prove the cause-effect relationship in CAD patients.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board and Ethical Committee of Centro Cardiologico Monzino (IRCCS). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PP and MD conceived the study. VM collected the informed consensus and the specimens. BP performed mass spectrometry evaluation. VA, VV, and IM performed the experimental evaluations. DM and PP performed statistical analyses and drafted the manuscript. GP prepared the illustration. MD, BP, GP, VV, VA, IM, AO, AD, VC, VM, and PS substantially revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00982/full#supplementary-material

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