CHAPTER TWO

# Mast Cells' Integrated Actions with Eosinophils and Fibroblasts in Allergic Inflammation: Implications for Therapy

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## Contents



## Abstract

Mast cells (MCs) and eosinophils (Eos) are the key players in the development of allergic inflammation (AI). Their cross-talk, named the Allergic Effector Unit (AEU), takes place through an array of soluble mediators and ligands/receptors interactions that enhance the functions of both the cells. One of the salient features of the AEU is the CD48/2B4 receptor/ligand binding complex. Furthermore, MCs and Eos have been demonstrated to play a role not only in AI but also in the modulation of its consequence, i.e., fibrosis/tissue remodeling, by directly influencing fibroblasts (FBs), the main target cells of these processes. In turn, FBs can regulate the survival, activity, and phenotype of both MCs and Eos. Therefore, a complex three players, MCs/Eos/FBs interaction, can take place in various stages of AI. The characterization of the soluble and physical mediated cross talk

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among these three cells might lead to the identification of both better and novel targets for the treatment of allergy and its tissue remodeling consequences.

## 1. INTRODUCTION

The term "allergy" was first used in 1906 by Von Pirquet. It derives from the Greek words "*allos*" (changed) and "*ergos*" (reaction) to describe the changes in the reactivity (Von Pirquet, 1946) or "uncommitted" biological responses of some individuals' immune system to certain substances in both protective immunity and hypersensitivity reactions (Kay, 2000). The development of specific signs and symptoms of reactivity is commonly referred to as "hypersensitivity reactions" and these individuals are described as atopic (Galli, Tsai, & Piliponsky, 2008). Over time, the meaning of the word changed and now allergy is frequently used synonymously for Immunoglobulin E (IgE) antibodies (Ab)-mediated allergic disease (Kay, 2001). The term allergic inflammation (AI) is used when it is important to stress the inflammatory course of the disease.

Atopy is an inherited disorder (Kjellman, 1977), usually attributed to an individual being prone to develop allergies because of a genetic state of hyperresponsiveness to otherwise harmless environmental substances (Galli et al., 2008). Atopic disorders encompass a vast range of common diseases such as allergic rhinitis, asthma, allergic conjunctivitis, food allergy, and atopic dermatitis (AD) (Anandan, Gupta, Simpson, Fischbacher, & Sheikh, 2009). Atopic response is defined by lymphocyte T-helper type 2 (Th2)-dependent immunological inflammation, where Th2-derived cytokines, including interleukin (IL)-4 and IL-13, lead to AI and the generation of allergen-specific IgE Ab (Rantala, Jaakkola, & Jaakkola, 2013). Atopic disorders are controlled by genetic factors (Chang, Wang, Chen, & Liu, 2012) balanced by environmental elements such as food and aeroallergen exposures, air pollution, and microbe infections (Hakimeh & Tripodi, 2013; Mbugi & Chilongola, 2010). Several studies relate asthma to susceptibility loci on chromosome 17q21 (genes for IL33, RAD50, IL1RL1, HLA-DQB1, DENND1B1, and IL2RB: Akhabir & Sandford, 2011) and on chromosome 20p (gene for ADAM33: Chi et al., 2013). Other chromosomes and genes which have been implicated in asthma are reviewed in Meng and Rosenwasser (2010). Therefore, no specific genetic marker for atopy has been described so far, leading to the conclusion that atopy is a polygenic disorder (Johansson et al., 2001).

Allergic diseases have recently increased in prevalence both in the Western world and in developing countries (Papadopoulos et al., 2012). The prevention of the initial development of AI might be achieved either through the generation of immunological tolerance against the allergens or through eventual manipulation of the immune response (Galli et al., 2008). Allergic symptoms and the underlying inflammation are commonly controlled in most patients. However, in contrast to the widespread belief that allergies are mild conditions, 15–20% of patients have a severe, debilitating disease and are under constant fear of death from a possible asthma attack or anaphylactic shock. Severe asthma together with AD is considered to be unmet clinical needs emphasizing the urgent necessity of therapeutics able to improve patient care, health care delivery, and disease prevention, as well as enhance the patient's quality of life (Papadopoulos et al., 2012).

AI is usually a two-phase process, the early and the late phases, characterized by peculiar aspects shared by all allergic diseases (Barnes, 2011). The early-phase response starts with the binding of specific allergen to IgE that are already attached to their high-affinity receptor FcεRI expressed on mast cells (MCs), which causes cross-linking of the receptor (Drinkwater et al., 2014). For decades, MCs and Eos have been regarded as the dominating cells in AI, MCs orchestrating the acute and Eos the late-phase and chronic outcome of the response (Galli et al., 2008). Nevertheless, many other inflammatory cells such as basophils, plasma cells secreting IgE, CD4+ Th2 cells (Moqbel & Odemuyiwa, 2008), neutrophils, monocytes/macrophages, CD8+ (cytotoxic) lymphocytes, invariant natural killer (NK) cells (Kara et al., 2014), and more recently ILC2 have also been implicated (Walker, Barlow, & McKenzie, 2013) in the development of allergy.

It is well accepted that activation of tissue-dwelling MCs caused by the allergen-induced complex of IgE bound to FcεRI (Drinkwater et al., 2014) results in their immediate degranulation and consequent release of an array of preformed and newly synthesized lipid mediators displaying mostly proinflammatory properties (summarized in Galli, Kalesnikoff, et al., 2005; Galli, Nakae, & Tsai, 2005; Mekori & Metcalfe, 2000; Minai-Fleminger & Levi-Schaffer, 2009; Sayed, Christy, Quirion, & Brown, 2008). Later on MCs produce and secrete a range of cytokines, chemokines, and growth factors that initiate and sustain the late-phase of allergy (Gri et al., 2012). During this phase, various inflammatory cells such as neutrophils, monocytes, basophils, Eos, and B and T lymphocytes are mobilized from the blood circulation into the site of inflammation and activated. In chronic conditions, these cells are also generated from the bone marrow precursors

(Ishmael, 2011). The Eos are the main "actors" of the late-phase of AI since they are classically increased in the peripheral blood (Wardlaw, Brightling, Green, Woltmann, & Pavord, 2000) and persist in the tissues longer than any other cell. Furthermore, their degranulation products including the granular basic proteins, several preformed cytokines, chemokines, and growth factors together with the newly synthesized lipid mediators have been shown to be correlated with the late/chronic symptoms of the allergic reaction (Munitz & Levi-Schaffer, 2004).

Cellular interactions of MCs with several other cells such as T cells, B cells, basophils, monocytes, neutrophils (summarized in Galli & Tsai, 2012), macrophages, dendritic cells and Eos with B cells (Wong, Doyle, Lee, & Jelinek, 2014), and platelets (Page & Pitchford, 2014) during the late and chronic phase might also be critical for the AI development (Lauzon-Joset, Marsolais, Langlois, & Bissonnette, 2014). Other cross-talks such as: airway epithelial cells/the underlying mesenchymal cells; MCs/Eos/FBs regulate the tissue remodeling in AI that has been well characterized in chronic asthma (Shifren, Witt, Christie, & Castro, 2012). Several of these cellular communications have been lately described for either mouse or human cells mostly *in vitro* and partially characterized (Galli et al., 2008).

Few years ago we put forward the hypothesis that a pivotal cross-talk could take place specifically between MCs and Eos in AI once the two cells are in the tissues (Minai-Fleminger & Levi-Schaffer, 2009; Piliponsky, Gleich, Bar, & Levi-Schaffer, 2002). This is because of peculiar aspects of MCs such as their reported long life, regenerative potential, and existence of a tissue precursor pool (Crapper & Schrader, 1983; Levi-Schaffer & Riesel, 1989), as well as of the Eos' continuous influx and prolonged life span in inflamed tissues (reviewed in Walsh, Stokes, & August, 2010). Bidirectional interaction between MCs and Eos, mediated by soluble mediators (Piliponsky, Gleich, Nagler, Bar, & Levi-Schaffer, 2003; Vliagoftis et al., 2004) and physical means (Elishmereni et al., 2011), has been investigated and described by us and termed the "Allergic Effector Unit" (AEU).

Moreover, MCs and Eos are central cells for the tissue remodeling consequences of AI, by interacting with various structural cells such as epithelial cells, endothelial cells, and smooth muscle cells and notably with the fibroblasts (FBs) that in turn also influence MCs and Eos behavior (reviewed in Bento & Hershenson, 1998; Vignola, Chanez, Bonsignore, Godard, & Bousquet, 2000). Therefore, another important cross-talk between MCs, Eos, and FBs takes place that influences not only tissue

remodeling but also the activity of MCs and Eos (Bainbridge, 2013). Notably, FBs produce a range of cytokines, chemokines, and lipid mediators (Xi et al., 2011) that contribute to the maintenance and activation of the inflammatory cells around the asthmatic airways, thus resulting in further chronicity/severity and tissue remodeling of the allergic disease (Meneghin & Hogaboam, 2007).

While some of the multiple aspects of MCs–Eos interactions and their cross-talk with FBs have been delineated, others are still awaiting further investigations. Nevertheless, it is evident that intervention in one or several of the various steps of these cross-talks might be important to prevent or at least ameliorate AI and its tissue remodeling outcome.

Here, we focus on the specific features of the interactions between MCs and Eos and of their cross-talk with FBs in the context of AI. We principally discuss human studies, especially those performed in asthma since more information has been accumulated in this disease than in the others, and will take into consideration supportive information from animal studies. Finally, we review some of the current therapeutic approaches that are being used or under contemplation for use to manage AI and its remodeling consequences.

## 2. MAST CELLS AND EOSINOPHILS IN ALLERGIC INFLAMMATION

MCs are highly granulated FcεRI bearing tissue-dwelling cells, which in humans develop from myeloid progenitors expressing CD34, CD117 (c-Kit), and CD13 (Gilfillan, Austin, & Metcalfe, 2011). MCs-committed progenitors are phenotypically $Lyn^-$ $cKit^+$ $Sca\text{-}1^-$ $Ly6c^-$ $Fc\varepsilon RI\alpha^-$ $CD27\text{-}\beta7^+$ and $T1/ST2^+$ in the bone marrow of adult mice (Chen, Grimbaldeston, Tsai, Weissman, & Galli, 2005). These progenitors disseminate the vascular space as mononuclear agranular cells and finally mature in peripheral tissues where they gain phenotypic diversity (Rodewald, Dessing, Dvorak, & Galli, 1996). MC maturation is exclusively dependent on Kit and influenced by stem cell factor (SCF) (Gilfillan et al., 2011). Bolstering the pivotal function of cKit/SCF for MCs, Kit-deficient mice ($Kit^{W\text{-}sh/W\text{-}sh}$ mice) have almost no MCs in their tissues (Katz & Austen, 2011). MCs are strategically localized near blood vessels, nerves, epithelia, and mucous membranes. They play crucial roles in host immune responses to pathogens, in inflammatory diseases, as well as in maintaining homeostasis during wound healing (Cemerski et al., 2012). In addition to allergy, MCs have been hypothesized to play a role in many inflammatory and fibrotic diseases

with various etiologies and in some instances it has been shown, but still much controversy exists regarding this issue (Rodewald & Feyerabend, 2012). In rodents, two major subclasses of MCs have been described based on the tissue in which they located: the connective tissue-type MCs (CTMCs) and the mucosal-type MCs differing in their immunocytochemical, biochemical, and functional characteristics (Rubinchik & Levi-Schaffer, 1994). In humans, MCs are defined according to their neutral protease content, i.e., respectively, MCs positive for tryptase and MCs containing both tryptase and chymase and carboxypeptidase A (Krishnaswamy, Ajitawi, & Chi, 2006; Pejler, Ronnberg, Waern, & Wernersson, 2010).

A hallmark characteristic of mature MCs is their intense staining with cationic dyes such as toluidine blue which detects stored proteoglycans carrying highly negatively charged sulfate groups on the glycosaminoglycan chains (Ronnberg, Melo, & Pejler, 2012). These negatively charged proteoglycans are vital for the storage of MCs mediators. The preformed MCs granule mediators also include histamine, an array of lysosomal enzymes, and the MCs-specific proteases (Pejler et al., 2010). MCs contain discrete amounts of preformed cytokines (Lundequist & Pejler, 2011), among them tumor necrosis factor (TNF-α) (Zhang et al., 2011) known to be stored as a preformed mediator uniquely by MCs (Olszewski, Groot, Dastych, & Knol, 2007). MCs synthesize in addition upon activation a number of cytokines, chemokines, and growth factors, i.e., IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-11, IL-13, GM-CSF, b-FGF, VEGF, and NGF (Bachelet, Levi-Schaffer, & Mekori, 2006; Bloemen et al., 2007; Prussin & Metcalfe, 2006) and with a fast kinetics lipid substances such as the arachidonic acid metabolites PGD2, LTB4, LTC4, HPETEs, HETEs, and others (Metcalfe, Baram, & Mekori, 1997). Histamine has been historically connected to MCs and basophils (being stored in these cells) whose pathophysiological effects, mediated through the four histamine receptors H1R–H4R, include the basis of many AI symptoms such as increased vascular permeability, smooth muscle contraction, and activation of nerves (Hallgren & Gurish, 2014). Since MCs express H2R and H4R, they can be affected by histamine themselves (Hallgren & Gurish, 2014) (various other MCs receptor has been reviewed in detail in Migalovich-Sheikhet, Friedman, Mankuta, & Levi-Schaffer, 2012). MCs homeostasis is controlled by IgE and the Th2-associated cytokines IL-4, IL-10, and TGF-β1, which modulate important effector proteins (e.g., c-Kit, FcεRI) in long-term MCs cultures (Ryan et al., 2007; Shelburne & Ryan, 2001). MCs can be activated

to degranulate and produce newly formed mediators by IgE-dependent and IgE-independent mechanisms (Brown, Wilson, & Metcalfe, 2008). IgE-independent mechanisms are transduced by MCs surface receptors unrelated to FcεRI, such as the ones for neuropeptides (i.e., substance P, neurotensin, and bradykinin), anaphylotoxin receptors (C3a and C5a), c-Kit receptor (SCF), adenosine receptors, Toll-like receptor (TLR), and scavenger receptors (Brown et al., 2008). We have recently found MCs to express other activating receptors (ARs) such as the CD48, belonging to the CD2 family and inhibitory receptors (IRs) such as CD300a and sialic acid-binding immunoglobulin-type lectin Siglec-7 of the immunoglobulin family. Other IRs are also functional on MCs (Karra & Levi-Schaffer, 2011). This would indicate a wider potential of MCs to respond to different stimuli. MC IgE-dependent activation, the classical one in starting allergy, is carried out through signal transduction initiated by phosphorylation of Lyn, Syk, and Fyn kinases from the Src family (Roth, Chen, & Lin, 2008).

Eos are bone marrow-derived granulocytes recruited from the peripheral blood to the inflamed tissue in parasitic helminthic infections (Klion & Nutman, 2004) and in allergic diseases (Martin, Kita, Leiferman, & Gleich, 1996). In addition, Eos have been implicated in several other conditions such as eosinophilic esophagitis, pulmonary hypertension, acute lung injury, endocarditis, and various solid cancers (Fukui et al., 2009; Handzel et al., 1998; Jacobsen, Helmers, Lee, & Lee, 2012; Lowe, Jorizzo, & Hutt, 1981). Eos differentiation from common myeloid progenitors (CMPs) is under the regulation of few key transcription factors including CCAAT/enhancer-binding protein (C/EBP family member), GATA-1 (a zinc finger family member), and PU.1 (an Ets family member) (Uhm, Kim, & Chung, 2012). CMPs, giving rise to Eos, commonly express IL-5Rα which can be considered the most specific and the earliest phenotypic marker acquired by this cell population at the commitment step of development pathway (Uhm et al., 2012) mediating Eos differentiation, maturation, survival, chemotaxis, and effector functions (Uhm et al., 2012). Eos are distinguished by their cytoplasmic crystalloid (also named secretory, specific, or secondary) granules storing diverse preformed cationic proteins such as Eos peroxidase (EPO), major basic protein (MBP), Eos cationic protein (ECP), and Eos-derived neurotoxin (EDN) (Muniz, Weller, & Neves, 2012). Basic mediators have toxicity to respiratory epithelial cells (Venge, Dahl, Fredens, & Peterson, 1988), may alter smooth muscle contraction (Coyle, Mitzner, & Irvin, 1993), and have the potential to promote the generation of reactive oxygen species, whose increase is associated with inflammation

(Dworski, 2000). Another population of small granules was initially reported (on the basis of their ultrastructure and cytochemically active arylsulfatase B activity) but are now believed to be vesicles derived from specific granules (Peter, 2013). Moreover, Eos both contain in a preformed fashion and can produce a large number of cytokines, chemokines, and growth factors (e.g., IL-4, IL-6, IL-8, IL-10, IL-13, GM-CSF, SCF, NGF, and TGF-β). Upon activation, Eos synthesize, as MCs do, several lipid mediators, i.e., PAF, LTC4, and PGE2 (Blanchard & Rothenberg, 2009).

Eos express receptors for IgA, IgE, IgG (Decot et al., 2005), for anaphylotoxins (Blom et al., 1998), and for chemokines such as CCR1 and CCR3 (Nagase et al., 2003). Furthermore, they display receptors for IL-3, IL-5, GM-CSF (Gregory et al., 2003), SCF (Yuan, Austen, Friend, Heidtman, & Boyce, 1997), IL-2 (Rand, Silberstein, Kornfeld, & Weller, 1991), IFN-γ (Neves et al., 2008), pattern recognition receptors belonging to TLRs, and NLR families (Kvarnhammar & Cardell, 2012). As for MCs also Eos express several ARs belonging to the CD2 family such as CD48, 2B4, CD58, and NTB-A (Munitz et al., 2005) and IRs such as CD300a, Siglec-7 and -8 (Munitz & Levi-Schaffer, 2007) and many others (summarized in Hogan, 2007; Rothenberg & Hogan, 2006) indicating the huge responsive potential of these cells not only in AI but also in other diseases. Various receptors expressing on Eos are described in detail in Driss, Legrand, and Capron (2013).

## 3. MAST CELL EOSINOPHIL CROSS-TALK: THE ALLERGIC EFFECTOR UNIT

Cells are known to communicate with each other through a variety of specific and nonspecific mechanisms. Cellular cross-talk can take place via released mediators through their specific receptors, physical contact mediated by ligand/receptor, and transfer of cellular contents from one cell to another cell (Lee et al., 2011; Rayner & Hennessy, 2013; Robbins & Morelli, 2014). The best example of a specific and therefore highly precise mechanism of cellular cross-talk is the immunological synapse among the interacting cells in the immune system, as demonstrated for T cells with antigen-presenting cells (APCs), and B cells and NK cells with the cells they recognize (Xie, Tato, & Davis, 2013).

It has been well accepted that a MCs–Eos cross-talk exists at the onset of AI when different MCs mediators cause the Eos tissue infiltration. IgE-activated MCs indeed release histamine, PGD2, and eotaxin that

recruit/activate Eos via the H4R, the chemoattractant receptor-homologous molecule on Th2 cells (CRTH2), and the chemokine receptor CCR3, respectively (Das, Flower, & Perretti, 1998; Hirai et al., 2001; Ling et al., 2004; Minai-Fleminger & Levi-Schaffer, 2009). Furthermore, the MCs mediators such as LTB4, LTC4, and LTE4 have been reported also to function as potent chemoattractants for Eos, promoting Eos adhesion and inducing IL-8 production (reviewed in Minai-Fleminger & Levi-Schaffer, 2009). MCs chymase was reported to induce Eos chemokinetic migration (CXCL8, CCL2, and CXCL1) and activation (release of IL-6) and to inhibit the Eos apoptosis (Wong, Ng, Lun, Cao, & Lam, Lam, 2009).

However, the interaction of MCs with Eos might take place not only in the recruitment phase of Eos but also afterward when Eos have reached the inflamed tissue where MCs reside. These interactions can be mediated both via paracrine mechanisms and via physical cell–cell contacts in addition to other communications means as described above (Fig. 1).

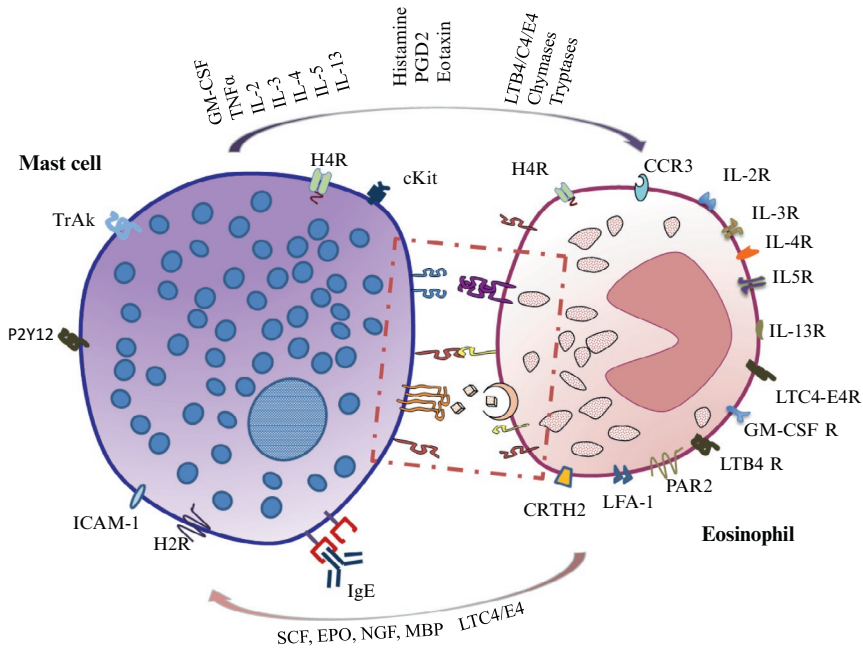


**Figure 1** The human Allergic Effector Unit (AEU). MCs and Eos cross talk is illustrated via both soluble mediators and respective receptors, and in the dotted box via receptor/ligand interaction shown in detail in Fig. 2. The mediators/receptors shown here are the ones characterized from our and other researchers' work in which a clear activity has been found in the frame of human MCs/Eos interaction.

### 3.1. Soluble interactions

MCs and Eos have been clearly shown to interact via soluble mediators (Fig. 1) with initial studies having been carried out to understand this phenomenon by adding soluble mediators from either MCs [primary MCs derived from mouse, rat, human tissues, human leukemic cell lines (HMC-1), mouse bone marrow-derived MCs (BMMCs), human cord blood derived MCs (CBMCs)] to Eos [derived from mouse bone marrow (BMEos), human peripheral blood Eos (pbEos), Eol-1 cell lines] or vice versa. Further to delineate the mechanism of these interactions, cellular sonicates containing all the preformed mediators were used followed more recently by cocultures of these two cell types. This last option is the best one since it better mimics the *in vivo* condition.

MCs synthesize GM-CSF, IL-3, and IL-5 that, as mentioned above, are responsible for survival, growth, differentiation, and activation of the Eos (reviewed in Shakoory, Fitzgerald, Lee, Chi, & Krishnaswamy, 2004). Freshly isolated human pbEos were incubated with either rat peritoneal or HMC-1 sonicates (Levi-Schaffer, Temkin, Malamud, Feld, & Zilberman, 1998; Temkin, Kantor, Weg, Hartman, & Levi-Schaffer, 2002), after 3 days, MCs significantly enhanced Eos survival. This was specifically attributable to stored MCs products (Levi-Schaffer et al., 1998). Among these, MCs TNF-α has been reported to inhibit apoptosis, prolong Eos survival, and induce Eos autocrine production of GM-CSF (Levi-Schaffer et al., 1998) through a mechanism involving nuclear factor-kappa B (Temkin & Levi-Schaffer, 2001). The contribution of TNF-α was demonstrated using neutralizing Ab to GM-CSF (Levi-Schaffer et al., 1998), and antagonist Ab to TNF-RI and TNF-RII (Temkin & Levi-Schaffer, 2001). They all substantially decreased the enhancing effect on Eos viability. Additionally, MCs sonicate also caused Eos to display morphologic signs of activation (Levi-Schaffer et al., 1998). Furthermore, the proteomic pattern of Eos incubated with MCs, TNF-α, or GM-CSF was evaluated. A strong proteomic pattern was expressed by the Eos GM-CSF providing the strongest signal and the highest rate of protein synthesis followed by TNF-α and HMC-1 sonicate indicating that the importance of the diverse mechanisms Eos can use to respond according to the stimulus they receive (Levi-Schaffer et al., 2002). Other authors have shown that human Eos incubated with activated human lung MCs supernatant release significant amount of ECP. The activation of Eos in these assays was inhibited by anti-IL-5, anti-TNF-α, and anti-GM-CSF neutralizing monoclonal Ab (mAb), emphasizing that MCs generation of such mediators as being an activatory signals for Eos (Okayama et al., 1997).

In other experiments, Eos activation carried out by both types of MCs sonicates occurred through β-tryptase, which probably cleaved PAR-2, and mediated the generation and release of IL-6 and IL-8 by Eos through MAPK/AP-1 pathways activation (Temkin et al., 2004, 2002). Other experiments showed that MCs tryptase can activate Eos to release EPO and β-hexosaminidase (Wong et al., 2009).

Eos were incubated with major MCs mediators (PGD2, LTB4, PAF, histamine, LTC4, IL-4, IL-5, IL-8, TNF, and GM-CSF), and in this study, it was observed that LTB4 and PAF were the only MCs products capable of inducing ECP release from Eos and an increase in $[Ca^{2+}]$ (Takafuji, Tadokoro, Ito, & Nakagawa, 1998). Interestingly, histamine has been found in another study to inhibit human Eos degranulation as assessed by EPO release. This result was attributed to the stimulation of HR2 (Ezeamuzie & Philips, 2000).

In addition to *in vitro* experiments to further assess the influence of MCs mediators on Eos functions, *in vivo* models of allergic diseases were performed. TNF-α was reported to be involved in Eos accumulation and reconfirmed to cause inflammatory mediators' release in a murine model of allergic peritonitis (Temkin, Pickholtz, & Levi-Schaffer, 2003). In peritoneal lavages, Temkin et al. (2003) found increased levels of TNF-α 1 h after WT mice challenge and consequent eosinophilia and EPO release 3 days later. In this model, the early elevation of TNF-α that happened concomitantly with histamine emphasized the functional correlation between MCs activation and Eos chemotaxis during the acute phase of allergy (Temkin et al., 2003).

Regarding the influence of Eos on MCs, it is noteworthy that Eos produce SCF (Bischoff & Dahinden, 1992; Dastych & Metcalfe, 1994; Hartman, Piliponsky, Temkin, & Levi-Schaffer, 2001; Meininger et al., 1992). SCF colocalization with MBP in pbEos suggested that its release, together with MBP and other granule-associated mediators, occurs in a prompt fashion upon Eos degranulation (Hartman et al., 2001). Moreover, Eos are capable of promoting MCs survival through their production of NGF (Solomon et al., 1998) via TrkA receptor (Hermes et al., 2001; Tam et al., 1997).

Bidirectional interactions between MCs and Eos comprise the influence that Eos have on MCs degranulation (Kaneko et al., 2009). As cited above, Eos are known to be a main source of LTC4, LTD4, and LTE4 (Bandeira-Melo, Bozza, & Weller, 2002; Bandeira-Melo & Weller, 2003). These potent cysteinyl leukotrienes have been shown to induce the activation of

BMMCs and since the same receptors are also present on BMMCs surface, this shows an autocrine signaling modality (Kaneko et al., 2009).

The group of Marone demonstrated that human heart MCs when incubated with ECP and MBP (Patella et al., 1996; Piliponsky et al., 2003; Zheutlin, Ackerman, Gleich, & Thomas, 1984) are activated to release histamine, tryptase, and PGD2.

Based on the observation that after an allergic inflammatory response, infiltrating Eos would come across MCs that previously underwent IgE-dependent activation, Piliponsky, Pickholtz, Gleich, and Levi-Schaffer (1999) cocultured rat peritoneal MCs with 3T3 FBs incubated with either sonicates of freshly isolated pbEos or with purified Eos mediators such as MBP, EPO, and EDN. In these experiments, it was shown that IgE-dependent activated MCs are still responsive to Eos sonicates and MBP indicating that in late-phase even in the absence of allergen, MCs/Eos interactions can result in the release of significant amounts of histamine and PGD2 (Piliponsky et al., 1999). MCs non-IgE-dependent activation mechanisms were also evaluated. It was found that freshly isolated highly purified human lung MCs or CBMCs (unresponsive to non-IgE-dependent activation) release histamine and PGD2 upon incubation with MBP only if they have been previously cocultured with human lung FBs displaying membrane-bound SCF (Piliponsky et al., 2003). Recently, the mechanism of MBP-induced activation of CBMCs has been identified as a result of MBP1–integrin β1 interaction on the MCs (Ben-Zimra, Bachelet, Seaf, Gleich, & Levi-Schaffer, 2013). Specifically, FBs-derived membranes prime CBMCs via a G-protein leading to upregulation in Hck protein level (Ben-Zimra et al., 2013). This study points out the interactions between MCs and Eos via novel described mechanisms not necessarily involving the IgE-dependent one that might be of pivotal importance in the late/chronic stages of AI and not well controlled by the novel anti-IgE therapies (see below).

## 3.2. Physical interactions

Our recent discovery that both human MCs and Eos possess many receptor/ligand couples (Fig. 2) has enabled us to hypothesize that these two cells could interact by physical binding and not only via soluble/released mediators. Surface molecules that might be implicated in the MCs–Eos contact and activation mechanism are the adhesion molecules ICAM-1 and LFA-1 and a number of AR/ligand couples of the CD2-family such as CD48 and its high-affinity ligand 2B4 (cited above) and DNAM-1, and Nectin-2. Of particular interest is 2B4 on Eos (Munitz et al., 2005) which functions

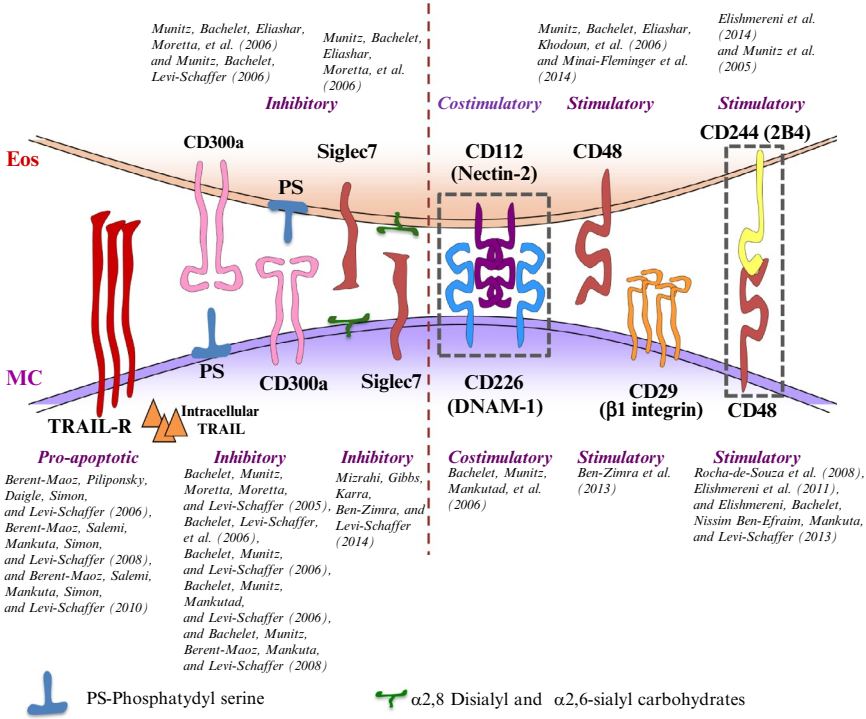


**Figure 2** Receptors/ligands in the AEU. MCs (down) and Eos (up) showing receptor/ligand interactions in the AEU. Receptors/ligands that might contribute to inhibitory functions/signals are on the left side and those that we have demonstrated to be responsible for stimulatory/costimulatory functions in the AEU are depicted on right side. The receptor ligand pairs that are boxed with dotted lines (CD48/2B4 and CD226/CD112) have a demonstrated function in binding between MCs and Eos and/or their activation. Other various receptors/ligands that are expressed on MCs and Eos are described in detail in Migalovich-Sheikhet et al. (2012) and Driss et al. (2013), respectively. (Berent-Maoz et al., 2006, 2010; Minai-Fleminger et al., 2014; Mizrahi et al., 2014; Munitz, Bachelet, Eliashar, Khodoun, et al., 2006; Munitz, Bachelet, & Levi-Schaffer, 2006).

as an activation molecule and its high-affinity ligand CD48, which is also a stimulating molecule expressed on these cells (Malaviya, Gao, Thankavel, van der Merwe, & Abraham, 1999; Malaviya & Georges, 2002; Minai-Fleminger & Levi-Schaffer, 2009). CD48 is also present on the membrane of CBMCs and was shown to be upregulated by invasive *Staphylococcus aureus* which, once internalized, also causes CBMCs to release TNF-α and IL-8 (Rocha-de-Souza, Berent-Maoz, Mankuta, Moses, & Levi-Schaffer, 2008). Through the CD48–2B4 pathway, MCs can bind to Eos and activate them to release EPO, IFN-γ, and IL-4 (reviewed in Minai-Fleminger &

Levi-Schaffer, 2009). While the expression of the CD48 molecule has been detected on both Eos and MCs, no 2B4 has been detected on human MCs (reviewed in Minai-Fleminger & Levi-Schaffer, 2009). Besides the CD48–2B4 axis, DNAM-1, together with its ligand Nectin-2 (that are highly expressed on MCs and Eos, respectively), enhances FcεRI-mediated activation of MCs and the modulation of the allergic response via MCs–Eos interaction (Bachelet, Munitz, Mankutad, et al., 2006). The DNAM-1/Nectin-2 interaction was found to contribute to MCs–Eos interface as a costimulatory signaling mechanism (Bachelet, Munitz, Mankutad, et al., 2006).

The leukocyte function-associated antigen 1 (LFA-1) molecule is a surface protein expressed by Eos and involved in cellular adhesion and interaction (Wacholtz, Patel, & Lipsky, 1989), which has been postulated to be part of an MCs activation pathway in the presence of activated Eos (Forbes et al., 2006; Inamura, Mekori, Bhattacharyya, Bianchine, & Metcalfe, 1998). LFA-1 is a ligand for ICAM-1 (CD54) receptor expressed on murine peritoneal and human uterine MCs (Forbes et al., 2006; Fox, Jewell, & Whitacre, 1994; Guo, Kagey-Sobotka, Lichtenstein, & Bochner, 1992; Inamura et al., 1998) whose activation had previously resulted in the recruitment of Eos to the site of inflammation.

It must be taken into account that, as described above, in addition to ARs and IRs, the death receptors and TNF-α-related apoptosis-inducing ligand (TRAIL) and their ligands are also present and functional on MCs (Karra & Levi-Schaffer, 2011) and Eos (Munitz & Levi-Schaffer, 2007). This is in spite of the activating phenotype that we have found so far in the AEU as described below.

We first looked for *in vivo* evidence of MCs–Eos couple formation. Several MCs–Eos pairs were indeed detected in allergic inflamed tissues such as human nasal polyps and asthmatic bronchi, as well as in mouse AD. Interestingly, in some of the couples, cells look degranulated (Elishmereni et al., 2011). Previously, *in vivo* evidence of coupled MCs and Eos had been reported in gastric carcinomas (Caruso, Fedele, Zuccala, Fracassi, & Venuti, 2007) but to the best of our knowledge not in AI. Since these phenomena were observed *in vivo*, we proceeded to characterize the AEU formation in an *in vitro* system using either murine or human cells. CBMCs were cocultured with pbEos for 1 h and the formation of heterodimeric couples was monitored. According to this, a well-defined interface between MCs and Eos was observed within 5 min and contacts lasted for about 3–4 min (Elishmereni et al., 2011). CBMCs and pbEos in coculture for

60 min were analyzed through transmission electron micrograph (TEM) (Minai-Fleminger et al., 2010) and interacting cell types were distinguished by their unique morphology as clearly linked to each other by "synapse-like" structures (Minai-Fleminger et al., 2010). In these TEM studies, it was noticed that interaction modified the morphology of both the cells since cocultured MCs exhibited electron-dense lipid bodies and Eos significantly expressed more vacuoles (Minai-Fleminger et al., 2010).

Interestingly, the transfer of EPO from Eos to MCs and tryptase from MCs to Eos has been also observed (Minai-Fleminger et al., 2010). Importantly, we found the role of CD48 on MCs and 2B4 on Eos to be strongly correlated to the interaction between the two cells.

The hypothesis that such communication might have an influence on cell survival was investigated. For this purpose, MCs and Eos were cocultured for up to 1 week with or without GM-CSF and SCF (Elishmereni et al., 2011). After 72 h, Eos were reported to be more viable in cocultures with MCs in SCF alone compared to GM-CSF containing monocultured Eos. Results indicated that while in the soluble route GM-CSF and TNF-α are involved in Eos survival, Eos increase in survival in transwell cocultures was solely associated to GM-CSF and partially mediated by physical communication between the two cell types (Elishmereni et al., 2011).

Our results confirmed that activation of Eos survival was achieved by mAb directed to either CD48 or 2B4 in monocultures and that the MCs induced rise in Eos viability in cocultures was partially reduced by blockade of 2B4 (Elishmereni et al., 2011). Therefore, the cocultured Eos are more viable in the physical cross-talk due to activation of 2B4 by CD48 on MCs and/or of the CD48 receptor by a yet unidentified MCs ligand (Elishmereni et al., 2011). Furthermore, human and murine MCs and Eos were cocultured under various conditions (1–2 h or 1–3 days) and it was found that the MCs–Eos couples, activated via 2B4–CD48 contact, degranulated and released β-hex, tryptase, EPO, and TNFα (Elishmereni et al., 2013).

The opposite route of MCs-induced Eos degranulation does not seem to need CD48–2B4 contact (Elishmereni et al., 2013) since blocking CD48 or 2B4 did not significantly decrease EPO release by the Eos. This might suggest that either other receptors/ligands are involved or that Eos activation is mostly under the control of soluble MCs-derived mediators. In this case, Eos-induced activation of MCs was shown through augmented release of tryptase and β-hex from BMMCs (Elishmereni et al., 2013).

In long-term cocultures (1–3 days), as a direct consequence of Eos–MCs contact, different changes in Eos features were reported. These

include an enhanced expression of ICAM-1 in Eos and increased phosphorylation of activation-associated signaling molecules, and enhanced release of TNF-α (Elishmereni et al., 2013). Recently, we found a similar AEU in the mouse system. BMMCs and BMEos cocultures resulted in couple formation, Eos increased survival, and both cells increased in activation. Moreover, BMMCs cocultured with BMEos obtained from 2B4 KO mice, where CD48/2B4 signaling is abolished, were less activated by IgE-dependent mechanism (Elishmereni et al., 2014). In the skin of a murine model of AD in 2B4 KO mice, it was found that significantly less MCs/Eos units were present and the disease was greatly reduced (Elishmereni et al., 2014). Taken together, all the above emphasize that the AEU is functional in maintaining Eos viability, promoting activation which was further shown by morphological changes occurring for both the cells, and making a critical contribution to the perpetuation of the inflammatory response in allergic conditions (Elishmereni et al., 2013) through both cells' activation.

Finally, it should be stressed again that we cannot rule out that at some later time point a downregulation of inflammation can start and resolution phenotype can take place (Karra, Haworth, Priluck, Levy, & Levi-Schaffer (*Accepted*)).

This, for example, might occur through the stimulation of the IRs. CD300a is constitutively expressed by both MCs (Bachelet et al., 2005) and Eos (Munitz, Bachelet, Eliashar, Moretta, et al. (2006)). Cross-linking of CD300a molecules by the specific anti-CD300a mAb on MCs surface resulted in the inhibition of IgE-induced degranulation and SCF-mediated survival through a mechanism involving tyrosine phosphorylation, phosphatase recruitment, and termination of cellular calcium influx (Bachelet et al., 2005). *In vitro* experiments on human pbEos showed that activation of CD300a abolished Eos activation and survival in response to IL-5 and GM-CSF (Munitz, Bachelet, Eliashar, Moretta, et al. (2006)). *In vivo* CD300a on MCs and Eos might be activated via the ligand phosphatidylserine (PS) that can be expressed by activated MCs (Simhadri et al., 2012) and by apoptotic Eos, respectively (Kankaanranta et al., 2014). Siglec-8 which has been investigated *in vitro* and *in vivo* on human Eos and MCs has been identified to cause selective apoptosis of Eos once stimulated, whereas *in vitro* engagement on human MCs inhibited degranulation (Bochner, 2009). MCs express TRAIL-R that we have shown to transduce proapoptotic signals (Berent-Maoz et al., 2008). They also contain TRAIL ligand (Berent-Maoz et al., 2008). Whether Eos express a functioning proapoptotic TRAIL-R1 is still unclear.

It is evident that the activating/inhibitory phenotype of the AEU needs still to be thoroughly investigated since many players besides AR and IR and their ligands can have different roles at different kinetics.

## 4. FIBROBLASTS FROM REPAIR TO FIBROSIS IN ALLERGIC INFLAMMATION

FBs are the fundamental cellular constituents of connective tissues, being located in virtually every tissue and organ and considered to be a cardinal and dynamic component of tissue biology (reviewed in Sorrell & Caplan, 2009). This heterogeneous population (Driskell et al., 2013) synthesizes and secretes structural (collagen and fibronectin, proteoglycans, etc.) and nonstructural (thrombospondins, osteopontin, etc.) extracellular matrix (ECM) molecules (Sorrell & Caplan, 2009). Moreover, through the generation of metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) and communication with nearby cells, FBs actively coordinate and remodel the ECM (Fig. 3) (Sorrell & Caplan, 2009). FBs express specific markers such as vimentin (Camelliti, Borg, & Kohl, 2005) and fibroblast-specific protein 1 (Souders, Bowers, & Baudino, 2009) and a vast range of receptors such as tyrosine kinase receptor for PDGF (Donovan, Shiwen, Norman, & Abraham, 2013), for 5-lipoxygenase, the terminal enzymes leukotriene $A_4$ hydrolase and leukotriene $C_4$ synthase, and receptors for leukotriene $B_4$ (BLT1) and cysteinyl-leukotrienes ($CysLT_1$) (James, Penrose, Cazaly, Holgate, & Sampson, 2006). Physiologically, tissue-resident FBs are in an inert state, although they fully exert their role of promoting the form and function of the organ they are located in under both metabolic and biomechanical points of view (Wynn & Ramalingam, 2012). To repair, regenerate, and restore homeostasis after injury, FBs proliferate and change into the activated form of myofibroblasts (MyoFBs) which express α-smooth-muscle actin (Hirai et al., 2014) and myosin bundles (Wynn & Ramalingam, 2012). In addition, MyoFBs have a highly contractile capacity (Kohan, Muro, White, & Berkman, 2010) that is fundamental in wound contraction (Baum & Duffy, 2011) and secrete abundant amounts of ECM making them vital players in the granulation tissue of the wound, aiding in contracture and closure, and orchestrating many aspects of the healing response (Wynn & Ramalingam, 2012).

MyoFBs are capable of producing higher levels of ECM-degrading proteases such as MMP2, MMP3, MMP9, and MMP14 (Ma et al., 2014) which contribute to ECM turnover and modification (summarized in Kalluri & Zeisberg, 2006) giving rise to tissue remodeling (Abel & Vliagoftis,

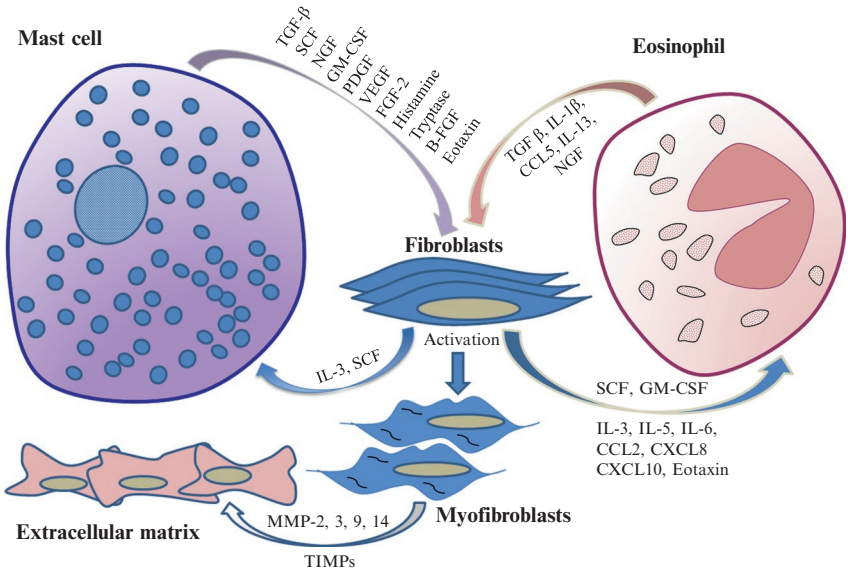


**Figure 3** Fibroblasts' bidirectional interaction with MCs and Eos in AI. The bidirectional interactions that have been described to take place between MCs, Eos, and FBs mediated via soluble factors are shown. FBs are influencing MCs and Eos survival and activation, and these two cells induce FBs activation steps that bring about the development of tissue remodeling/fibrosis.

2008). Numerous secreted, soluble, and physical factors in the milieu support MyoFBs activation, proliferation, and survival, such as cytokines (IL-1, TNF, TGF-β1 (Midgley et al., 2013), and IL-13), growth factors (CTGF and PDGF) and matrix factors (hyaluronan fragments, HA synthase-2 (Li et al., 2011)), and mechanical stress and/or stiffness (Wynn & Ramalingam, 2012). During normal wound healing, when the tissue is repaired α-SMA expression is diminished and MyoFBs disappear by apoptosis (Hinz, 2007). In pathological wound healing, MyoFBs are known to be resistant to programmed cell death (Hinz, 2007). Their perpetuating activity contributes to overexuberant formation of fibrous connective tissue which impairs tissue architecture and function, eventually resulting in aberrant wound-healing mechanisms representing the main components of the pathogenesis of fibrosis (Sakai & Tager, 2013). Evidences of such a mechanism were reported in biopsies of asthmatic patients characterized by increased numbers of FBs–MyoFBs which might be attributed to both the growth and chemotaxis of these cells to the site of AI (Smith & Levi-Schaffer, 2000) as well as higher counts of MyoFBs in the bronchial mucosa compared to healthy individuals (Smith & Levi-Schaffer, 2000). In distinct fibrotic

diseases, such as idiopathic pulmonary fibrosis (IPF), both FBs and MyoFBs accumulate in "fibroblastic foci" in lungs, where an excess production of the ECM takes place (Sakai & Tager, 2013). It is possible that in AI especially in late-phase and chronic disease, MCs and Eos and AEU continuous activation fuel the necessary growth factors for the survival and activation of the MyoFBs as described below.

Indeed, MCs and Eos have been hypothesized and shown to be directly involved in modulation of fibrotic processes. In particular, *in vitro* data have provided clear-cut evidence for these two inflammatory cells in increasing proliferation, and either augmenting or reducing collagen synthesis in human FBs from different anatomical locations (Levi-Schaffer & Weg, 1997). For example *in vivo* studies in murine and human cGVHD, the activation of MCs has been shown to have profibrotic effects, suggesting that MCs stabilization therapy might be relevant in controlling the disease. Specifically, Nedocromil ameliorated the skin features of cGVHD as shown in a murine model (Levi-Schaffer, Goldenhersh, Segal, & Nagler, 1997).

Regarding Eos, their contribution to the development of tissue remodeling and fibrosis is widely acknowledged (Ackerman, 2013). Studies using two strains of Eos-deficient mice (PHIL and ΔdblGATA) strongly reinforce the consideration that Eos contribute to the pathology of airway remodeling in asthma (Ackerman, 2013). In addition to what has been affirmed in mouse models, clinical trials using anti-IL-5 Ab to abolish Eos in bone marrow, blood, and tissues of patients with eosinophilic asthma successfully reversed some features of Eos-mediated tissue damage, remodeling, fibrosis, and airway dysfunction and pathologies associated with the hypereosinophilic syndrome (Ackerman, 2013).

Since Eos activation is usually subsequent to MCs engagement, a proper intervention on the MCs and Eos activation might have a prophylactic/therapeutic role in the very early development of fibrotic processes as described below.

## 5. MAST CELLS AND FIBROBLASTS: BIDIRECTIONAL INTERACTIONS

In 1879, Ehrlich described the disappearance of MCs in acute inflammation and a concomitant increase in the number of these cells with the presence of increased FBs (Wagner, Edwards, Moncrieff, & Wagner, 1984). MC infiltration and number are found to be directly associated with degree of fibrosis as observed in various disorders (Grizzi et al., 2013;

Hiromura, Kurosawa, Yano, & Naruse, 1998; Konttinen et al., 2000). MCs play a role in the pathogenesis of fibrotic lung diseases since elevated numbers of MCs have been counted in the lungs of patients with sarcoidosis (Ohrn et al., 1995), cryptogenic organizing pneumonia, hypersensitivity pneumonitis (Schildge, Klar, & Hardung-Backes, 2003), and IPF (Wygrecka et al., 2013).

The presence of FBs and specifically of MyoFBs has been detected in inflammatory tissues in asthmatic patients where the AEU takes place (Smith & Levi-Schaffer, 2000). Therefore, it can be postulated that MCs–Eos interactions are one of the ways to promote the activation of FBs leading to fibrotic events. In homeostatic conditions, MCs histamine, proteases, lipid mediators, and specific growth factors, such as TGF-β, SCF, NGF, GM-CSF, PDGF, VEGF, and FGF-2, influence different stages of wound healing (Fig. 3) (Artuc, Steckelings, & Henz, 2002) directly promoting growth and differentiation of FBs (Fig 3), keratinocytes, and other resident cells (Artuc et al., 2002). A similar scenario can take place in allergic diseases.

To assess the specific influence of MCs in fibrosis, studies were carried out *in vitro* by coculturing, for example, HMC-1 sonicates with human skin FBs revealing that the interaction enhances collagen synthesis, activity of MMPs, TIMP-2, and collagen gel contraction (Garbuzenko et al., 2002). The release of histamine and tryptase by HMC-1 increased synthesis of collagen by human skin FBs as well as their own proliferation. HMC-1 also increased TIMP-2 and collagen gel contraction, confirming that MCs have a direct and potentiating role in skin remodeling and fibrosis (Garbuzenko et al., 2002). The interaction between MCs and FBs has been investigated by both coculture experiments in monolayers and tridimensional collagen structures to simulate *in vivo* interactions between cells and ECM (Yamamoto, Hartmann, Eckes, & Krieg, 2000). MCs have been reported to tightly attach to FBs when cocultured in monolayer systems (Levi-Schaffer et al., 1985; Levi-Schaffer, Austen, Gravallese, & Stevens, 1986). In three-dimensional collagen lattices cultures, addition of MCs to FBs significantly enhanced gel contraction (Yamamoto et al., 2000). Both in the monolayer model (Adachi et al., 1992) and in the three-dimensional one, this mechanism was reported to be mediated, in part, by SCF/c-Kit interaction (Yamamoto et al., 2000), but still some doubts persist on other receptors involved. In a murine model of bleomycin-induced pulmonary fibrosis, MCs role in the development of fibrosis has been characterized. MCs release renin into the interstitial space that contributes to the formation of angiotensin in which it is further cleaved by MCs-derived chymase giving rise

to the local production of ANG II (reviewed in Veerappan et al., 2013). ANG II triggers the local activation of neighboring FBs through ANG II receptor resulting in fibrinogenesis (reviewed in Veerappan et al., 2013). Analogously, MCs release of histamine activates FBs via histamine receptor (H1R) (Pinheiro et al., 2013) providing another link which may be crucial for the development of fibrosis.

To investigate the influence of FBs on MCs, cocultures of rat or human lung MCs with 3T3 FBs were used as a system that mimics MCs microenvironment *in vivo*. FBs were shown to affect MCs development, survival, phenotype, and their responsiveness to IgE-dependent and -independent activation (Levi-Schaffer et al., 1985; Levi-Schaffer, Austen, et al., 1987). FBs release IL-3 and SCF which are required for MCs development (Lantz & Huff, 1995). Furthermore, *in vitro* rat peritoneal CTMCs exhibited a viability of 30 days when cocultured in the presence of mouse 3T3 FBs, whereas MCs cultured alone revealed a significant loss in viability (Levi-Schaffer et al., 1985). Additionally, under these conditions, BMMCs changed their phenotype toward CTMCs as demonstrated by the increase in their histamine content and a pronounced increase in the biosynthesis of heparin proteoglycan (Levi-Schaffer, Dayton, et al., 1987). Specific activation of these cells by anti-rat IgE Ab led to compound exocytosis morphologically similar to freshly isolated cells suggesting that *in vivo* differentiated rat HP-MCs, cocultured with living FBs, maintain their histology, morphology, immunologic responsiveness, histamine content, and capacity for synthesis of heparin proteoglycan (Levi-Schaffer et al., 1985).

Other studies conducted on MCs grown from cultures of bone marrow cells with SCF and IL-3 and primary cultures of murine FBs showed increases in both histamine and eotaxin release suggesting that this interaction is crucial for the increase of histamine release and eotaxin production (Hogaboam et al., 1998). Furthermore, in addition to its critical role for MCs expansion, differentiation, and survival, SCF, both soluble and membrane-bound (expressed by FBs), has recently emerged as a promoter of MCs activation in mouse models (Ito et al., 2012). Mouse BMMCs chronically exposed to SCF showed a consistent attenuation of FcεRI-mediated degranulation and cytokine production which is likely to be caused by ineffective cytoskeletal reorganization associated to a downregulation of expression of the Src kinase Hck (Ito et al., 2012). Thus, the reciprocal influence of MCs and FBs in terms of proliferation and activation is a critical step in the fibrotic process (Fig. 3) and might be targeted to intervene in early stages of fibrotic processes.

## 6. EOSINOPHLS AND FIBROBLASTS: BIDIRECTIONAL INTERACTIONS

Eos, like MCs, have been associated with a number of fibrotic conditions (Levi-Schaffer et al., 1999) such as eosinophilic angiocentric fibrosis (Deshpande, Khosroshahi, Nielsen, Hamilos, & Stone, 2011; Li et al., 2013), idiopathic chronic eosinophilic pneumonia (Yoshida et al., 1994), endomyocardial fibrosis, scleroderma and scleroderma-like conditions, and IPF (Levi-Schaffer et al., 1999). Eos were shown to directly modulate human lung and dermal FBs proliferation (Fig. 3), collagen synthesis, and lattice contraction (Levi-Schaffer et al., 1999). In particular, Eos-derived MBP has been reported to induce the activation of FBs (Rochester, Ackerman, Zheng, & Elias, 1996) and to act synergistically with IL-1 and TGF-β to increase the production of the profibrogenic cytokines IL-11 and LIF-1 in lung FBs (Rochester et al., 1996). TGF-β (Levi-Schaffer et al., 1999) and IL-1β also exert profibrogenic effects, inducing a fibrogenic FBs phenotype (Gomes et al., 2005). Gomes et al. (2005) demonstrated that cocultures of Eos–FBs induced consistent FBs IL-6 secretion and expression at mRNA levels. To further confirm these results, a neutralization Ab was used to identify IL-1β (>60%), as the principal Eos-derived mediator inducing FBs IL-6 expression (Gomes et al., 2005).

The interaction between FBs with Eos (Fig. 3) has been shown to affect also Eos properties (Dolgachev, Berlin, & Lukacs, 2008). In particular, FBs produce GM-CSF and SCF that, as mentioned above, stimulate differentiation, activation, adherence (Solomon et al., 2000), and survival of both the Eos and MCs (Dolgachev et al., 2008). SCF plays a role in Eos recruitment in AI through both the production of CC chemokines from Eos such as CCL5 and CCL6 and the stimulation of adhesion to matrix and VCAM-1 via VLA4 (Dolgachev et al., 2008). An increased expression of SCF by FBs occurs in allergic airway providing a significant stimulus for Eos (Fig. 3) activation during an allergic response (Dolgachev et al., 2008). *In vitro* experiments showed that cocultures of pbEos with human conjunctival FBs determined prolonged Eos survival mediated by IL-3, IL-5, and GM-CSF and a higher secretory function of Eos. This is relevant in allergic eye diseases such as vernal keratoconjunctivitis in which a continuous Eos inflammation is connected to intense FBs proliferation (Solomon et al., 2000). Furthermore, nasal FBs from human biopsy tissue have been stimulated with both IL-4 and lipopolysaccharide and shown to induce major

production of eotaxin from FBs. This might be responsible for Eos recruitment and development of AI (Nonaka et al., 2004). Another inflammatory condition in which the interaction between Eos and FBs has been identified is AD (Wong et al., 2012). Primary human Eos and dermal FBs both express the functional complex for IL-31 receptor ST2 and IL-33 receptor ST2 which has been found in its constitutive expression in Eos (Wong et al., 2012). These two cytokines have been reported to stimulate the coculture of Eos and FBs to secrete high amounts of proinflammatory IL-6 and the AD-related chemokines CXCL1, CXCL8, CXCL10, CCL2, and CCL5 (Wong et al., 2012), with Eos being the main source of CCL5 and FBs the most important producer of IL-6, CCL2, CXCL8, and CXCL10 upon IL-31 and IL-33 stimulation (Wong et al., 2012). Another cytokine which has recently drawn attention is IL-13, mainly produced by Eos which according to both *in vitro* and *in vivo* studies has a key role in airway remodeling (Aceves & Ackerman, 2009). Experimental evidences suggest that this Th2 cytokine is associated to consistent tissue fibrosis and airway mucous production in murine asthma models (Aceves & Ackerman, 2009).

Eos and FBs interactions are, as for MCs and FBs ones, a crucial process in the induction of the pathological wound healing in AI. Therefore, Eos together with MCs should be targeted for AI-associated fibrosis.

## 7. THERAPEUTIC IMPLICATIONS OF MAST CELLS, EOSINOPHILS, AND FIBROBLASTS CROSS TALKS FOR ALLERGIC INFLAMMATION

As we extensively described, MCs, Eos, and FBs are the three main players of AI and associated fibrosis. Many established and new drugs, some of them still under preclinical and clinical studies, exist to control AI with various degrees of success. Part of these drugs target the MCs, and others the Eos. However, there are very few drugs for fibrosis by itself (reviewed in Cohen-Naftaly & Friedman, 2011; Rockey, 2008), and nothing to reverse the fibrotic tissue has been discovered as yet. The existing antifibrotic drugs are based on inhibiting the effects of growth factors/cytokines influencing the FBs such as IFN-γ, TNF-α, and TGF-β, on inhibiting MMPs by, for example, Doxycyline (Rafii, Juarez, Albertson, & Chan, 2013), and on inhibiting collagen synthesis, for example, Pirfenidone (5-methyl-1-phenyl-2-(1*H*)-pyri-) (Cohen-Naftaly & Friedman, 2011). However, for AI-associated fibrosis currently no specific treatment option in available.

We can prevent the AI-associated fibrosis by managing the inflammation. Therefore, the best strategy is to have a drug able to stop either the first one, i.e., MCs, or the second one, i.e., Eos. If this is not successful, the FBs should be directly targeted. Here, we limit the description to some drugs available and some under preclinical and clinical studies that in our view could be the preferred ones taking into account both the inflammatory stages and the fibrotic ones of an allergic disease.

It is clear from the previous sections that the main effector cells of AI are the MCs, the Eos, and probably the self-fuelling AEU, and that the FBs are target/effector cells that should also be limited in their profibrotic activity. Therefore, the best way to downregulate the AI is to control these main players by means that either suppress or stabilize these cells and/or block their proinflammatory/profibrotic released mediators.

Glucocorticosteroids (GCs) are the most effective drugs for treating allergic diseases because of their multiple suppressing effects on inflammation comprising their activity on all the three key players of this allergic milieu (Klion et al., 2006). Although GCs do not stabilize MCs, they decrease their cytokine production (Barnes, 1998; Brattsand & Linden, 1996; Obojski & Kraus-Filarska, 2004), decrease Eos numbers in blood and tissues, interfere with multiple cellular and humoral mechanisms of the inflammatory network (Barnes, 1998; Obojski & Kraus-Filarska, 2004), and reported to reduce Eos profibrogenic effect in asthma (Puxeddu, Lack, Smith, & Levi-Schaffer, 2004). Notably, GCs have also some anti-fibrotic direct activity by inducing FBs apoptosis (Mendoza-Milla et al., 2013; Szabo et al., 2010). Studies in murine models of allergen-induced airway remodeling have demonstrated that GCs significantly reduce allergen-induced peribronchial collagen deposition and total lung collagen levels (Cho et al., 2004) and also reported to prevent accumulation of MyoFBs and airway remodeling (Miller et al., 2006). Nevertheless, some patients require high doses of GCs with consequent high incidences of serious side effects or are even unresponsive to them.

The next approach to control AI and fibrosis is to block the *primum movens* of the reaction, i.e., the MCs, and this has been proven not to be an easy task. MCs activation can be prevented by MCs stabilizers such as cromolyn sodium and nedocromil sodium, which inhibit the release of allergic mediators from MCs by still a not completely characterized mechanism (Howell & Altounyan, 1967; Finn & Walsh, 2013; Lal, Malhotra, Gribben, & Hodder, 1984). These drugs have been used as anti-asthma drugs for decades often with minimal results though probably due to limited

absorption by topical application (Bernstein et al., 1972; Edwards & Stevens, 1993; Howell & Altounyan, 1967; Rainey, 1992). Interestingly, some H1 antihistamines have dual-action of both antihistaminic and MCs-stabilizing activities (Cook, Stahl, Barney, & Graziano, 2002; Levi-Schaffer & Eliashar, 2009). Furthermore, in the past, we have shown that ketotifen, an anti-H1 drug with MCs-stabilizing properties, decreased the fibrosis in the skin of cGVHD patients (Nagler, Segal, Slavin, & Levi-Schaffer, 1995). Another promising candidate is azathioprine that inhibits MCs activation (Molderings, Brettner, Homann, & Afrin, 2011) and is often used in combination with GCs for the treatment of pulmonary fibrosis (Dheda, Lalloo, Cassim, & Mody, 2004; Raghu et al., 1991; Rogliani, Mura, Assunta Porretta, & Saltini, 2008). The next generation of MCs-stabilizing drugs isolated from natural sources such as from simple phenols, alkaloids, terpenes to simple amino acids is currently under research (reviewed in Finn & Walsh, 2013).

Another way to prevent MCs degranulation is to block IgE-dependent stimulation of MCs. The IgE–FcεRI interaction can be prevented either via blocking the FcεRI receptor on MCs or blocking the Fc portion of the IgE. Immunotherapies using anti-IgE Ab directed to the Fc portion of the Ab (Omalizumab) have been showing promising results in treatments of several allergic disorders not only by blocking IgE binding to the FcεRI receptor but also by reducing plasma IgE levels, as well as FcεRI expression on MCs (Asero, Casalone, & Iemoli, 2014; D'Amato et al., 2014; Domingo, 2014; Jerzynska, Sztafinska, Woicka-Kolejwa, & Stelmach, 2014). Other approaches to target MCs could be the inhibitors of SCF/c-Kit by anti-SCF or anti-c-Kit Ab. Until now, the only drugs used clinically to block c-Kit-dependent MCs activation are tyrosine kinase inhibitors (reviewed in El-Agamy, 2012; Jensen, Metcalfe, & Gilfillan, 2007), such as Imatinib, Nilotinib, Dasatinib, Masitinib, and Midostaurin that are used mostly in mastocytosis and in severe non-GCs-responsive allergy (Harvima et al., 2014).

Another strategy that is being used in AI is to block the MCs released mediators' activity that can stop early allergy symptoms, subsequent Eos tissue infiltration and activation, and finally FBs activation. Histamine released by MCs should be a very good candidate influencing all the steps of AI especially via H1 and the newly discovered H4 receptors. Histamine can be downregulated by several existing anti-H1R antihistamines (Simons, 2004; Simons & Simons, 2011) possibly together with anti-H4R to block both the symptoms of allergy, Eos chemotaxis, and FBs proliferation

(see above). This combination has shown already important synergistic therapeutic effects in a mouse model of chronic dermatitis (Ohsawa & Hirasawa, 2012), and clinical trials are underway for asthma and other allergic diseases (reviewed in Salcedo, Pontes, & Merlos, 2013).

The next relevant targets are tryptases and chymases, the major enzymes released during MCs degranulation since they have been shown to be active both in AI and fibrosis (Caughey, 2007, 2011). The pathophysiologic role of β-tryptase is not clear, but it has been associated with the promotion of inflammation and matrix remodeling (Caughey, 2007; Harvima & Nilsson, 2011). JNJ-27390467, a β-tryptase inhibitor, also reduces airway inflammation in experimental models of asthma (Costanzo et al., 2008). RWJ-58643, a competitive inhibitor of both MCs β-tryptase and pancreatic trypsin, was able to significantly reduce the symptoms, Eos numbers, and IL-5 levels at a low dose. Tryptase inhibitor (MOL 6131) is reported to decrease airway inflammation in murine asthma (Oh et al., 2002). However, no data exist on the effectiveness of these drugs on fibrosis. Chymase, a potent chymotrypsin-like serine proteinase, causes matrix destruction and inflammation (Caughey, 2007; Huttunen & Harvima, 2005). Several potent chymase inhibitors such as SUN C8077 (Tomimori et al., 2003), NK3201 (Sawaguchi et al., 2012), and TY-51469 (Takato et al., 2011) have been tested in a variety of animal and *ex vivo* models with proven anti-inflammatory and antifibrotic effect. Cathepsin G is another chymotrypsin-like serine proteinase (Caughey, 2007), and several existing chymase inhibitors also inhibit cathepsin G to some extent (D'Orleans-Juste et al., 2008). Since chymase and cathepsin G share similar biological functions, it might be therapeutically useful to develop inhibitors that inactivate both enzymes simultaneously, such as RWJ-355871, which has shown efficacy in rat, mouse, and sheep models of lung or paw inflammation (Maryanoff et al., 2010). These studies are suggesting that chymase inhibitors might be useful for treatment of fibrosis associated with AI.

The preferred approach to inhibit AI has long been to target Eos rather than the MCs since these cells have been viewed as the main contributors of the late/chronic and damaging stages of allergy. Moreover, they have been supposed to be an easier target than tissue-resident MCs. One of the strategies to control Eos is to inhibit their differentiation, maturation, and in consequence, numbers in blood and tissues by targeting IL-5 (reviewed in Landolina N & Levi-Schaffer F, 2014). This has been done by means of either anti-IL-5 Ab such as Mepolizumab and Reslizumab (Leckie

et al., 2000; Radinger & Lotvall, 2009; Rosenberg, Dyer, & Foster, 2013; Walsh, 2013) or by blocking IL-5 receptor with mAb such as Benralizumab to inhibit the IL-5-mediated receptor activation (Wechsler et al., 2012). Eos migrate to the inflamed tissue following chemoattracting signals. The Eos trafficking into the tissue is regulated by various adhesion molecules, for example, VLA-4, VCAM-1, ICAM-1, ICAM-2, ICAM-3, and various integrins such as α4β7 (CD49d/β7)/α4β1(CD49d/29), etc. (Barthel, Johansson, McNamee, & Mosher, 2008; Driss et al., 2013) and by various cytokines and chemoattractant receptors such as CCR3/CCL11, PGD2, etc. (Driss et al., 2013; Kita, 2011). PGD2 or CRTH2 (Pettipher, Hansel, & Armer, 2007), blocked by OC000459 (a selective CRTH2 antagonist), was found to inhibit PGD2 mediated Eos chemotaxis in addition to preventing the activation of Th2 lymphocytes (Pettipher et al., 2012). This compound was reported to reduce allergic symptoms in asthma (Singh et al., 2013) and in other allergic diseases, for example, grass pollen-induced nasal and ocular allergy (Horak et al., 2012). An interesting compound is TPI-ASM8, a mixture of two modified phosphorothioate antisense oligonucleotides: one blocks CCR3 and the other targets the common β chain of GM-CSF, IL-3, and IL-5 receptors (Nguyen & Casale, 2011). Eotaxin triggered MCs infiltration and contributed to AI-associated fibrosis. The eotaxin has a profibrogenic effect on human lung FBs (Kohan, Puxeddu, Reich, Levi-Schaffer, & Berkman, 2010; Puxeddu et al., 2006). Therefore, targeting eotaxin with mAb, such as Bertilimumab, could reduce MCs infiltration and associated fibrosis (Mangieri et al., 2012; Zweifel, Matozan, Dahinden, Schaffner, & Mohacsi, 2010). Another selective and competitive CCR3 antagonist NCT01160224 is under evaluation in phase II trials for its effectiveness in reducing sputum eosinophilia in mild to moderate asthma. However, no results have been reported as yet (GlaxoSmithKline, 2012). Ab against adhesion molecules also showed promising results. Natalizumab is a humanized mAb against VLA-4 that reversibly binds to α4 and is in phase III clinical trials for inflammatory diseases (Stuve & Bennett, 2007). Several competitive inhibitors targeting integrins have been reported to reduce Eos recruitment, thus reducing airway eosinophilic inflammation in asthma (Vanderslice & Woodside, 2010). However, two orally active, dual α4β1/α4β7 antagonists, TR14035 and Valategrast (R411) tested in trials with other α4β1 antagonists (GW-559090 or RBx-7796), performed poorly in human asthma despite significant effects in animal models (Barthel et al., 2008).

Intracellular signaling for MCs and Eos survival, and activation pathways are other promising novel therapeutic targets in AI. Activation of MCs and Eos can be blocked by inhibitors that act on signaling pathways transduced from plasma membrane receptors to cytoplasmic effectors such as spleen tyrosine kinase, phosphatidylinositol 3-kinases, Src homology 2 domain-containing inositol 59 phosphatase 1, Bruton tyrosine kinase, the protein tyrosine kinase Kit, and sphingosine kinases (reviewed in Harvima et al., 2014).

One of the most appealing approaches to prevent AI and fibrosis is possibly with anti-IL-4/anti-IL-13 or anti-receptor Ab, for the roles of these cytokines not only in AI but also in fibrosis are seen especially in asthma (Dasgupta, Neighbour, & Nair, 2013; Steinke, 2004). IL-13 is a major cytokine involved in airway inflammation and remodeling in asthma and remains a highly relevant new target for the treatment of chronic severe asthma, utilizing either recombinant human IL-13α2 receptor as a molecule decoy or blocking mAb (Snell, 2000). Human and humanized blocking IL-4 mAb are currently in clinical development (Holgate, 2004). TNF-α is a potent proinflammatory cytokine that has wide-ranging effects on Eos, MCs, and in fibrosis as we have reported above. Therefore, TNF-α has been considered as a new target for therapy in chronic asthma. However, a recent large multicenter trial with the Ab Golimumab showed no beneficial effect on lung function, symptoms, or exacerbations, and there were increased reports of pneumonia and cancer (Wenzel et al., 2009). The possibility to target pharmacologically TNF-α in AI/tissue remodeling should be further investigated.

## 8. FUTURE DRUGS

AI is a multicellular response, and it is therefore almost impossible to control the function of all the cells involved for the unique benefit of the patient. The current drugs/therapy are not satisfactory as yet. New therapeutic strategies involving mechanism-based approaches are needed. MCs, Eos, and the AEU can be inhibited by activating their IRs or by blocking their activating ones. A promising strategy concerning MCs and Eos activation could be to stimulate the IR expressed on these cells, such as CD300a, FcγRIIB, or Siglec-8 by mAb or by the specific ligands (recently reviewed in Harvima et al., 2014). In a mouse model of cutaneous anaphylaxis cross-linking of CD300a with c-Kit by a bi-specific Ab fragment was

reported to abrogate MCs degranulation (Bachelet et al., 2008). Linking of CD300a with FcεRI abolished the allergic and inflammatory responses in two different animal models (i.e., IgE-dependent PCA and ovalbumin-induced acute experimental asthma) (Bachelet, Munitz, & Levi-Schaffer, 2006). Among ARs, CD48, and 2B4 are the main interacting receptors involved in the formation of AEU. By specifically blocking one or both receptors AEU formation can be prevented. Moreover, CD48 is highly expressed on MCs and Eos, its levels on pbEos increase in asthmatics, it is one of the asthma signature genes in murine asthma, and it is a novel target in asthma therapy (Munitz, Bachelet, & Levi-Schaffer, 2007). In a mouse model of allergen-induced asthma, CD48 was found to be upregulated. By specifically blocking this receptor, the AI can be downregulated (Munitz, Bachelet, Finkelman, Rothenberg, & Levi-Schaffer, 2007).

Regarding fibrosis, a novel option is to use microRNAs. Van Rooij et al. (2007) gave an account for the first time of the role for miRNAs in the fibrogenic response to tissue injury. Since then, many recent studies have revealed a critical role for various miRNAs for which aberrant expression drives the initiation and progression of the fibrosis process in response to persistent tissue injury (Bowen, Jenkins, & Fraser, 2013; Milosevic et al., 2012; Putta et al., 2012; Zhou et al., 2013). These miRNAs, which are sometimes called FibromiRs, have been reported to be used as new anti-fibrotic therapies (Pottier, Cauffiez, Perrais, Barbry, & Mari, 2014).

A recent review shed some light on the possibility that tissue fibrosis and FBs-to-MyoFBs differentiation can indeed be reversed, through a new therapeutic approach for fibrotic disorders (Yang, Chen, Liu, & Chen, 2014). Therefore, research in this direction is critically needed and should be encouraged.

## 9. CONCLUSIONS

MCs and Eos creating a functioning AEU seem to be even more important cells in AI than in the past. Their interactions with FBs are also pivotal for the existence of this reaction and its fibrosis consequence. Some drugs are available to downmodulate these cross-talks vicious cycles. But still much more research should be carried out to define the role of these interactions *in vitro* but mostly *in vivo*. This will result in more defined data that will lead to new drug discovery.

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