Pharmacological opportunities to control inflammatory diseases through inhibition of the leukocyte recruitment

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Abstract

Leukocyte recruitment to tissues is a highly orchestrated process and is one of the pillars of the inflammatory process. The contribution of leukocytes to tissue damage is very clear, suggesting that targeting leukocyte accumulation in tissue to be relevant for the development of novel therapies to treat chronic inflammatory diseases. Here, we review briefly known mechanisms of leukocyte recruitment and suggest potential targets for the development of novel anti-inflammatory therapies.

Pharmaceutical companies spend a considerable part of their budget in basic and applied research aiming at the development of new anti-inflammatory drugs. In the last decade, several new anti-inflammatory drugs, mostly antibody-based (such as anti-TNF-a and IL-6), have emerged. However and despite the introduction of these novel medications, a significant proportion of patients still have an inadequate response to current medications, indicating that further efforts are necessary to provide better quality of life worldwide. This is compounded by the increase of incidence of chronic autoimmune and inflammatory diseases, including type 1 diabetes, rheumatoid arthritis and lupus. Therefore, it is clear that there is still need for the development of novel anti-inflammatory drugs.

The identification of novel targets to reduce or control inflammation in humans is probably the main challenge that impairs the development of new anti-inflammatory drugs. As the recruitment of leukocytes is a key feature of chronic inflammation and contributes significantly to tissue damage, much focus has been placed in the reduction or inhibition of leukocyte infiltration as potential strategies for the design of new drugs. Indeed, a few drugs that affect leucocyte recruitment have been approved in recent years, including anti–α4 integrin monoclonal antibody (mAb) natalizumab (Tysabri) for multiple sclerosis. Furthermore, basic research on the mechanisms of leucocyte recruitment using experimental models of inflammatory diseases has added significantly to our knowledge of the key check points controlling leukocyte accumulation and tissue damage. Once these targets are identified, they provide the rationale for the pharmaceutical industry to develop subsequent selective agents, such as mAbs or small molecules, that inhibit or reduce the functional activity of the molecules involved in the leucocyte recruitment cascade (e.g.: chemoattractant receptors). In this review, we detail the events involved in leucocyte recruitment, suggesting possible targets for therapeutic intervention in each step of the process.

Multisteps of the leukocyte recruitment process

In the bloodstream, leukocytes normally circulate in the center of the lumen and tether the endothelium in a random rate. Such endothelium-leukocyte interaction is a very important aspect of vascular physiology and for the inflammatory response, and will govern crucial steps of proper leukocyte recruitment. In non-inflamed vasculatures, this rapid contact with endothelial cells will last only for milliseconds, and leukocytes return to the main flow. However, inflamed tissues have a very well regulated strategy to recruit leukocytes: they release inflammatory mediators, including TNF-α, LTB4, IL-6, CXCL1 and CXCL2, that will modulate endothelium function, enhancing its adhesiveness and, therefore, the contact time with circulating leukocytes [1-6]. During this process, different adhesion-dependent intracellular pathways are stimulated, culminating in a progressive state of leukocyte activation. Of note, proper spatial and chronological leukocyte activation will govern the fate of the inflammatory process; i.e. overt intravascular leukocyte stimulation is associated with collateral endothelial damage, while emigrating leukocytes that are progressively recruited to damage tissues will add to proper tissue healing. It is important to note that even in the steady state, endothelial cells express several adhesion molecules, including P- and E-selectin (CD62P and CD62E, respectively), ICAM-1 (CD54), ICAM-2 (CD102) and ICAM-3 (CD50), VCAM-1 (CD106), PECAM-1 (CD32) [7]. Upon stimuli, endothelial cells display a quantitative change in their profile of expression of adhesion molecules. P-selectin is pre-formed and stored in endothelial cells in α-granules. In contrast, E-selectin is not normally expressed by endothelium, except in skin vessels, but is rapidly up-regulated by inflammatory cytokines [2,4,8-10].

In this way, selectins bind to leukocytes using carbohydrate groups presented on proteins, including the P-selectin glycoprotein ligand-1 (PSGL-1). Selectin-mediated

adhesion (specially L-selectin) requires shear stress to sustain leukocyte rolling on endothelium [11,12]. While selectins adhere cells to the endothelium in a low affinity state, the shear stress created by the constant blood flow can also push the cells along the endothelium, detaching them like a “velcro”. Almost immediately, the same leukocyte may re-adhere in the next micrometres, and this “tug of war” played by the blood flow against the adhesive forces from selectins creates a well known leukocyte behavior called “rolling”. Rolling is not only important to keep the cells closer to the endothelium, but also cause leukocyte activation due to intracellular pathways that are triggered via selectin adhesiveness [2]. For instance, leukocytes that rolled on the endothelium express more integrins, including β2 integrins (CD11/CD18) and VLA4 (CD49d/CD29), and these adhesion molecules might also be more adhesive to the ligand itself [7,13]. The rolling process puts leukocytes in close contact to endothelial cells and allows them to “find” chemoattractant molecules. There is some debate of whether the chemoattractant receptors, which are on the surface of leukocytes, actually bind to chemoattractant molecules that are seating on the surface of endothelials cells or whether these chemoattractants diffuse through endothelial cells and interact with their receptor on passing leukocytes. Because local flow is intense, the former possibility (binding to chemoattractants on the surface of endothelial cells) appears more likely. Indeed, there is now much evidence suggesting that chemoattractant molecules interact with glycosaminoglycans (GAGs) present on the surface of endothelial cells and that GAG binding is important for leukocyte adhesion and subsequent migration. The interaction of GAGs to chemokines has been investigated as putative sites for the development of novel anti-inflammatory drugs [14,15]. Chemoattractants bound to the surface of endothelial cells will support leukocyte adhesiveness and subsequent leukocyte migration to the target sites [16]. More recently, it was demonstrated that adhered leukocytes do not always emigrate in the original retention site; these cells search for “hot spots” to emigrate. This is supported by intravital microscopy studies showing

that leukocytes (mainly neutrophils and monocytes) crawl along the vessel wall and emigrate preferentially in the intercellular junction of endothelial cells, a region which is also richer in adhesion molecules relevant in the context of diapedesis, including PECAM-1 [17]. Crawling is a multistep process, and blockade of adhesion molecules on leukocytes or endothelial cells prevent these cells to reach hotspots to emigrate. It has been shown that antibodies against integrins LFA-1 (CD11a/CD18; αLβ2 integrin) and Mac-1 (CD11b/CD18, αMβ2 integrin) on monocytes and integrin ligands ICAM-1 and ICAM-2 on endothelial cells inhibited monocyte crawling [16,18,19]; however despite the absence of adhesion molecules, these cells adhered and polarized normally but could no longer crawl on the endothelium. Importantly, crawling is now considered as a crucial step in the leukocyte recruitment cascade, since strategies that inhibited crawling also prevented leukocyte extravasation [18-20].

Once leukocytes are exposed to the chemotatic gradient, several intracellular signaling pathways are activated aiming to shape the cell to the final step of migration: diapedesis [1,21]. There is a well-orchestrated cascade that involves kinases, phosphatases and GPCRs, and the collaboration between enzymes and signaling molecules, including PI3K, SHIP and p38, will induce structural changes in the cytoskeleton that will allow leukocytes to squeeze themselves within endothelial junctions and then reach extravascular compartments. For this, leukocytes extend pseudopodia, projecting the cell membrane to pass through gaps between endothelial cells. This is necessary to use minimal space between endothelial cells therefore avoiding plasma leakage [17,22,23].

Once gone through the endothelium, leukocytes start a new phase in their migratory behavior. Now, while there are no shear forces to fight against, they need to digest the extracellular matrix to reach their destination. To accomplish this, leukocytes are

loaded with several proteolytic enzymes in their granules. One of the major enzyme families involved in this process are the matrix metalloproteinases (MMPs) [24]. These enzymes degrade extracellular matrix proteins, opening a tunnel for leukocyte locomotion within connective tissue, with minor damage to the host. There are different kinds of MMPs, including collagenases (MMP1, 8 and 13), gelatinases (MMP2 and 9), metalloelastase (MMP12) and many others. At this point, integrins are still very important for adequate leukocyte movement in the direction of the inflammatory spot. Once released from leukocyte granules, MMPs can also process a number of bioactive molecules, enhancing or inhibiting their activity. Depending on the nature of the stimuli, different leukocyte subtypes will emigrate to tissue, and will also modulate which cells will arrive next, or which cells are no longer required [25].

In general, leukocyte recruitment comprises a multistep process, which includes firstly the participation of selectins followed of activity of chemo-attractant receptors signaling and consistent adhesion to endothelium by integrins action (**Figure 1**). Subsequently, leukocytes migrate into chemotactic gradients. Therefore, the inhibition of the participants of this process, such as selectins, chemoattractants and integrins, affect the leucocyte recruitment and the establishment of the inflammatory response, providing the basis for the development of new therapeutic approaches for inflammatory diseases. In addition, several studies have now investigated the role of signaling molecules, such as PI3K, SHIP and p38, for diapedesis and locomotion activities. The next sections describe many of the promising cell migration targets for several subsets of leukocytes.

Selectin inhibitors

Selectins have a relevant and well-established role in inflammation and immune responses, since they mediate the first steps required in the process of leukocyte binding to vascular endothelium and initialization of the leucocyte recruitment

process, characterized by leukocyte tethering and rolling. There are three types of selectins: L-selectin, P-selectin and E-selectin and these may be induced by inflammatory conditions, especially E-selectin. It has been described that the blockade of selectins or the selectin ligand P-selectin glycoprotein ligand-1 (PSGL-1) ameliorated inflammatory responses in a variety of experimental models, including models of atherosclerosis, ischemia-reperfusion conditions, psoriasis and asthma [26-29]. In most experiments involving animals, a recombinant human PSGL-1-Ig fusion protein (rPSGL-1-Ig) was given in order to prevent neutrophil infiltration in the site. Additionally, it also was demonstrated that administration of rPSGL-1-Ig was effective to prevent allograft and xenograft rejection [30]. In accordance, several human trials were carried out evaluating the potential of administration of selectins inhibitors. The pan-selectin inhibitors Bimosiamose (develop by Revotar Biopharmaceuticals) and Rivipansel (develop by Pfizer) showed encouraging results in clinical trials for obstructive pulmonary disease (COPD), psoriasis, asthma and vaso-occlusive crisis (VOC) [31]. Bimosiamose has been discontinued and Rivipansel is now undergoing phase 3 clinical trials for VOC.

Additionally, it has well been described that some components of humoral innate immunity such as pentraxin 3 (PTX3) can also affect leucocyte recruitment through interactions with selectins [32,33]. In fact, PTX3 binds to P-selectin and may limit inflammation by inhibiting neutrophil rolling, acting as a competitive inhibitor of the P-selectin–PSGL-1 interaction [33]. In the context of inflammatory conditions, it has been reported that the genetic ablation of PTX3 promotes more myocardial damage associated with more neutrophil infiltration in a model of cardiac ischemia-reperfusion [34]. In sepsis, it was observed high levels of circulating PTX3, which accounts for systemic anti-inflammatory effects [33]. However, the role of PTX3 in the majority of human diseases still remains poorly understood and clinical studies are being carried out to clarify its implications for several inflammatory diseases, including rheumatoid

arthritis and type II diabetes (ClinicalTrials.gov identifier: nCT02503046; ClinicalTrials.gov identifier: nCT01678521; ClinicalTrials.gov identifier: nCT00674596). In summary, although the use of selectin inhibitors is promising, results from phase 3 clinical trials are eagerly awaited to define long term efficacy and safety of these products in humans.

Chemoattractant receptors antagonists

Chemoattractants are molecules crucial for the process of activation of rolling leukocytes and constitute the largest family of molecules that contribute to the process of leukocyte migration. Upon activation, leukocytes adhere firmly to endothelial cells and then migrate into the tissue and then towards the site that initiated the stimulation. Chemoattractants are very diverse, ranging from lipid molecules, short chain fatty acids, small peptides and proteins (**Table 1**). Chemoattractants bind to a family of G-protein-coupled seven transmembrane receptors and provide the largest diversity that account for the selective recruitment of leukocytes to sites of inflammation. Examples of the potential of antagonists of chemoattractant receptors for the treatment of chronic inflammatory diseases are given below. By no means, we attempt to compile a comprehensive list of all of the potential use of antagonists of chemoattractant receptors.

<u>CXCR2 and CXCR1</u>

CXCR1 and CXCR2 are the major chemokine receptors expressed by neutrophils. Human neutrophils express both receptors whereas murine neutrophils express mainly CXCR2 [35]. CXCR2 is a receptor that contains the sequence Glu-Leu-Arg near the N terminus and mediates neutrophil influx to inflammatory sites in response to CXCL1, 5, and 7 [36]. Data from animal models of inflammation show that CXCR2 inhibitors are effective to attenuate neutrophil influx to sites of inflammation. For

example, Reparixin, a non-competitive allosteric inhibitor of CXCR1 (but also active on CXCR2) [37], promotes a decrease of neutrophil influx and tissue injury in models of peritonitis and ischemia-reperfusion (I/R) injury [37-40]. Furthermore, DF2162, a synthetic compound with similar effects to Reparixin, was also effective to diminish the neutrophil influx and inflammatory hypernociception in mice [41].

CXCR2 antagonists appear to ameliorate inflammation in a range of animal models of chronic inflammatory disorders, including arthritis, EAE, psoriasis, COPD and asthma [42-46], suggesting that CXCR1/2 receptor antagonists may be useful in the context of chronic autoimmune diseases in humans. The effectiveness of inhibitors of CXCR1/2 activity is currently being assessed in clinical trials of autoimmune diseases (reviewed in [46]).

Moreover, the beneficial effects mediated by CXCR1/2 inhibition have also been demonstrated in the attenuation of other inflammatory conditions. The injury caused by I/R is linked with a severe inflammatory response marked by intense recruitment of the neutrophils and others leucocytes in the inflammatory site [47]. In this way, the inhibition of CXCR1/2 using selective antagonists has been shown to prevent experimental I/R injury in the intestine and transplanted organs, providing new perspectives concerning the development of drugs that may be effective at amelioration I/R injury in relevant clinical situations [38,48].

The most promising compounds tested in clinical trials have focused on CXCR2, since there are more chemotactic ligands described to this receptor than to CXCR1 and experimental animals mostly used to model human disease (i.e. mice and rats) express mainly CXCR2. There are no compounds that have been approved as yet, but there are several companies pursuing the effects of CXCR1/2 antagonists in the context of transplantation and cancer (Reparixin, Dompe Corporate,

ClinicalTrials.gov identifier: nCT01817959; ClinicalTrials.gov identifier: nCT02370238) and COPD (Danirixin, GlaxoSmithKline, ClinicalTrials.gov identifier: nCT00504439; MK-7123, Merck, ClinicalTrials.gov identifier: nCT01006616).

CCR2

CCR2 is a chemoattractant receptor expressed in a range of cell types, such as monocytes, basophils, activated T lymphocytes, dendritic cells, and endothelial and vascular smooth muscle cells. It is the receptor for several chemokines: CCL2, CCL7, CCL8 and CCL3 [49]. The activity of CCR2 and its ligands has been associated with the development and maintenance of several diseases, including rheumatoid arthritis, multiple sclerosis, atherosclerosis and cancer [50-53]. In rheumatoid arthritis, experimental and clinical data showed that CCR2 has a critical role in promoting neutrophil migration to the joint, even though CCR2 is not expressed in neutrophils under physiological conditions. However, during autoimmune diseases this cell type becomes CCR2 positive (**Figure 2**). Interestingly, the inhibition of CCR2 or its ligand CCL2 protected mice against the infiltration of neutrophils and destruction of the joints [54,55]. In this way, many companies developed antagonists of CCR2 and performed clinical studies to confirm the efficacy of CCR2 antagonists in the treatment of rheumatoid arthritis, multiple sclerosis and atherosclerosis. Merck and Millennium found negative results as the effectiveness of its compounds in rheumatoid arthritis (ClinicalTrials.gov identifier nCT00542022 and nCT00239655), but many clinical trials are still ongoing assessing the effects of novel CCR2 antagonists in other disorders, including atherosclerosis, COPD and type 2 diabetes (ClinicalTrials.gov identifier: nCT02388971; ClinicalTrials.gov identifier: nCT01215279; ClinicalTrials.gov identifier: nCT00699790).

CXCR4

The chemokine receptor CXCR4 is expressed by several leukocyte subsets and has a high similarity with the human and mouse protein. The most studied ligand for this receptor is the chemokine CXCL12 (formerly called SDF-1), which is also highly conserved between human and mouse. CXCL12/CXCR4 axis is well studied in inflammatory responses in different organs, including brain and heart, but also in embryonic development because it mediates progenitor cell migration under homeostatic conditions (reviewed in [56]). In addition, interaction of CXCL12 with CXCR4 is the major pathway involved in migration of hematopoietic progenitors from the bone marrow to circulation under both physiological and pathological conditions [57]. Therefore, this axis plays a pivotal role on the maintenance of circulating number of leukocytes and rapid mobilization upon stimuli. However, more recently, alternative ligands and activation patterns for CXCR4 were reported. The chemokine MIF (Migration Inhibitory Factor) – which is highly secreted following diverse inflammatory stimuli - was identified as an alternative ligand for CXCR4 [58]. Furthermore, a complex formed between HMGB1 and CXCL12 enhances CXCR4 activation, thus the blockage of this interaction might be a pharmacological alternative to treat ischemic and trauma patients since the initial phase of leukocyte recruitment under these conditions is partially dependent on HMGB1 [59].

C5a receptors

The C5a receptor (C5aR) is the receptor for the complement component C5a, which is chemo-attractant for neutrophils [60]. The expression of C5aR and exacerbated production of C5a are associated with the pathogenesis of multiple diseases, such as rheumatoid arthritis, sepsis, neuropathic and inflammatory pain [61-63]. Compounds and monoclonal antibodies against C5aR are effective to control the inflammatory response in experimental models of arthritis [64,65]. Furthermore, the oral administration of a novel potent allosteric inhibitor of the C5aR synthetized by Dompe Corporate presented a strong effect in attenuating inflammatory and neuropathic

pain, suggesting that targeting C5aR is a coherent avenue for the development of new therapies [63]. Currently, there are advanced clinical trials addressing the efficacy of C5aR inhibitors is for anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) (CCX168, ChemoCentryx, ClinicalTrials.gov identifier: nCT02222155) and atypical hemolytic uremic syndrome (CCX168, ChemoCentryx, ClinicalTrials.gov identifier: nCT02464891). In addition, Novo-Nordisk is establishing the safety and tolerability of single intravenous (i.v.) and subcutaneous (s.c.) doses of NNC0215-0384 (a anti-C5aR mAb) in subjects with active rheumatoid arthritis on background methotrexate (MTX) treatment (ClinicalTrials.gov identifier: nCT01611688; ClinicalTrials.gov identifier: nCT01955603). Therefore, subsequent clinical studies addressing the efficacy of C5aR inhibitors in the treatment of inflammatory diseases, such rheumatoid arthritis, must confirm the evident potential of the C5aR inhibitors as strategy for the development of new drugs.

Receptors involved in T cell–mediated inflammatory diseases

T cell subsets express receptors for chemotactic molecules according to their functional requirements. Indeed, whereas naive T cells express L-selectin and CCR7 that allow the migration across high endothelial venules, during T cell differentiation, the T *helper* subtypes acquire the expression of distinct chemoattractant receptors that allow them to migrate to sites of inflammation outside lymph nodes. These receptors are reliable markers and provide the functional properties of these specifics sub-types of T cells. For instance, CXCR5 marks T follicular helper (Tfh) cells, CCR5 and CXCR3 mark Th1 cells, CCR3 and CRTh2 (PGD2 receptor) mark Th2 cells, and CCR6 preferentially marks Th17 cells [66]. The differential expression of chemokine receptors explains why differentiated T cells migrate to different sites of inflammation and interact with different leucocytes types. Therefore, the specific expression of the chemoattractant receptors identifies an important target to develop new drugs to treat

disorders that are driven by specific T helper cell lineages. Below, we will exemplify the potential of blocking specific chemokine receptors for treating T cell dependent inflammatory diseases.

CXCR3 and CCR5. The blockade of the receptors CXCR3 or CCR5 attenuates a range of experimental inflammatory conditions mediated by Th1 cells, including autoimmune diseases, atherosclerosis, sepsis, graft-versus-host disease and transplant rejection [67-75]. CXCR3 has been suggested to play a critical role in the development of inflammatory conditions because it promotes recruitment of Th1 cells [76]. CXCR3 expression and its ligands CXCL10 (IP-10) and CXCL11 (ITAC) are upregulated in several disorders characterized by Th1 lymphocyte activation, including rheumatoid arthritis [77]. Notably, several preclinical experiments have been conduced to address the potential therapeutic effects of CXCR3 inhibitors and pharmaceutical companies have developed potent CXCR3 antagonists in the recent past. For instance, the small molecule AMG-487 developed by ChemoCentryx and Tularik (now part of the Amgen group) has excellent potency, high selectivity and good oral bioavailability [78]. The compound was taken to Phase II clinical trial in patients with moderate-to-severe psoriasis, but was not shown to be effective. Further development of the drug has been discontinued due to lack of efficacy in the human diseases [79]. Additionally, other antagonists of CXCR3 have been developed by Merck and Sanofi. These compounds were synthetized from arylpiperazines and carboxylic acids precursors and some of them also were identified through microbial, plant, and marine extracts. Indeed, three classes of naturally occurring small molecule CXCR3 antagonists with micromolar activity in a radioligand binding assay have been identified [80]. However, there are no reports of further development concerning these naturally and synthetic compounds with subsequent tests in humans.

CCR5 is the natural receptor for the ligands CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES) [81]. Notably, as previously mentioned, experimental data using CCR5-deficient animals or antagonists of the receptor have shown to be a promising target for the amelioration of inflammatory disorders such inflammatory arthritis [75]. However, clinical data have shown that the administration of Maraviroc, a small molecule inhibitor of CCR5 developed by Pfizer, failed to demonstrate efficacy in a trial of patients with rheumatoid arthritis (ClinicalTrials.gov Identifier: nCT00427934). Of note, the rationale for these controversial findings in humans could be explained by the fact that CCR5+ T cells express others chemokine receptors such as CCR1, which are also capable of responding to CCL3 and CCL5 ligands and thus to mediate leucocyte migration into inflammatory site [82,83]. Currently, there are a few clinical trials being conducted using CCR5 inhibitors in patients suffering by autoimmune diseases. For instance, one of them is particularly for patients with sarcoidosis and as adjuvant therapy for rheumatoid arthritis patients in clinical remission established by anti-TNF administration (ClinicalTrials.gov identifier: nCT02134717; ClinicalTrials.gov identifier: nCT00979771). Despite the apparent lack of efficacy in chronic inflammatory diseases, Maraviroc is approved for the use in HIV patients. This use stems from the use of CCR5 by the virus to infect leukocytes [84,85]. Several other CCR5 antagonists are currently being investigated for use in HIV patients.

CCR3 and CRTh2. CCR3 is considered a promising targets for allergic diseases and notably asthma, in part because it is expressed and its functions are associated with recruitment of a range of leucocyte types involved in the pathogenesis of these disorders, including eosinophils, basophils, mast cells and subpopulations of Th2 cells [86,87]. Indeed, during the trigger of allergic responses, Th2 cells release IL-4 and IL-13, which induce the expression of several chemokines, such as CCL11, CCL24 and CCL26, which mediate the attraction of more Th2 cells and eosinophils

to the inflammatory site via CCR3 [86]. It has been shown that deficiency or inhibition of CCR3 promoted reduction of clinical features in mouse models of allergic airway inflammation [88-90]. Differently from experimental models in which leucocytes types that participate of allergic conditions are well established, a central issue in human allergic diseases, particularly asthma, is defining what cell type is the most 'pathogenic'. CCR3 is expressed by eosinophils, mast cells, basophils and only by a subset of Th2 cells in humans [89] Oral administration of the compound GW766994 developed by GlaxoSmithKline in patients with asthma did not improve the clinical features of asthma in treated patients as compared to placebo, suggesting that other mechanisms in addition to leucocyte recruitment mediated by CCR3 could be involved in the development of airway hyperresponsiveness in humans [91] (ClinicalTrials.gov identifier: nCT01160224).

The receptor for prostaglandin PGD2, CRTh2 (chemoattractant receptor–homologous molecule expressed on Th2 cells), is present only in allergic-type inflammatory cells, including Th2 cells, eosinophils and basophils. CRTh2-mediated signaling causes the chemotaxis of Th2 cells and eosinophils to inflammatory sites [92]. Thus, it is rational that compounds that are able to inhibit CRTh2 could be promising targets for allergic disorders. Indeed, administration of selective CRTh2 receptor antagonists significantly reduced airway inflammation in experimental models of asthma [93]. Clinical studies have also been conducted using CRTh2-selective antagonists. In this context, monotherapy with OC000459, a potent CRTh2 antagonist developed by Oxagen presented an improvement in symptom scores for patients with moderate asthma over placebo (ClinicalTrials.gov identifier: nCT00890877). The oral administration of another selective CRTh2 antagonist, ARRY-502 developed by Array BioPharma, also reduced Th2 mediators and clinical features in patients with mild to moderate Th2-driven asthma, providing promising insights about the insertion of CRTh2 antagonists to treat Th2-driven allergic

diseases, such as asthma, atopic dermatitis and allergic rhinitis (ClinicalTrials.gov identifier: nCT01561690).

CCR6 and CCR4. Th17 cells comprise a distinct T helper cell lineage involved in the pathogenesis of various autoimmune diseases. Th17 cells are characterized by expression of CCR6 and CCR4, and this combination of receptors marks this subset of T cells, whose functions are related to driving the migration of Th17 lymphocytes to inflammatory sites [94]. In this context, the inhibition of CCR6 attenuated several experimental Th17-mediated diseases, such as rheumatoid arthritis, multiple sclerosis, psoriasis and cancer [95-98]. Despite many evidences demonstrating the potential of the blockade of this receptor for treatment of Th17 mediating autoimmune diseases, there are few clinical trials being conducted at the moment. Therefore, it is necessary to perform more studies to clarify whether CCR6 inhibition could be a robust target to develop new drugs.

There are many evidences that support the pivotal role of CCR4 in the progression of disorders mediated by Th17 cells trafficking [99]. In this context, the genetic ablation of CCR4 has been reported to reduce EAE [100]. Furthermore, it has also been demonstrated that patients with systematic lupus erythematosus (SLE) and multiple sclerosis (MS) have a high expression of this receptor on T CD4+ producing IL-17 cells, indicating that the development of CCR4 inhibitors could be emerging therapeutic targets for these disorders [101,102]. However, until the moment there are no reports from clinical trials assessing the possible therapeutic effects of compounds able to inhibit CCR4 activity during the course of autoimmune diseases.

On the other hand, recent studies have shown that the blockade of CCR4 is an emerging treatment for cancer. Controversially, Th17 cells share common chemokine receptors and homing properties with another population of CD4+ T cells classified

as regulatory T (Treg) cells, responsible to orchestrate suppressive responses in the tumoral site, facilitating tumor growth [94]. Among these shared receptors, CCR4 is present in the surface from both cell types and it is implicated in the participation of Treg cells recruitment into tumoral site [94]. In this way, it has been demonstrated that the blockade of CCR4 prevents the migration of regulatory T (Treg) cells to tumor site [103]. Additionally, other investigations have described the beneficial effects of CCR4 administration controlling tumor progression by others cellular mechanisms. It was demonstrated that CCR4 antagonists induce an antigen-specific CD8+T cells associated with the control of tumor progression [98]. Moreover, studies using a mAb human against CCR4 (mogamulizumab), which exhibits a potent cytotoxicity via antibody-dependent cellular cytotoxicity for cells expressing CCR4, demonstrated to be promising in the earlier phase of the trials involving patients affected by leukemia/linfoma (Clinicaltrials.gov identifier nCT00355472). In fact, the efficacy of administration of mogamulizumab is associated with the notion that around 90% of leukemia patients have expression of CCR4 on tumor cells [104], and therefore mogamulizumab promotes the depletion of the majority of the neoplastic cells [105].

CCR9. CCR9 comprises the only receptor for CCL25, which is expressed on lymphocytes of intestinal lamina propria, dendritic cells (DCs) and thymocytes [106,107]. For instance, the interaction between CCL25 and CCR9 contributes to T cell and DC migration and movement of T cells in the thymus [108,109]. In addition, it was reported that there is high CCR9 expression on leucocytes during inflammatory conditions such as RA [110]. Experimental studies have shown the potential in the development of new drugs using the blockade of CCR9 as therapeutic strategy to treat several inflammatory disorders, including arthritis and colitis [108,111]. Subsequently, clinical studies were conducted addressing the therapeutic efficacy of CCR9 antagonists in the context of human inflammatory diseases. Vercinon (also

known as Traficet-EN or CCX282) developed by ChemoCentryx is a Phase III-ready drug candidate for the potential treatment of patients with moderate-to-severe Crohn's disease and one of two forms of inflammatory bowel disease (IBD) [112]. In accordance, other pharmaceutical companies have developed CCR9 antagonists, such as GSK1605786 developed by GlaxoSmithKline, which is in phase 2 for the treatment of small bowel and Crohn's disease (ClinicalTrials.gov identifier: nCT01658605; ClinicalTrials.gov Identifier: nCT01316939).

Integrin inhibitors

The integrins, as previously mentioned, have a crucial role for interactions of leukocytes with endothelial cells, other cell types and with matrix structures during leucocyte extravasation and activation [113]. Therefore, several new therapeutic strategies have been developed for inflammatory diseases aiming to inhibit integrin function/activity and, subsequently to prevent cellular adhesion of leucocytes to endothelial cells in the transmigration process. In this context, natalizumab is a mAb against both α4β1 (VLA-4) and α4β7 (developed by Biogen-Idec and Elan) that already is inserted in the schedule of the treatment for multiple sclerosis and Crohn's disease, mainly recommended for resistant individuals to available first-line disease-modifying therapy [114-116]. Additionally, Firategrast (a small molecule α4 antagonist developed by GlaxoSmithKline) recently completed phase I in clinical trials for the treatment of multiple sclerosis. Further Phase II studies are being conducting to assess the effects on white blood cell counts in the cerebrospinal fluid [117]. Still, IVL745 and IVL745, small molecule antagonists of α4β1, were previously tested on allergen-induced bronchoconstriction in patients with asthma [118]. However, the clinical trials aimed to treat asthmatic patients failed to demonstrate efficacy greater than that observed with placebo [117].

Another blocker of integrins approved for the use in humans is efaluzimab, which comprises a humanized mAb against CD11a-CD18 (LFA-1), an important integrin that binds to endothelium ICAMs and that is needed for T cells activation [119]. Efaluzimab was approved for psoriasis treatment and it is pointed as one of most effective treatments for this disorder [120-122]. At the moment, other studies are being conducting to validate the efficacy of the efaluzimab treatment for others inflammatory diseases, such as type I diabetes (ClinicalTrials.gov identifier nCT00737763).

Interestingly, others novel targets have been identified and shown to control leucocyte recruitment by mediating leukocyte adhesion to vascular surfaces. Indeed, the developmental endothelial locus-1 (Del-1), a glycoprotein secreted by endothelial cells originally described for its role in embryonic vascular development [123], inhibits the interaction of integrin LFA-1 with intercellular adhesion molecule ICAM-1 on endothelial cells, limiting the firm adhesion of leukocytes on vascular endothelium and subsequent reduction of leucocyte recruitment [124]. In this way, some studies have suggested that Del-1 is a potential target to develop new drugs to treat inflammatory diseases such multiple sclerosis and periodontitis [125,126]. There are no reports confirming the usefulness of blocking Del-1 in humans.

In general, a range of promising approaches in pharmacological targeting integrins are being developed and many other drugs soon will be approved. However, clinical studies in this area must be conducted carefully addressing the safety and toxicity. This attention is also important for antagonist of chemotactic receptors and selectins, since these molecules are crucially involved in several physiological systems and in the maintenance of tissue homeostasis. As blockers of leukocyte recruitment, prolonged blockade may cause immunossupression. Indeed, administration of large

doses or wrong schedule of administration may cause increase of susceptibility to infections.

Inhibitors of intracellular signaling cascade

The complex phenomenon of leucocyte recruitment requires the activation of a well-orchestrated intracellular signaling cascade consisting of kinases, phosphatases and other pathways triggered by GPCRs. PI3K signaling, for example, has an important role in facilitating the production of inflammatory mediators in damaged tissue, including the chemokines CCL2, CCL3, and CCL5, and still has a direct effect in mediating leukocyte rolling, adhesion and diapedesis [127]. PI3Ks can be divided into three classes based on their structure, regulation and lipid substrate specificity: class I, class II and class III. Most studies, however, have focused on class I PI3Ks [128]. Genetic deficiency of class I isoform PI3Kγ leads to the attenuation of several experimental models of inflammation, including experimental autoimmune encephalomyelitis (EAE) and inflammatory arthritis [129,130]. Moreover, several novel compounds that inhibit the activity of inhibition of PI3K have been developed and assessed in a variety of models of inflammatory diseases and cancer [131,132]. It has been demonstrated that these agents can also inhibit the function of several different chemoattractant receptors simultaneously, providing an advantage in the utilization of PI3K inhibitors for all isoforms, which control more efficiently the inflammatory process. Effects of some pan-inhibitors of PI3K are being used for early stage clinical trials in oncology [133,134].

Additionally, p38 mitogen-activated protein kinase (MAPK) is a crucial signaling pathway that governs the multistep leucocyte recruitment process[135]. p38 MAPK activity affects cytoskeleton functions, providing changes in the cellular machinery that implicate in diapedesis and transmigration into inflammatory site [136]. The

activation of p38 MAPK has been described to be associated with cross-linking of L-selectin on neutrophils, exposure of neutrophils to TNF-α or LPS and stimulation of neutrophils by chemotactic factors such as fMLP [136-139]. Consistently, pharmacological inhibition of p38 MAPK signaling was previously shown by ameliorating inflammatory disorders such as asthma, inflammatory arthritis, inflammatory bowel disease, systemic lupus erythematosus and other autoimmune diseases [140-146]. Therefore, p38 MAPK inhibition also is suggested as a promising therapeutic target [147]. In this context, pharmaceutical companies developed potent inhibitors of p38 MAPK and some of them are in the phase II of clinical trials. One of these compounds developed by Hoffmann-La Roche is being evaluated in patients with rheumatoid arthritis that have an inadequate response to methotrexate (clinicaltrials.gov identifier: nCT00316771). Still, in another trial performed by Pfizer, the results addressing the efficacy of a p38 MAPK inhibitor denominated PH-797804 were promising to future directions in the therapy of patients with rheumatoid arthritis (clinicaltrials.gov identifier: nCT00383188; clinicaltrials.gov identifier: nCT00620685).

Therefore, the inhibition of the intracellular signaling pathways may be an interesting way to develop new drugs to treat inflammatory diseases. Besides the signaling pathways previously described, others are also essential for the complex process of leukocyte recruitment, including SHIP and ERKs [148,149]. Thus, inhibition of these pathways may also represent targets for development of new therapeutic strategies. However, the experimental and clinical studies aimed to develop inhibitory of intracellular signaling pathways to treated inflammatory diseases must be carefully addressed since the systemic inhibition of these pathways can affect the functionality and homeostasis of several other physiological systems, with the potential for devastating side effects. Indeed, the adverse effects reported through clinical studies focusing on inhibitors of intracellular pathways, such as PI3K and p38 MAPK, include neurotoxicity and hepatotoxicity associated with hyperglycaemia, maculopapular

rash, gastrointestinal intolerance (anorexia, nausea, vomiting, dyspepsia, diarrhoea), and stomatitis [150,151]. For example, one of the consequences provided by administration of PI3K inhibitors is the development of a hyperglycemic state, since PI3K is a key component in the insulin signaling pathway and inhibition of insulin signaling leads to insulin resistance and increased glucose levels in blood [152,153]. Thus, despite their potential, full knowledge of efficacy and side effects is necessary for these drugs to find their potential in clinical practice.

Conclusion and perspectives

Chronic inflammatory diseases in humans are complex diseases of long duration and frequently diagnoses when there is a considerable amount of tissue dysfunction and, hence, a degree of irreversibility. Potential targets based on expression in disease, ex vivo and in vitro studies and preclinical models are many as discussed above and briefly overviewed in **Table 2**. However, the ultimate testing is in humans. Clinical trials in chronic diseases are usually costly, especially because chronic end points are necessary and there is some treatment (even if not fully effective) for many, if not most, chronic inflammatory diseases. The need to compare against existing therapies enhances significantly the cost of clinical trials. An “ideal drug” should: 1) preferentially be given and active by the oral route ; 2) cause a minimum of adverse events; 3) be easy to manufacture; 4) be effective for the treatment of multiple diseases [154].

Chemoattractant receptors belong to the GPCR family and are, thus, traditionally seenas good ‘druggable’ candidates for small molecules. This is a reason why pharmaceutical industry has embraced chemoattractant receptors, especially chemokine receptors, with optimism. The potential for the development of new drugs using this approach is clear, but there have been few advances. It is not clear why

these targets have not met the expectation initially raised. Perhaps there is a need for better knowledge of the participation of the various chemoattractants and their receptors in various diseases. In addition, the idea that antagonists at these receptors may yield broad acting anti-inflammatory strategies may be unrealistic, as chemokine (and other chemoattractant) expression is tightly regulated, with a degree of redundancy and various participations in different organs and diseases [155]. Intracellular targets are of significant interest, potentially broader acting and with greater anti-inflammatory potential. Side effects may be more common and their use needs to find the correct place in clinical practice. Antibodies against cytokines or adhesion molecules have revolutionized treatment of multiple inflammatory diseases and have received much attention from companies. These are relatively easier to develop and known to be effective in various diseases, depending on the target. Antibody-based therapies have removed much of the interest and resources in developing small molecules, but we believe that there is still a potential for development of novel orally-active novel anti-inflammatory compounds. In addition, the field of inflammation resolution is just in its early ages and may also unravel novel targets for drug development [156,157].

Figure Legends

Figure 1 – Schematic representation multiple processes involved in the leucocyte recruitment.

The general paradigm of leukocyte recruitment involves initial contact with endothelium (tethering) and weak cellular adhesion. This step is mainly mediated by selectins. Shear forces lead to leukocyte movement along the endothelium (rolling) with further leukocyte adhesion, which are mediated by different integrins. At this time point, leukocytes crawl intravascularly seeking for “hot spots” for migration. Once emigrated, these cells digest the connective tissue components adjacently to

the vessel to reach the inflammatory focus. Of note, these steps might differ within the multiples vasculatures within the tissues.

Figure 2 – Administration of CCR2 antagonist in the improvement of clinical features in an experimental arthritis model. Increase of CCR2 mRNA expression in blood neutrophils isolated from RA patients compared to healthy controls (left painel). Markedly reduction of the neutrophil recruitment to the joints of mBSA-immunized mice challenged with mBSA or CCL2 under influence of CCR2 blockade (with RS504393, 2 mg/kg subcutaneously) (right painel). From Talbot et al., 2015 (License to publish number: 374259014418).

Tables

Table 1 – Variety of chemoattractants types and their target receptors.

Table 2 – Molecules involved in the leucocyte recruitment process as therapeutic targets for inflammatory diseases.

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Figure 1

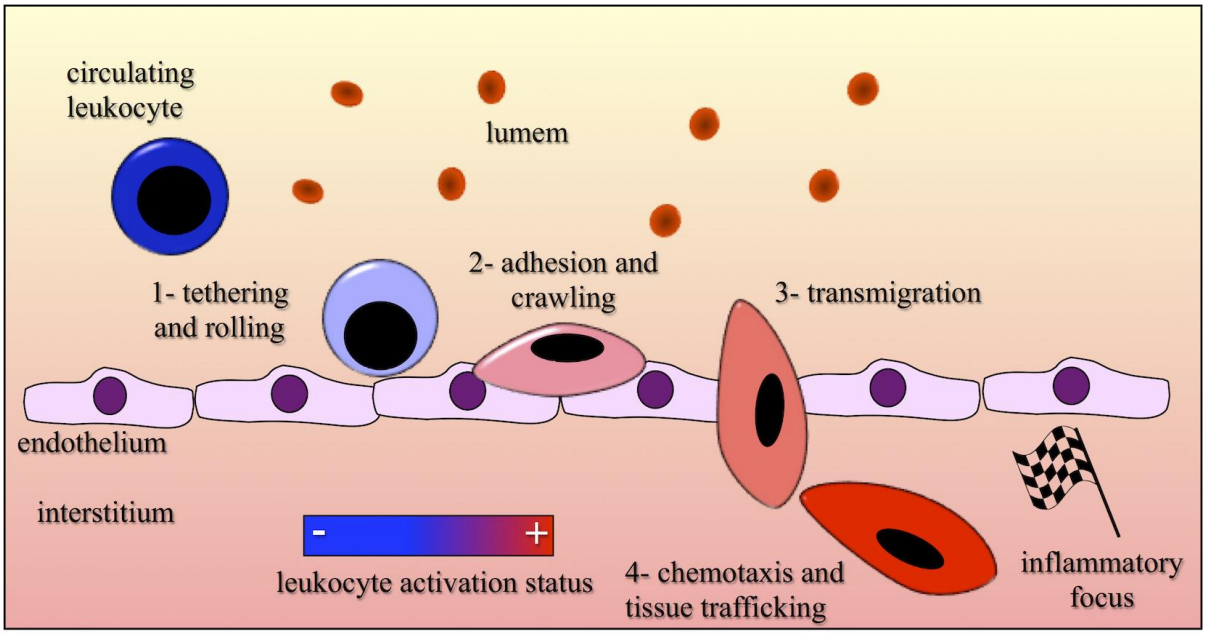
circulating
leukocyte
lumem
1- tethering
and rolling
2- adhesion and
crawling
3- transmigration
endothelium
interstitium
-
+
leukocyte activation status
4- chemotaxis and
tissue trafficking
inflammatory
focus

Table 1 – Variety of chemoattractants types and their target receptors.

Chemoattractant type	Examples	Target receptor
Lipids	LTB_4	BLT1, BLT2
	PGD_2	DP1, DP2
	PAF	PAFR
	5-oxo-ETE	OXE receptor
Short chain fatty acids	Acetate	GPR43
	Proprionate	GPR43
	Butyrate	GPR43
Small peptides and proteins	CXCL1 (KC)	CXCR2
	CXCL2 (MIP-2α)	CXCR2
	CXCL3 (MIP-2β)	CXCR2
	CXCL4 (PF4)	?
	CXCL5 (ENA-78)	CXCR2
	CXCL6 (GCP-2)	CXCR1, CXCR2
	CXCL7 (NAP-2)	CXCR2
	CXCL8 (IL-8)	CXCR1, CXCR2
	CXCL9 (Mig)	CXCR3
	CXCL10 (IP-10)	CXCR3
	CXCL11 (I-TAC)	CXCR3
	CXCL12 (SDF-1)	CXCR4
	CXCL13 (BLC, BCA-1)	CXCR5
	CXCL14 (BRAK)	?
	Cxcl15 (Lungkine)	?
	CXCL16	CXCR6
	CCL1 (I-309)	CCR8
	CCL2 (MCP-1)	CCR2
	CCL3 (MIP-1α)	CCR1, CCR5
	CCL4 (MIP-1β) CCR5	CCR5
	CCL5 (RANTES)	CCR1, CCR3, CCR5
	CCL6 (C-10)	?
	CCL7 (MCP-3)	CCR2, CCR3
	CCL8 (MCP-2)	CCR1, CCR2, CCR3, CCR5
	Ccl9/10(MIP-1γ)	?
	CCL11 (Eotaxin-1)	CCR3
	CCL12 (MCP-5)	CCR2
	CCL13 (MCP-4)	CCR2, CCR3, CCR5
	CCL14 (HCC-1)	CCR1
	CCL15 (Leukotactin-1)	CCR1, CCR3
	CCL16 (HCC-4)	CCR1, CCR2, CCR5
	CCL17 (TARC)	CCR4
	CCL18 (PARC)	CCR8
	CCL19 (ELC)	CCR7
	CCL20 (MIP-3α)	CCR6
	CCL21 (SLC)	CCR6, CCR7
	CCL22 (MDC)	CCR4
	CCL23 (MPIF-1)	?
	CCL24 (Eotaxin-2)	CCR3
	CCL25 (TECK)	CCR9
	CCL26 (Eotaxin-3)	CCR3, CX3CR1
	CCL27 (CTAK)	CCR10
	CCL28 (MEC) CCR3, CCR10	CCR3, CCR10
	XCL1 (Lymphotactin α) XCL2	XCR1
	(Lymphotactin β)	XCR1
	CX3CL1 (Fractalkine)	CX3CR1
	C3a	C3aR
	C5a	C5aR

Figure 2

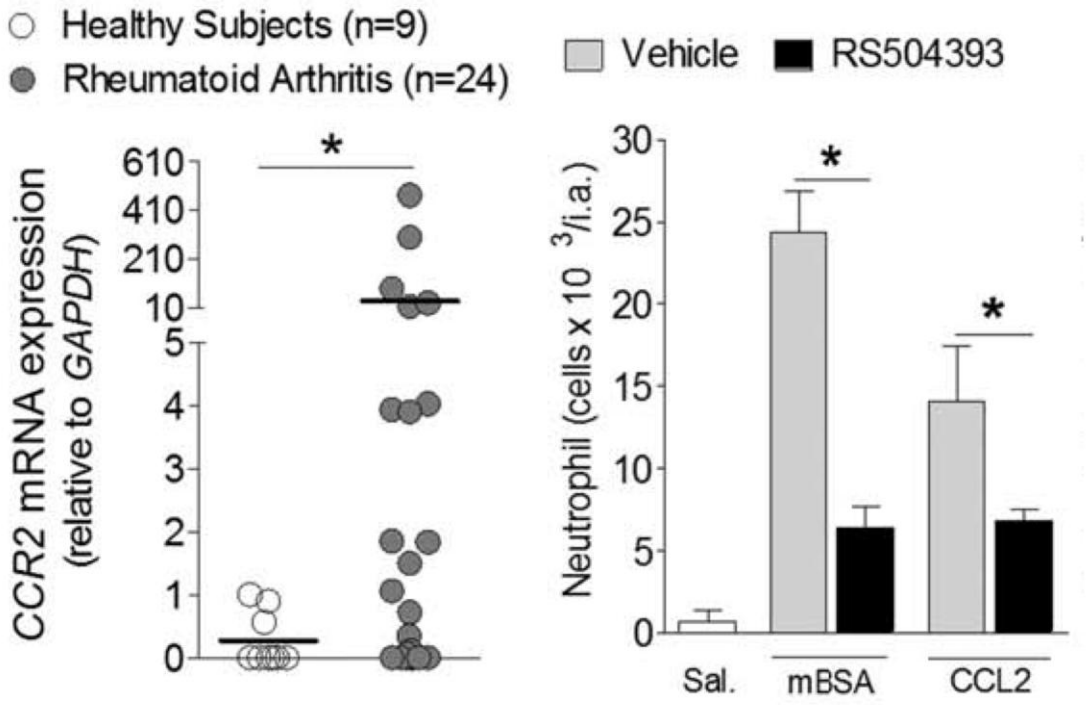
Healthy Subjects (n=9)
Rheumatoid Arthritis (n=24)
CCR2 mRNA expression (relative to GAPDH)
610
410
210
10
5
4
3
2
1
0
*
Vehicle
RS504393
Neutrophil (cells x 10 3/i.a.)
30
25
20
15
10
5
0
*
*
Sal.
mBSA
CCL2

Table 2 – Molecules involved in the leucocyte recruitment process as therapeutic targets for inflammatory diseases

Disease Indication	Target molecules	References
Rheumatoid Arthritis	CXCR2	Jacobs et al., 2010
	CCR2	Ogata et al., 1997
		Szekanecz et al., 2006
		Talbot et al., 2015
	C5aR	Andersson et al., 2014
		Moriconi et al., 2014
	CCR5	Doodes et al., 2009
	CCR6	Hirota et al., 2007
	CCR9	Yokoyama et al., 2014
	PI3Kγ	Camps et al., 2005
	p38 MAPK	Page et al., 2010
		Guma et al., 2012
Multiple sclerosis	α4 integrin	Polman et al., 2006
		Coisne et al., 2009
		Kappos et al., 2011
		Millard et al., 2011
	CXCR2	Kerstetter et al., 2009
	CCR2	Charo et al., 2006
	CCR4	Forde et al., 2011
	CCR6	Liston et al., 2009
	PI3Kγ	Berod et al., 2011
Psoriasis	Selectins	Friedrich et al., 2006
	LFA-1	Gordon et al., 2003
		Lebwohl et al., 2003
		Leonardi et al., 2005
	CXCR2	Sumida et al., 2014
	CXCR3	Johnson et al., 2007
	CCR6	Hedrick et al., 2009
Asthma	Selectins	Rosen et al., 2005
	α4β1 integrin	Norris et al., 2005
	CXCR2	Leaker et al., 2013
	CCR3	Ponath et al., 1996;
		Ma et al., 2002
		Fulkerson et al., 2006
		Neighbour et al., 2014
	CRTh2	Pettipher et al., 2007
	p38 MAPK	Haddad et a., 2001
		Lea et al., 2015
Cancer	CCR2	Fang et al., 2012
	CCR4	Bayry et al., 2014
	CCR6	Pere et al., 2011
	PI3K	Rodon et al., 2013
		Thorpe et al., 2015
Atherosclerosis	Selectins	Cayatte et al., 2001
	CCR2	Boring et al., 1998
	CCR5	Zernecke et al., 2006
		Tacke et al., 2007
COPD	Selectins	Watz et al., 2013
	CXCR2	Rennard et al., 2015

*Graphical Abstract (for review)

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