HISTORY OF CHEMOATTRACTANT RESEARCH

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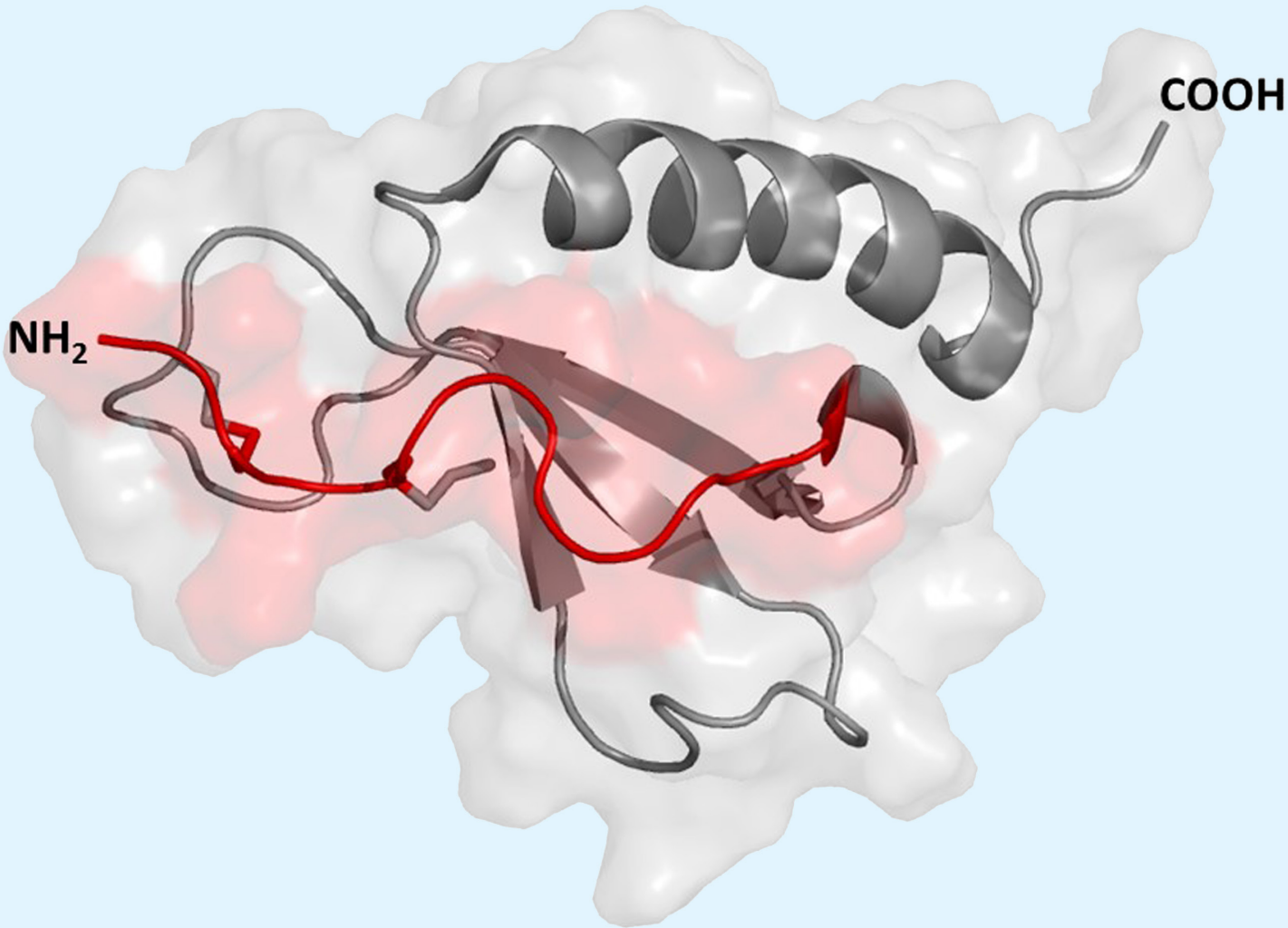

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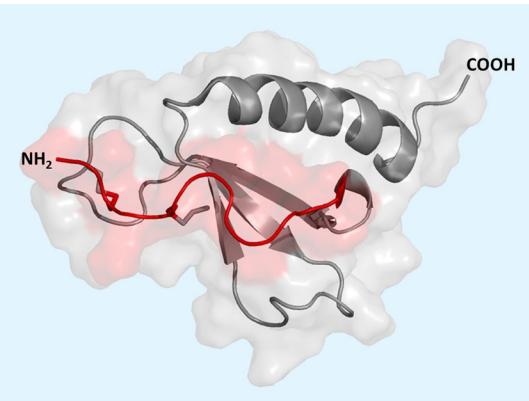
HISTORY OF CHEMOATTRACTANT RESEARCH

Topic Editor:
Bernhard Moser, Cardiff University, UK



Spacefilling and ribbon composite diagram of human CXCL14. Protein structure data stored in RCSB Protein Data Bank (http://www.rcsb.org/pdb/) were analyzed using the PyMOL programme. The help of Dr. David Cole, Cardiff University, in preparing this figure is acknowledged.

In the Research Topic "History of Chemoattractant Research" we will portray some of the key discoveries that helped to transform cell migration research into a global playing field within immunology (and beyond). Early progress had a profound effect on both, academia and industry. Today, numerous academic laboratories are fully engaged in compiling a detailed road map describing the highly complex network of immune and tissue cells that respond to chemoattractants. Industrial research, on the other hand, centers on drugs that interfere with immune cell traffic in inflammatory diseases and cancer.

The following series of "short stories" provide personal accounts on key discoveries. The individual molecular discoveries enabled numerous research laboratories worldwide to unravel their significance in steady-state or pathological immune processes. Although ground-breaking in their own right, it is therefore worth emphasizing that rapid progress in chemoattractant research was made possible by many other laboratories who were not directly involved in the original discovery process. Therefore, the authors of this mini-series are discussing their findings in the context of time, place and subsequent progress enabled by their discoveries. It is hoped that a wide readership will find these accounts entertaining as well as educational although those who wish to gain a more detailed knowledge are referred to the many outstanding reviews on chemokines and other chemoattractants.

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Editorial: History of Chemoattractant Research

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Keywords: history, chemokines, chemoattractants, migration, immunity

This Research Topic entitled "History of Chemoattractant Research" collects a series of personal stories by numerous experts in the field of chemoattractant research. The individual contributions portray some key discoveries that helped to transform cell migration research into a global playing field within immunology (and beyond). Early progress had a profound effect on both academia and industry. Today, numerous academic laboratories are fully engaged in compiling a detailed road map describing the highly complex network of immune and tissue cells that respond to chemoattractants. Industrial research, on the other hand, centers on drugs that interfere with immune cell traffic in inflammatory diseases and cancer.

By definition, chemoattractants include early ("classical") chemoattractants of variable chemical composition and the large family of chemokines (chemotactic cytokines) that greatly outnumber the former compounds. As inferred from their name, all chemoattractants share the ability to induce cell migration (chemotaxis) via binding to a single class of G-protein-coupled receptors on target cells. Chemoattractant research was originally viewed as a specialty subject within cell biology. However, due to the increasing number of chemoattractants being discovered and their effect on every type of immune cells distributed throughout our body, it became quickly clear that chemoattractants constitute essential regulators of all aspects in immunity. Defects in the chemoattractant system are frequently associated with immunodeficiencies or autoimmunity/chronic diseases. We now know that the complexity of the chemokine and classical chemoattractant system perfectly mirrors the multitude of immune cells distinguished by lineage relationship, function, and tissue location. In fact, chemokine receptor profiling turned out to be highly useful for defining immune cell subsets as exemplified by the numerous T-helper subsets that we know today. Indeed, such work has led to a fundamental paradigm linking the functional specialization of distinct immune cells with their migratory behavior. No doubt, the principal and unifying function of chemoattractants is their ability to induce directional cell migration, involving processes as complex as immune cell transendothelial migration as well as chemokine gradient-controlled immune cell migration within tissues. In addition, some chemokines are able to costimulate T-cell differentiation, promote immune cell survival, or act as antimicrobial peptides in peripheral epithelial tissues. A few constitutive chemokines are essential for organ development during embryogenesis and some of these even control tumor cell relocation to secondary sites. Their importance is further emphasized by the realization that viruses have hijacked host genes encoding chemokines and their receptors in order to interfere with antiviral immunity or have evolved to use certain chemokine receptors as entry coreceptors.

The following series of "short stories" provide personal accounts on key discoveries. The individual molecular discoveries enabled numerous research laboratories worldwide to unravel their significance in steady-state or pathological immune processes. Although groundbreaking in their own right, it is worth emphasizing that rapid progress in chemoattractant research was only made possible by many other laboratories whose work attached "meaning" to these early findings. The authors of this miniseries are discussing their findings in the context of time, place, and subsequent progress enabled by their discoveries. It is hoped that a wide readership will

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find these accounts entertaining as well as educational although those who wish to gain a more detailed knowledge are referred to the many outstanding reviews on chemokines and other chemoattractants.

The field of chemokines really started in 1987 with the cloning of the human gene encoding CXCL8, which occurred in parallel in the laboratories of five independent international groups. Two stories, one by Marco Baggiolini (1) and the other by Teizo Yoshimura (2), summarize this groundbreaking discovery and give a vivid account about the friendly race that ensued from the realization that activated monocytes secreted neutrophil-specific chemoattractant activity to the molecular discovery of CXCL8. Unfortunately, and probably due to the enthusiasm shared by the research community at that time, it was decided to call CXCL8 an interleukin (IL-8), which turned out to be a misleading denomination. The three-dimensional structure of CXCL8 is a hallmark of all members of the chemokine superfamily and indicated that, in fact, chemokine-like proteins have been identified several years before CXCL8. These include IP10 (CXCL10) (3), LD78 (CCL3) (4), and TCA3 (5), the mouse ortholog of human I-309 (CCL1) (6). However, their chemoattractant activity remained obscure until well after the discovery of CXCL8. Also, platelet factor 4 (CXCL4) (7–9), the first peptide featuring a prototypical chemokine fold, was actually never shown to be a chemoattractant. The identification of CXCL8 immediately initiated a highly competitive search for its receptor(s) and the receptors for the well-described classical chemoattractant agonists, including the formylated bacterial peptide fMLP and the complement protein C5a. Norma and Craig Gerard summarize these early events from their own, personal perspective (10). By the early 1990s, the new field of chemokines took off in unprecedented speed, and it became quickly clear that the newly discovered chemokines not only targeted neutrophils but also monocytes and many other innate cells and even T and B cells. Tim Williams tells the exciting story about the discovery of eotaxin (CCL11) and its involvement in eosinophil recruitment during allergic diseases (11). Chemokines are implicated not only in infections and inflammatory diseases but also in homeostatic processes. The first such chemokine is SDF-1 (CXCL12), and Takashi Nagasawa tells his story about the importance of SDF-1 in embryogenesis, hematopoiesis, and even HIV infection (12). Unlike SDF-1, most homeostatic chemokines do not display a lethal phenotype in gene-deficient mice yet play an essential role in the traffic control of immune cells. Early work with orphan chemokine receptors in mice provided a first indication of the importance of chemokine receptors, notably BLR1 (CXCR5) (13) and BLR2 (CCR7) (14), in controlling cellular interactions within secondary lymphoid tissues (lymph nodes and spleen). I tell the story about how we at the Theodor-Kocher Institute and Martin Lipp's group in Berlin identified CXCR5 as the specific marker for the novel T-helper cell subset termed follicular B helper T (T_{FH}) cells (15). Not all chemokine receptors are capable of mediating cell migration responses, and these "non-signaling" receptors are now collectively called atypical chemokine receptors (ACKR). Three of these, ACKR1, ACKR2, and ACKR3, with unique functional features are subject of extensive investigations. ACKR3, previously known as RDC1 or CXCR7, binds CXCL12 with higher affinity than its primary receptor CXCR4 and, in addition, binds also CXCL11, one of the three CXCR3-specific chemokines (16, 17). ACKR3-deficient mice die *in utero* (18), suggesting a vital role in embryogenesis similar to what has been reported for CXCR4. Richard Horuk tells the story about ACKR1, also known as DARC or Duffy Antigen on red blood cells (19). It is an entry receptor for the malaria parasite *Plasmodium vivax* and, surprisingly, acts as a transcellular transporter of CXCL8 and many other chemokines in endothelial cells. The story about ACKR2, also known as D6, is told by Gerard Graham (20) and highlights yet another facet in chemokine research, namely the modulation of inflammatory milieus by ACKR2 via binding, uptake, and intracellular degradation of inflammatory chemokines. Chemokines do not act like normal cytokines do. In fact, immune cells expressing the corresponding chemokine receptors need to sense a chemokine gradient, and Amanda Proudfoot highlights the importance of glycosaminoglycans present on extracellular matrices in this process (21). The story by Paolo Lusso (22) describes how his groundbreaking discovery led to the immediate fusion of two seemingly unrelated fields of research, "chemokines" and "HIV infection," fostering unprecedented collaborations between many international laboratories. His discovery of CCL3, CCL4, and CCL5 that acted as HIV-suppressor factors demonstrated that certain chemokines and possibly their receptors were involved in HIV infection. Almost simultaneously, fusin was reported to be the first HIV coreceptor, and this story is told by Edward Berger (23). Fusin turned out to be identical with the orphan chemokine receptor LESTR that we have published previously. In collaboration with Conrad Bleul, we then "deorphanized" LESTR by showing that this new chemokine receptor (CXCR4) is specific for CXCL12. Fernando Arenzana-Seisdedos summarizes these events and tells the story about CXCL12 and its HIV-suppressor activity (24). CXCR4 is not the only HIV coreceptor. Indeed, CCL3, CCL4, and CCL5, the HIV-suppressor factors, previously discovered by Paulo Lusso do not bind to CXCR4. The story by Marc Parmentier fills this gap and reveals that several groups worldwide, including his own, discovered CCR5 as the specific receptor for CCL3, CCL4, and CCL5 (25). CCR5 is the coreceptor primarily involved in person-to-person transmission of HIV, and individuals lacking CCR5 are largely protected against HIV. In clear contrast to HIV, many viruses carry genes that target the chemokine system, encoding either inhibitors that interfere with the function of chemokine receptors present on host immune cells or chemokine-neutralizing proteins with similarities to chemokine receptors, and Philip Murphy's story touches on this important aspect of chemokine research (26). Chemokines and their receptors play a crucial role not only in viral diseases but also in all other inflammatory diseases as well as cancer. It is, therefore, obvious that chemokine receptors were selected as primary targets in translational research. Detailed structural data of chemokine receptors are of paramount importance for the design of small-molecular-weight inhibitors, and Tracy Handel's story tells about the difficult journey she undertook to accomplish a high-resolution crystal structure of CXCR4 (27). Despite incredible investments by all major drug companies (as well as many small start-up businesses), the yield of approved chemokine receptor-specific drugs is still modest. In fact, the two success

stories about FDA-approved compounds are not related to the treatment of inflammatory diseases. The first one by Elna van der Ryst tells the development of Maraviroc, a CCR5 antagonist used to treat HIV-infected individuals (28), and the second one by Erik de Clerk summarizes the discovery of the CXCR4-specific inhibitor AMD3100 and its use in hematopoietic stem cell mobilization (29).

Chemokine research goes on unabated although the race of molecular discoveries as highlighted here has well past its zenith. All major activities are now focused on understanding what all these original findings really mean. The field has progressed along so many different and seemingly unrelated routes that writing comprehensive reviews that cover all aspects of chemokine research has become a monumental task. One thing is certain, however, the last two decades have demonstrated once and for all that chemoattractant research can no longer be considered a subspecialty of cell biology.

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OPINION
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CXCL8 – the first chemokine

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Keywords: CXCL8, chemokines, neutrophil leukocytes, Naf, IL8

After spending 2 years at the Rockefeller University in New York, as a research associate in the laboratory of Christian de Duve, I accepted an offer from Sandoz Ltd., which was attractive in terms of space, equipment, and research facilities, and returned to Switzerland. *Beatrice Dewald*, joined my laboratory after several years at NYU and Vanderbilt University, and we continued our studies of the enzymes of neutrophil granulocytes initiated at Rockefeller, with particular attention to neutral proteinases, their release and their role in tissue damage and inflammation. We went back to academia in 1983, when I became director of the Theodor Kocher Institute, a unique institution for graduate studies, associated with the Faculties of Sciences and Medicine of the University of Bern, which everybody called TKI. Theodor Kocher, both surgeon and scientist, was awarded the Nobel Prize in 1909 "*for his work on the physiology, pathology, and surgery of the thyroid gland*." He firmly believed in the role of basic research for medical progress, and donated his prize money as an initial contribution to the construction of a dedicated interfaculty institute.

At the TKI, research on human leukocytes in inflammation and host defense continued with new colleagues, *Alfred Walz*, head of a laboratory for biochemistry and molecular biology, working on interferon and cytokines, and two physicists, *Dave Deranleau* and *Vinzenz von Tscharner*, who developed methods and instruments for the real-time analysis of cell activation. My experience in the pharmaceutical industry was an asset, but the TKI had something more to offer: outstanding Ph.D. students and postdoctoral fellows, a major resource for innovation. The young associates and the students kept me close to the bench and open to lateral thinking.

I am expected to narrate how the first chemotactic protein was discovered. In Bern, it all began with a surprising encounter. One evening, on the stairs of the TKI, I bumped into *Paul Imboden*, a Ph.D. student in the laboratory of *Alfred Walz*, who told me he had found in human monocyte cultures an agent that stimulates neutrophil leukocytes. No real surprise, there, I thought. Still, I proposed to test on neutrophil leukocytes the effects of the new substance and of chemotactic agonists that were known, i.e., C5a, fMet–Leu–Phe, platelet-activating factor, and leukotriene B_4. The new substance was a protein that triggered responses similar to those induced by common chemotactic agonists, but acted through a yet unknown G-protein-coupled receptor. Unlike the common agonists, which induce migration of different granulocytes and even of monocytes, the novel protein was specific for neutrophils, and we thus called it NAF, for "neutrophil-activating factor." The observed selectivity for a single type of white cells was an important new finding. We imagined, with some optimism, that NAF could be a prototype for a novel class of chemotactic proteins, and thought that the search for proteins related to NAF was going to pay off. Analogs were indeed identified, and the laboratories that pioneered such progress agreed to name the new proteins "chemokines," in abbreviation of "chemotactic cytokines."

The characterization of NAF had to be completed first. *Alfred Walz* prepared a highly purified sample of the protein, and I arranged for micro-sequencing at the Sandoz Research Institute in Vienna. To our surprise, we were not alone! Between December 1987 and April 1988, the newly discovered protein was presented in four independent papers (1–4). It was an unusual, choral announcement of four matching partial amino-acid sequences. Furthermore, the sequence data were in agreement with the cDNA-deduced sequence of a secretory protein of 99 residues that had been published a few months before. The protein was homologous to β-thromboglobulin, but its properties and function were not identified (5).

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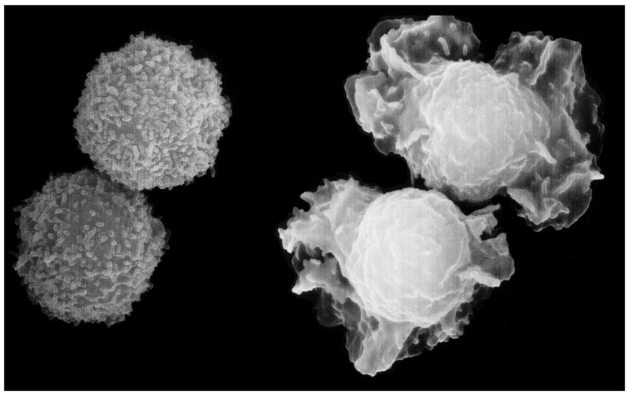

FIGURE 1 | Shape change of neutrophils, the most elegant response to chemo-attractants, observed within seconds of challenge, and characterized by the protrusion and retraction of ectoplasmic extensions, due to polymerization and breakdown of actin. The protrusions appear to function like a swimmer's arms and legs. Turbidity recordings suggest that the changes are synchronized, possibly to facilitate migration (11). Scanning electron micrograph by *Matthias Wyman*, a former Ph.D. student at TKI, now professor at the University of Basel.

Ivan Lindley, *Heinz Aschauer*, and other colleagues at the Sandoz Research Institute in Vienna, who had sequenced our purified protein, went a few steps further. They synthesized a gene coding for the 72-residue NAF, which they cloned and expressed. The recombinant protein was analyzed at the TKI, and found to be identical in activity and potency to purified, natural NAF (6).

The first chemokine had been thoroughly characterized, but still did not have a name. None of the acronyms used in the papers reporting isolation, sequencing, cloning, and expression (MDNCF, NAF, MONAP, LYNAP, etc.) being suitable, the new protein ended up with a fancy but misleading name: "interleukin 8." It was the first and the last chemokine to be taken for an interleukin.

Four papers putting forward the same message indicated that the chemokine area was important and competitive. I went to Frederick to see *Ed Leonard*, *Jo Oppenheim*, and colleagues and discuss possible collaborations. For me, that visit was also a chance to meet *Teizo Yoshimura* and *Kouji Matsushima*. We decided to continue our friendly relations without a formal collaboration, which was reasonable since there was no way of knowing how things would develop in the field and how research in our laboratories would evolve. In line with TKI traditions, we characterized NAF/IL8 using biochemical and biophysical methods, as shown in the comparison of neutrophil responses to NAF/IL8 and fMet–Leu–Phe by Thelen et al. (7). Real-time recordings of changes in cell shape, cytosolic free calcium levels, superoxide formation, and granule enzyme release showed that the responses to both chemo-attractants followed similar kinetics. In addition, the effects of both agonists were inhibited to a similar extent by pretreating the neutrophils with *B. pertussis* toxin and other inhibitors of signal transduction. Despite the similarities in response quality, we observed a clear difference between the two agonists in terms of potency, with NAF/IL8 being 10–30 times more effective than fMet–Leu–Phe.

I emphasized that NAF/IL8 is highly selective for neutrophils, but I cannot end this brief account without pointing out that the first chemokine had, in fact, additional attractant properties, with unique scientific implications: for one thing, it attracted two brilliant scientists from Vancouver to Bern, *Bernhard Moser* and *Ian Clark-Lewis*. They were both primarily interested in NAF/IL8 and wanted to identify its receptor. They also expected to find new chemokines and new receptors, and eventually to study the structural determinants for receptor recognition and activation. *Bernhard Moser* went to school in Bern, studied at ETH Zurich, and obtained a PhD degree at the University of British Columbia before returning to Bern to clone and characterize chemokine receptors, and to study white cell traffic in immune defense. *Ian Clark-Lewis* was introduced to immunology at the famous Walter and Eliza Hall Institute of Medical Research in Melbourne and specialized in chemical protein synthesis in the US and in Canada. At the Biomedical Research Centre of the University of British Columbia, he established an impressive facility for solid-phase protein synthesis. Chemokines impressed him as a promising area for studying structure-activity relations and for the design of chemically-modified analogs including receptor antagonists.

From among the thirty and more publications witnessing the productive collaboration between *Ian*, *Bernhard* and other scientists at the TKI for more than a decade, I shall quote three highlights relating to NAF/IL8. Early structure-activity relation studies identified the short amino-terminal sequence preceding the first cysteine as the site for receptor binding and triggering (8), a principle that turned out to be valid for the whole chemokine family, underscoring the prototypical value of NAF/IL8. A very extensive study using a large sample of synthetic analogs with single amino-acid exchanges revealed that except for the cysteines and the ELR motif no other residue appeared to be required for NAF/IL8 receptor binding and activity (9). The same study showed, in addition, that IP10 (a CXC chemokine that does not activate neutrophils) can be modified to a potent attractant of neutrophils by insertion of discrete sequence domains taken from the NAF/IL8 amino-terminal loop (9). *Ian* also answered a fundamental question that was raised after observing that NAF/IL8 forms dimers in solution: do chemokines act as monomers or dimers? By replacing Leu in position 25 with N-methyl-Leu in the NAF/IL8 sequence, he created a derivative that could not dimerize but nevertheless retained full activity (10), indicating that NAF/IL8 binds to its receptors and trigger responses as a monomer. For *Bernhard Moser* the work on IL8 and receptors was a sort of high-level warming up. Bernhard's major achievements came a few years later, with *Marcel Loetscher* and *Pius Loetscher* as associates, and several Ph.D. students, after moving on to arenas, which were increasingly populated by lymphocytes.

Ian Clark-Lewis died prematurely in 2002. He is much missed by those who worked with him, and saw him as a distinguished TKI-member from the West coast. He did not mind long-distance travel, and visited us regularly. He frequently took time to discuss scientific issues on the phone, in long, remarkable conversations. From Vancouver, he had set up a productive network of contacts and friendly relations with many of us.

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Discovery of IL-8/CXCL8 (the story from Frederick)

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The infiltration of leukocytes is a specific, rather than a random, event, and regulated by the production of chemoattractants that specifically attract a certain type of leukocytes. By early 1980s, chemoattractants, now referred as "classical chemoattractants," including the bacterial peptide *N*-formyl-methionyl-leucyl-phenylalanine (FMLF), the C5a fragment of serum complement and the lipid mediator leukotriene B4 (LTB4), had been identified. However, FMLF and C5a lacked cell specificity and attracted both neutrophils and monocytes. LTB4 exhibited both chemotactic and chemokinetic activities for phagocytes. There were several reports suggesting the existence of other cytokine-like chemoattractants. Lymphocyte-derived chemotactic factor (LDCF) was detected in the culture supernatant of mitogen-activated peripheral blood mononuclear cells (PBMC) (1). A neutrophil chemoattractant was also present in the culture supernatant of activated monocytes or macrophages (2). However, these cytokine-like chemoattractants were not purified or cloned.

The early 80s was the time when various cytokines were finally purified and/or cloned by immunologists owing to the technological advance in protein purification and molecular cloning. The laboratory of Joost J. Oppenheim was focused on investigations of interleukin 1 (IL-1) and established that it was produced by many cell types and had multiple biological activities by also stimulating a great variety of cell types. Subcutaneous injections of IL-1 had been shown to induce acute inflammatory responses with rapid margination of neutrophils followed by their extravascular infiltration (3). They therefore tested the chemotactic effects of partially purified human epithelial cell-derived thymocyte-activating factor (ETAF); an epithelial cell-derived IL-1. ETAF purified by elution from Sephadex gels was found to attract both polymorphonuclear and mononuclear leukocytes *in vitro* (4). Since ETAF was biochemically identical to IL-1, it was concluded that IL-1 was chemotactic. These findings were confirmed by Dinarello and his colleagues (5). In addition to IL-1, recombinant human TNF was shown to be chemotactic for neutrophils by another group (6). These findings suggested that cytokine-like chemoattractants produced by activated mononuclear leukocytes reported earlier could be attributed to IL-1 and/or TNF. However, the Oppenheim laboratory was concerned about the fact that their preparations of ETAF/IL-1were not pure and pursued opportunities to investigate more purified preparations of IL-1 (personal communication with Joost J. Oppenheim).

I studied inflammation in Hideo Hayashi's laboratory in Kumamoto, Japan from 1979 to 1985. My research was focused on three putative macrophage chemoattractants partially purified from skin extracts of guinea pig delayed hypersensitivity reaction, one of which was thought to be the guinea pig version of LDCF. Unfortunately, it was never purified to determine its identity. I met Ed Leonard in 1984 when he came to Japan to attend The 10th International Reticuloendothelial System Congress. His lab was interested in identifying the nature of chemoattractants derived from activated mononuclear leukocytes or bacteria. He expressed his interest in our guinea pig chemoattractants and recruited me to join his laboratory. In 1985, I completed my PhD project and came to Ed's lab on a fellowship supported by the Japanese Foundation for Promotion of Cancer Research. I brought partially purified guinea pig LDCF to study, but that plan was immediately scratched when Ed and Tibor Borsos, chief of our lab, found out that guinea pig skin was treated with acetone before protein extraction. They were concerned about possible artifacts due to acetone treatment.

We were very much experienced in evaluating the activity of chemoattractants by examining their potency and efficacy using the 48-well multi-well chemotaxis chambers developed by Ed (7) (**Figure 1**). In 1986, I published my first paper in Ed's lab examining the oxidation of FMLF by neutrophils and I was ready for a larger task. Since we were very intrigued to learn that the cytokine IL-1 was chemotactic for neutrophils and monocytes, we decided to examine the chemotactic activity of IL-1 in more detail. In collaboration with the Oppenheim lab, we examined the chemotactic activity of highly purified native IL-1 or recombinant IL-1. To our surprise, neither of them was chemotactic for neutrophils or monocytes. We speculated that IL-1 might indirectly attract neutrophils by inducing the release of a chemoattractant such as LTB4 by neutrophils. We tested this hypothesis, but there was no chemotactic activity in the supernatant of IL-1-stimulated neutrophils. We also examined whether IL-1 could augment neutrophil migration induced by other chemoattractants, such as FMLF, or activate basophils to release neutrophil chemoattractants. Again, IL-1 showed no effects. These results led us to conclude that the partially purified IL-1 preparation was contaminated by a neutrophil chemoattractant, which was different from IL-1. The supernatant of LPS-activated PBMC or monocytes, which

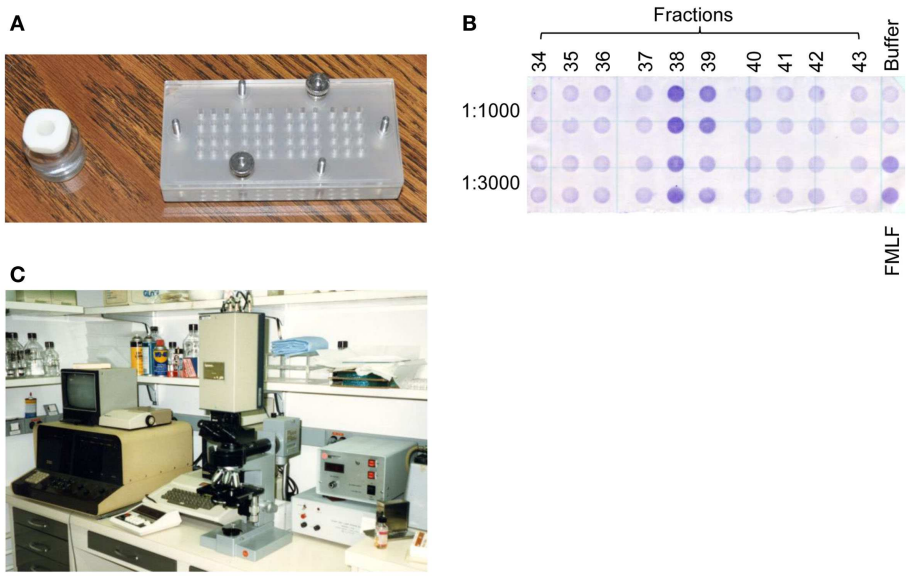

FIGURE 1 | A chemotaxis assay system used to purify MDNCF. **(A)** Previously used individual chemotaxis chamber (left) vs. 48-multi-well chemotaxis chamber (right). **(B)** Consecutive fractions eluted from a reversed-phase HPLC column were assayed in duplicate at two different dilutions (1:1000 and 1:3000) in a single chamber with a negative (buffer) and positive (FMLF) control, and migrated cells on the bottom of the filter were stained (from the original notebook). The fraction 38 had the peak activity. **(C)** The percentage of migrated cells was analyzed using an image analyzer.

are rich sources of IL-1 and used as a source of partially purified IL-1, indeed also contained a potent neutrophil chemotactic activity, further supporting our conclusion (8).

Kouji Matsushima, head of a group in the Oppenheim lab, had a vast knowledge and experience in protein purification and had just succeeded in purifying IL-1. Because the Leonard and the Oppenheim lab were both interested in identifying the protein, which contaminated crude IL-1 preparations, Kouji and I quickly established collaboration and decided to biochemically identify this activity. Since we were both from Japan, we had no language barrier. We first isolated neutrophil chemotactic activity from IL-1 activity contained in the culture supernatant of LPS-activated PBMC (8), and then achieved the first purification by the end of 1986. This protein was the first chemoattractant with target cell specificity and we termed the protein monocyte-derived neutrophil chemotactic factor (MDNCF) based on its activity and cellular source (9).

The N-terminal amino acid sequence of MDNCF was determined in Ettore Appella's laboratory, NCI. It was realized that MDNCF exhibited considerable amino acid sequence similarity at the N-terminus to β-thromboglobulin (β-TG), platelet factor 4 (PF4), and γIP-10. The β-TG precursor CTAPIII (connective tissue activating protein) stimulated replication of connective tissue cells, suggesting its role in wound healing. PF4 was a chemoattractant. γIP-10 was an interferon-induced product of the U937 monocyte-like cell line. Thus, MDNCF not only structurally but also functionally belonged to this family of small proteins involved in inflammation (9). β-TG, PF4, and γIP-10 were subsequently determined as the members of the chemokine family (10). When we submitted our manuscript to a journal for publication, we had considerable difficulty overcoming the criticisms of one of the reviewers, who maintained that detection of a cell-derived chemoattractant for neutrophils was redundant with already well known chemotactic factors such as FMLF and C5a, and was therefore not of any importance. It has recently become clear that a signal relay of multiple chemoattractants is critical for the precise trafficking of leukocytes to sites of tissue injury (11); thus, chemoattractants have a non-redundant role.

Shortly after the publication of our report in 1987, the laboratories of Marco Baggiolini and Jo Van Damme also reported the purification of the identical protein (12, 13). At a meeting in Baden, Austria, it was agreed by the co-discoverers of this cell-derived neutrophil chemoattractant to name this activity neutrophil-activating peptide (NAP-1). However, Larsen and coworkers in the Oppenheim's laboratory subsequently discovered that NAP-1 also chemoattracted a subset of T lymphocytes and they therefore proposed to rename it as interleukin-8 (IL-8) (14). Although this proposal was eventually accepted, it was disliked by the interleukin aficionados because they did not consider chemotactic effects very important (personal communication with Joost J. Oppenheim).

During the purification of MDNCF, we noticed that a monocyte chemoattractant was also present in the culture supernatant of LPS-activated PBMC. However, purification of the monocyte chemoattractant was more difficult because the amount of this chemoattractant in the supernatant was lower than that of MDNCF. Subsequently, both the Leonard and the Oppenheim laboratories independently purified the protein, monocyte chemoattractant proten-1 (MCP-1) or macrophage chemotactic and activating factor (MCAF), respectively, from the culture supernatant

of tumor cell lines and published a paper in 1989 (15, 16). We believed that MCP-1 was identical to LDCF. Unexpectedly, amino acid sequence analysis of this MCP-1 revealed the presence of four half cysteine residues at almost identical locations to those of MDNCF, although the overall amino acid sequences between the two proteins were not highly conserved. There was no extra amino acid between the first two half cysteine residues in MCP-1 (CC vs. CXC). By this time, there were reports of additional proteins belonging to this protein family, including RANTES, pLD78, TCA3, MIP, and JE. Although these proteins were later determined as chemokines (JE turned out to be the mouse ortholog of human MCP-1) (10), MCP-1 was the only protein identified based on its biological capacity to selectively attract monocytes. Identification of IL-8 and MCP-1 thus pointed out the existence of a family of chemotactic cytokines with leukocyte specificity and led to the identification of the protein family chemokines with at least two CXC and CC subgroups. This family was subsequently referred to as chemokines, an abbreviated version of "chemoattractant cytokines," by Oppenheim.

As noted above, we began our study by asking a simple question whether IL-1 was indeed a chemotactic factor. The results of the study led us to the discovery of IL-8, the first cytokine-like chemoattractant with cell specificity, and most importantly, which in turn led to the discovery of a novel protein family with chemotactic activity. This was an example of a successful collaboration between two laboratories with complementary strength. We were extremely fortunate to be the first to identify IL-8 and MCP-1 as evidenced by being awarded the patents for each, because the field was ripe for the discovery and many laboratories were just behind us.

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Cloning of the human C5a anaphylatoxin receptor, and more

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The initial observation in 1973 that the complement C5 activation product, C5a, has the ability to stimulate human neutrophils led to the concept of a specific C5a receptor (1). In the mid-1986, a groundbreaking paper published in Nature announced the cloning of the beta-adrenergic receptor, and for the first time established seven transmembrane (7TM) G protein-coupled receptors as members of the rhodopsin superfamily (2). Almost simultaneously, Feltner and colleagues demonstrated that the FMLP, C5a, and LTB4 activities on rabbit neutrophils could be inhibited by pertussis toxin, indicating coupling to GTP binding proteins (3). In 1987, Masu and colleagues used an oocyte expression cloning system to isolate a cDNA encoding the neuropeptide substance K receptor; and later, the same group identified the related substance P receptor (4, 5).

In 1989, we realized that the pertussis sensitivity of the fMLP, C5a, and LTB4 receptors suggested that they would also be members of the rhodopsin superfamily. When we aligned the handful of structures for the then known 7TM receptors (adrenergic, serotonin, dopamine, FSH/LH, and substance P and K receptors), we recognized homologies in both the transmembrane segments and intracellular loops, which presumably facilitated interactions with G proteins. This observation actually presented us with an opportunity to attempt to clone receptors by homology to the superfamily as "orphan receptors."

We constructed an antisense oligonucleotide with minimal degeneracy that encompassed a highly conserved NPXXY motif in the seventh transmembrane segment of the known rhodopsin family members. In order to enrich in C5a receptors, we took advantage of the fact that the receptors were induced by cyclic-AMP in U937 cells, and in retinoic acid differentiated human HL60 cells. By summer of 1990, we had isolated ~20 cDNAs using this approach from the cAMP induced U937 cell library. About half of these clones were an identical cDNA that we named NPIIY-18. Using this as a probe, we demonstrated that NPIIY-18 recognized a ~2.2 kb mRNA only in cAMP differentiated U937 cells. Northern blot analyses showed that NPIIY-18 was present only in cells known to express the C5a receptor. As NPIIY-18 was not a full-length cDNA, we then probed the retinoic acid differentiated HL60 cell library. We isolated a full-length DNA from this library that encoded a 7TM receptor with 25% homology to the substance K receptor and 35% homology to the human fMLP receptor (FPR1), which was cloned by Francois Boulay in May 1990 (6). When expressed in COS cells, we showed that NPIIY-18 encoded a high-affinity receptor for human C5a (**Figure 1**) (7). This work was accepted for publication in Nature in December 1990. In the summary paragraph of this manuscript, we pointed out that our approach should be helpful to clone the receptors for the leukotrienes, platelet activating factor, interleukin-8, and adenosine receptors as these are all present on cAMP differentiated U937 cells. Almost simultaneously, Francois Boulay confirmed our identification of the human C5aR, which his group accomplished by expression cloning of differentiated HL-60 cells (8). His work was published in March 1991, some 4 months after ours.

In November 1990, within months of the identification of the human FPR1, Thomas et al. reported the cloning of the rabbit receptor for fMLP, F3R (9), which had almost no significant homology to the human receptor. This was troubling, because we knew that when the ligand

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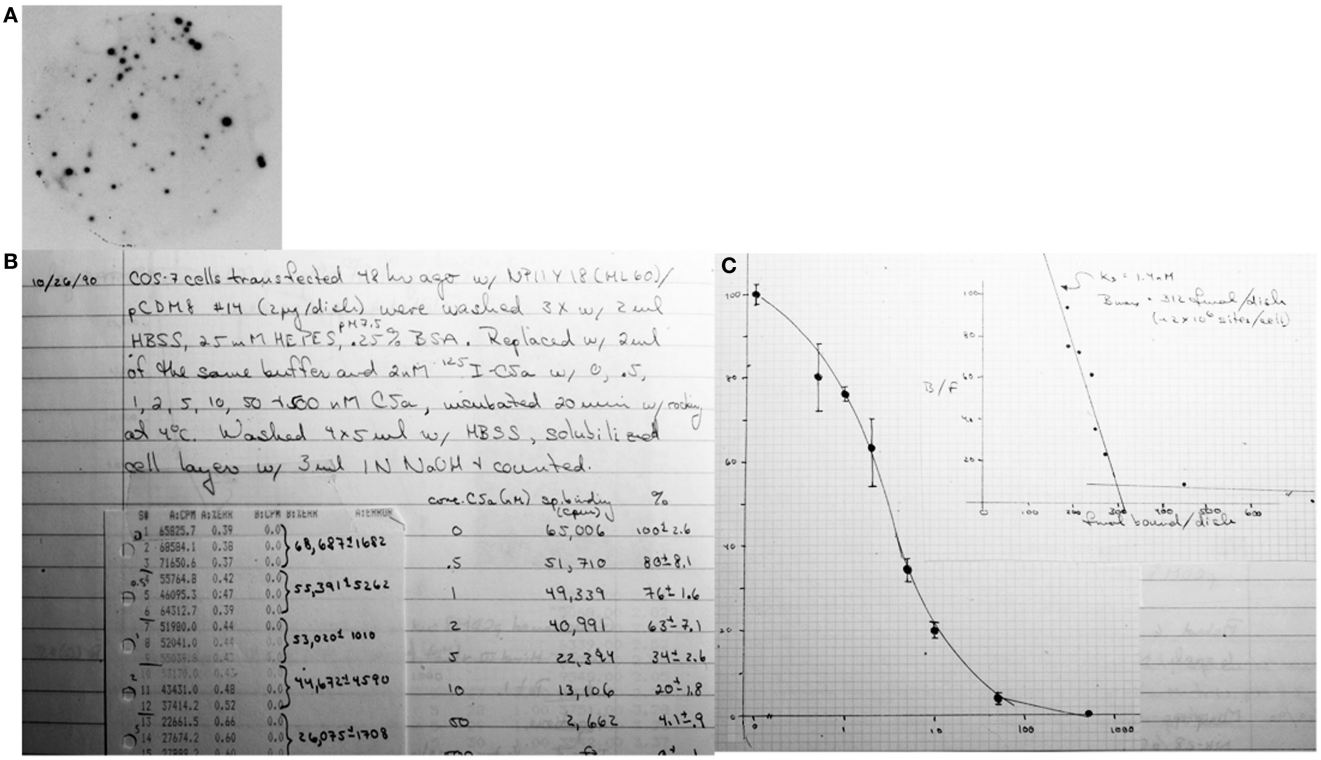

FIGURE 1 | Final steps in cloning and identifying the human C5a receptor. **(A)** Tertiary screen of clone NPIIY-18 using ^{32}P-labeled oligonucleotide probe. **(B)** NPIIY-18 was transfected into COS7 cells and tested for binding of ^{125}I-C5a under competitive binding conditions. **(C)** The resulting binding isotherm and Scatchard analysis proving the clone's identity.

was identical from species to species, the receptors were generally highly conserved. Thus, the adrenergic, dopamine, serotonin, and histamine receptors are >90% identical across species. Curiously, the cDNA was reported to bind the FPR1 radioligand and transduce calcium transients. Because the expression of the claimed rabbit F3R formyl peptide receptor was restricted to neutrophils, we wondered if, in fact, the Navarro lab had misidentified an interleukin-8 receptor. One of us (Craig Gerard) actually traveled to the Navarro lab to obtain the F3R cDNA to establish a collaboration and test its identity as a receptor for IL-8. At that time, there was no radioligand IL-8 commercially available. Henry Showell, of Pfizer Central Research, was able to provide us with a custom iodinated IL-8, which we demonstrated to bind F3R. Unfortunately, we did not have sufficient quantities of the reagent to perform comprehensive studies to publish our findings. We disclosed our result to Javier Navarro, but were left in silence. Unbeknownst to us, Dan Witt, at Repligen, had reportedly approached the Navarro lab with a similar idea. Thomas et al. went on to publish F3R as an IL8 receptor, without retracting the previous paper (10). During this time, Tom Schall and I met at a FASEB meeting with Phil Murphy, and suggested to him that he use F3R to clone a human homolog from HL60 cells and test it against IL8. The landmark Murphy and Tiffany paper resulted (11). Phil offered one of us (Craig Gerard) coauthorship for the helpful suggestion but because of intellectual property concerns at our institution, we requested an acknowledgment instead.

Over the next decade, the orphan receptor approach led to the identification of a wide variety of chemoattractant receptors, including most of the chemokine receptor system. The most notable events in the area of chemokines occurred when CXCR4 and CCR5 were identified as HIV coreceptors. It was known from the work of Ed Berger that CXCR4 was the obligate coreceptor with CD4 for laboratory-adapted strains of HIV (12). However, the wild type, the so-called macrophage tropic strain used an unknown coreceptor. In December 1995, it was reported in the New York Times that the Gallo laboratory had identified Mip1α, Mip1β, and RANTES as substances that inhibited HIV infections (13). Coincidentally, at the Fourth International Chemokine Symposium, held June 27–30, 1995, Izzy Charo described an orphan receptor identified as CCR5, linked to CCR2, which bound Mip1α, Mip1β, and RANTES (14). Thus, an international race began as five chemokine labs partnered with HIV labs to prove the hypothesis that CCR5 was the HIV coreceptor.

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Eotaxin-1 (CCL11)

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The eosinophil was first named by the brilliant German scientist Paul Ehrlich in 1879, while he was experimenting with aniline dyes to stain blood cells and tissues. He also discovered neutrophils, basophils, and mast cells. The highly basic proteins in cytosolic granules of a small subpopulation of cells in human blood stained vivid pink with the acid dye eosin (from the Greek "eos" meaning dawn), hence "eosinophils." He subsequently observed high numbers of these cells in the sputum of asthmatic patients and recognized the close relationship between eosinophilia and the severity of asthma. Pertinent to our story was his proposition that a "material which attracts eosinophils" exists. Further, he postulated that eosinophils and neutrophils possess different "chemotactic irritability" and that eosinophils only migrate to sites where a "specific stimulating substance" is present (1).

This could have been the inspiration behind the Eotaxin project but, in truth, its origins were more prosaic. To provide a brief background, my Ph.D. project on mechanisms of inflammation involved the measurement of microvascular plasma protein leakage in rabbit and guinea pig skin using ^{125}I-albumin as a marker. This led to an investigation of endogenous mediators that increase the permeability of venules *in vivo* using intradermal zymosan as the inflammatory stimulus. Alternatively, zymosan was administered intraperitoneally in rabbits and the skin system was used as an *in vivo* bioassay for peritoneal exudates collected at intervals. The major finding from all these studies was that the principle permeability-increasing mediator was extravascularly generated C5a. Further, C5a-induced leakage was dependent on a rapid interaction between neutrophils and venular endothelial cells, as evidenced by neutrophil depletion experiments (2): (followed up recently in *J Exp Med*, 2014). We then began experiments with ^{111}In-neutrophil trafficking *in vivo*, and the purification and identification of C5a brought us into contact with an expert protein sequencing group in London. In a paper published in 1986, we noted that there was a small amount of permeability-increasing activity, other than C5a, in 2 h zymosan-induced peritoneal exudates. Some time later, we assayed 6 h exudates in the skin in the presence of a C5a neutralizing antibody and identified two potent activities. Purification using HPLC, followed by microsequencing, revealed that these were the rabbit equivalents of IL-8 (CXCL8) and MGSA (CXCL1); results published in 1990 and 1991. Thus, at this stage, our journey had taken us from an interest in the barrier function of the venular endothelium, to the complement system and neutrophils, and then on to chemokines.

By this time, I had moved to the National Heart & Lung Institute in West London to take up a professorial chair funded by a charity, the National Asthma Campaign, later renamed Asthma UK. We seemed to be on another planet; clearly, the world of asthma was orbiting around the eosinophil. There was little interest in the neutrophil (although eventually this changed with a growing emphasis on the heterogeneity of the disease, some asthma subtypes being clearly neutrophilic). To redress the balance, I introduced Lucia Faccioli, a visitor from Brazil, to eosinophil expert Redwan Moqbel in the Institute and we developed a method to measure ^{111}In-eosinophil accumulation in guinea pig skin *in vivo*. I later recruited David Griffiths-Johnson who had specialized in lung lavage of allergen-challenged sensitized guinea pigs. The plan to combine the two techniques as an *in vivo* generating and *in vivo* bioassay system to identify endogenous eosinophil chemoattractants was submitted to the asthma charity as a project grant, but sadly this was rejected with not unreasonable reservations about feasibility. Despite this, we continued using funds raised for another project. After several "false dawns," the pursuit proved successful and in 1992 we were regularly detecting activity in lung lavage fluid, indicated by a strong ^{111}In-eosinophil signal in bioassay skin samples. Unfortunately, at this point, we had lost our biochemist, Peter Jose, who had developed the methodology for the purification of rabbit IL-8 and MGSA. Peter had abandoned the hunt for the elusive eosinophil chemoattractant and moved out of science to a rural retreat in Marmande in France. I flew to France clutching the new data and met Peter who seemed more interested in the ripening of his strawberry crop, but was persuaded to return to London to take on the challenge. The lavage fluid was put through a series of HPLC purification stages and within a relatively short time Peter had purified the protein for microsequencing. Within 2 weeks, the sequencing group had an N-terminal sequence of a novel CC chemokine. Soon, they had sequenced peptide proteolytic fragments of the protein and had assembled the full 73-aa sequence. We called this protein "Eotaxin" (condensed from "eosinophil chemotaxin"). We submitted a manuscript to *Nature* and were pleased with the positive reports that came back from two of the referees, which betrayed a North American flavor ("flavor"). The third, more critical, referee appeared to

be from the "United" Kingdom and stated that the molecule had not been cloned and that chemotaxis had not been demonstrated *in vitro*. These statements were true, but seemed to miss the point. There was considerable interest in academia and industry in the eosinophil as a therapeutic target in asthma. Activated eosinophils release their highly basic, tissue-damaging proteins, and a range of mediators that can exacerbate lung inflammation. Despite the interest, cloning had not revealed the Eotaxin sequence. We had tried eosinophil chemotaxis *in vitro* as an assay, but the cells were confused by a whole gamut of non-specific stimulants in lavage fluid. In contrast, the *in vivo* skin assay excelled in picking up the chemoattractant "needle" in a "haystack" of irrelevant molecules. After two more unsuccessful attempts at submission to *Nature* with more data, the paper was redrafted and appeared, considerably delayed, in the *Journal of Experimental Medicine* in March 1994 (3).

In early 1994, I received a phone call from Tim Springer inviting me to give a talk at Harvard. After the talk, Tim remarked that Henry Dale (who deduced that histamine was released *in vitro* from tissues of allergen-sensitized guinea pigs in the early 1900s) would have appreciated our approach. I took this as a compliment but, on reflection, this more resembled understandable sarcasm at our "retro" methodology. However, one advantage with our approach was that we could immediately set our molecule in a disease context and give it a rational name. With the encouragement of Craig Gerard, Tim invited me to join the scientific advisory board of LeukoSite Inc., the company that he had recently founded with Eugene Butcher. The Eotaxin patents were licensed to the Company and a successful relationship ensued. Based on the protein sequence (**Figure 1**) we cloned guinea pig Eotaxin in a previously established collaboration with Christine Power at GSK in Geneva. Marc Rothenberg at Harvard then used the guinea pig protein sequence to clone mouse Eotaxin and embarked on a series of experiments with allergy models in Eotaxin knockout mice (4) followed by important papers demonstrating a role for Eotaxin, particularly in diseases of the GI tract. Paul Ponath and colleagues at LeukoSite cloned human Eotaxin (5) and its receptor CCR3. There were several publications on these sequences around this time. Subsequent discoveries in other laboratories published in 1997 and 1999 revealed two more Eotaxins signaling through CCR3, Eotaxin-2 (CCL24), and -3 (CCL26), with low sequence similarity to the renamed Eotaxin-1, designated CCL11 of the CC chemokine family. We used immunoassays to investigate the role of Eotaxin-1 in the guinea pig allergy model (6), and Alison Humbles then moved to Craig Gerard's laboratory to investigate mouse models using CCR3 knockout mice. Many groups, including ours, published papers showing the expression of Eotaxin in human asthma.

A recurring question from eosinophil experts concerned the relationship between Eotaxin and the cytokine, IL-5, which was reported to be an eosinophil chemoattractant. Contrary to the reports, we found that IL-5 did not induce eosinophil accumulation when injected into guinea pig skin. However, intravenous IL-5 induced the release of eosinophils from the bone marrow reserve and the dramatic increase in circulating cells in the blood markedly enhanced eosinophil recruitment induced by intradermal Eotaxin-1 (7). We subsequently developed an *in situ* bone marrow perfusion system to study mechanisms of eosinophil release in detail.

As at LeukoSite, many companies developed CCR3 antagonists. Ian Sabroe in my laboratory devised a technique to measure responses of human eosinophils to chemokines (the "GAFS" Shape Change Assay, now widely used in academia and industry) and discovered a subpopulation of donors whose cells responded to CCR3 and CCR1 agonists. This led to the first publication on a CCR3 antagonist (8), one that could also antagonize CCR1. There have been clinical trials of CCR3 antagonists in asthma patients but, as yet, no drug has reached the market. Cambridge Antibody Technology (now MedImmune) produced a potent therapeutic antibody (CAT-213, iCo-008, Bertilimumab) that neutralizes Eotaxin-1. This antibody, tested initially in allergic rhinitis, has been licensed to iCo Therapeutics and to Immune Pharmaceuticals, for testing in further clinical trials. As well as being evaluated as a therapeutic target in asthma and in allergic diseases in general, Eotaxin-1 is used as a biomarker in clinical trials. There is also interest in diseases of the GI tract where

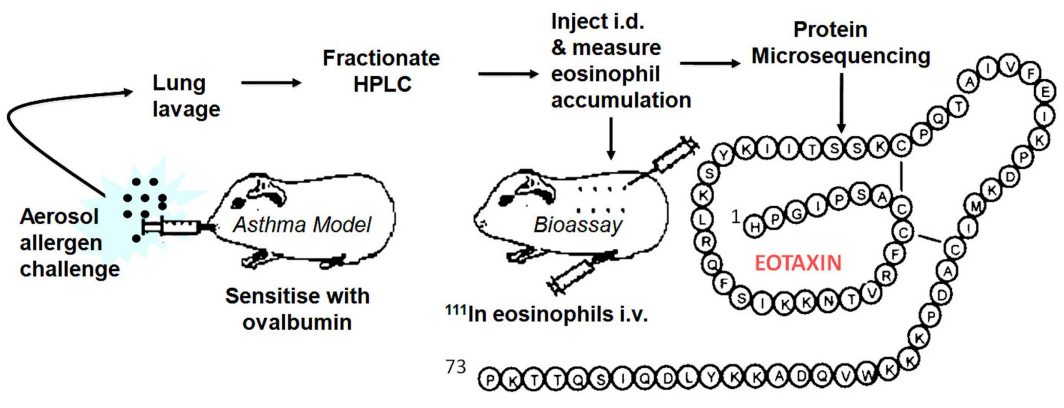

FIGURE 1 | Generation, bioassay, purification, and sequencing of Eotaxin-1.

Rothenberg's work has been particularly influential, with trials planned in ulcerative colitis and Crohn's disease. In addition, Eotaxin-1 is implicated in diseases, such as atherosclerosis, apparently independently of its action on eosinophils. Interestingly, as published in *Nature* in 2009, CCR3 is expressed on endothelial cells in vessel overgrowth of the macula in age-related macular degeneration (AMD) and locally produced Eotaxins are thought to mediate angiogenesis in this condition [see Ref. (9)]. Thus, Bertilimumab is being considered for the treatment of AMD and other eye diseases. There is also evidence, from cross-circulation studies between old and young mice that circulating Eotaxin-1 rises during aging and this suppresses neurogenesis and cognitive function, as published in *Nature* in 2011 [see Ref. (9)], raising possibilities for future therapy in dementia.

Thus, from humble origins, the work on Eotaxin-1 has raised tantalizing opportunities for therapy ranging across several diseases. These possibilities have not yet translated into effective therapy, but we are not alone in this in the chemokine field (9).

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CXCL12/SDF-1 and CXCR4

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Chemokines are a large family of structurally related chemoattractive cytokines, which have four conserved cysteines forming two disulfide bonds, and act through seven-transmembrane-spanning receptors coupled to heterotrimeric GTP-binding proteins (G-protein-coupled receptors). Chemokines were thought to be signaling molecules that attract leukocytes to sites of inflammation; however, CXC chemokine ligand (CXCL)12 [also known as stromal cell-derived factor (SDF)-1α and pre-B-cell-growth-stimulating factor (PBSF)] is the first member that was shown to be critical for developmental processes, including hematopoiesis (1), cardiogenesis (1–3), vascular formation (2), and neurogenesis (3), as well as the maintenance of tissue stem cells (4).

Identification of CXCL12

Our interest is how bone marrow microenvironments regulate hematopoiesis, including B lymphopoiesis. To address this, we tried to identify a cytokine, which was important for B cell development in the marrow. In 1988, Namen et al. identified interleukin 7 (IL-7) produced by a bone marrow-derived stromal cell line as a cytokine, which enhanced the proliferation of B cell precursors. However, several studies suggested that IL-7 was not sufficient to support B lymphopoiesis. Hayashi et al. speculated that at first stage in B cell development, progenitors depended on unidentified molecules produced by the stromal cell line called PA6 alone for proliferation and differentiation into the second stage, where progenitors depended on both PA6-derived factors and IL-7 for proliferation (5).

It was unclear whether PA6-derived factors were soluble factors or not in Hayashi's model (5). To address this issue, we cultured bone marrow hematopoietic cells in the absence or presence of PA6 cells separated by a membrane filter, allowing the passage of proteins but not cells. We showed that while very few viable B cell precursors were present 7 days after the culture of bone marrow hematopoietic cells in the presence of IL-7 and absence of PA6 cells, the proliferation of B cell precursors were enhanced in the presence of PA6 and IL-7. These findings suggested the existence of soluble factors produced by PA6 cells that stimulated the proliferation of B cell precursors in the presence of IL-7 (6).

We tried to develop more simple culture system suitable for molecular cloning and found that a stromal cell-dependent B cell precursor clone, DW34, which was established from Whitlock-Witte-type culture by limiting dilution on a stromal cell line, could proliferate in the presence of a conditioned medium from PA6 cells (6). An expression cDNA library was prepared from PA6 cells using the vector pME18S, and then more than 10^4 pools were screened for the activity to stimulate the growth of DW34 cells after enforced expression in COS-7 cells, and positive pool was subdivided until a single positive clone was identified. We revealed that a conditioned medium from the positive clone-transfected COS-7 cells had DW34 growth stimulating activity and termed this molecule PBSF (6). The nucleotide sequence and deduced amino acid sequence of the clone were determined and its product was identical to a chemokine called SDF-1α (6, 7). We felt these results somewhat disappointing because chemokines were thought to be rather inflammatory mediators at that time. In 1993, Tashiro et al. developed a method for molecular cloning of cDNAs that contain

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signal sequences, such as those encoding secreted proteins and receptors without the use of specific functional assays, and identified SDF-1α; however, its function was unclear (7). Thus, we revealed that SDF-1α/PBSF (now formally named CXCL12) stimulated the proliferation of B cell precursors (6).

Identification of a Receptor for CXCL12

All known chemokine receptors are G-protein-coupled receptors (GPCR) and amino acid sequence is conserved among these molecules. Based on this, we synthesized four degenerate oligonucleotides corresponding to conserved amino acid sequences in transmembrane regions of the chemokine receptors, including murine CXCR2, CCR2, and human HUMSTR, and used them as primers in PCR experiments to identify chemokine receptors abundantly expressed by murine CXCL12 responsive DW34 cells (8). The deduced amino acid sequence of a cDNA yielded by this approach shared 90% amino acid identity with previously identified human HUMSTR/HM89/LESTR/fusin, a HIV-1 entry co-receptor and designated murine HUMSTR/HM89/LESTR/fusin (now formally named CXCR4) (8). CXCL12 induced an increase in intracellular free Ca^{2+} in DW34 cells and CXCR4-transfected Chinese hamster ovary (CHO) cells, suggesting that CXCR4 is a receptor for CXCL12 (8). On the other hand, Bleul et al. and Oberlin et al. demonstrated that human HUMSTR/HM89/LESTR/fusin is a receptor for human CXCL12 (9, 10). The majority of chemokine receptors recognize more than one chemokine, and many chemokines bind to more than one chemokine receptor. However, we and others revealed that mice lacking CXCR4 showed hematopoietic and cardiovascular phenotypes strikingly similar to those of CXCL12 deficient mice, as described below, indicating that CXCR4 is the primary physiologic receptor for CXCL12 in mammals (1–3).

Essential Physiological Roles of CXCL12–CXCR4 Signaling

To determine the role of CXCL12 in hematopoiesis, we generated and analyzed CXCL12 and CXCR4 deficient mice, which died perinatally. Consistent with the activities of CXCL12 in promoting the proliferation of B cell precursors (6), CXCL12-CXCR4 signaling was essential for the development of B cells from the earliest precursors in fetal liver and bone marrow (1, 11). Surprisingly, CXCL12-CXCR4 signaling was also essential for homing of hematopoietic stem cells (HSCs) and neutrophils to fetal bone marrow during ontogeny (1–3, 12). Subsequently, we generated CXCR4 conditionally deficient mice and revealed that CXCL12-CXCR4 signaling was essential for the maintenance of HSCs, the production of immune cells, including B cells, plasmacytoid dendritic cells (pDCs), which expressed high levels of type I interferon (IFN), and were thought to play important roles in antiviral immunity, and NK cells and homing of end-stage B cells, plasma cells into bone marrow (4, 11, 13). In addition to hematopoiesis, we found that CXCL12-CXCR4 signaling was essential for homing of primordial germ cells (PGCs) to gonads, a cardiac ventricular septal formation and vascularization of the gastrointestinal tract during ontogeny (1–3). In the meantime, Littmann's group described that CXCR4 was essential for migration of granule cells in appropriate positions in the cerebellum during neurogenesis (3), and besides these additional physiological roles of CXCL12-CXCR4 signaling, other groups revealed its relevant pathological roles. In 1996, Feng et al. found that CXCR4 acted as an essential co-receptor for T cell-tropic strains of human immunodeficiency virus type-1 (HIV-1), and Bleul et al. and Oberlin et al. demonstrated that CXCL12 had HIV-suppressive activities (9, 10). Furthermore, CXCL12-CXCR4 signaling has been reported to be involved in migration of cancer cells, including presumptive cancer stem cells, to sites of metastasis and increased their survival and/or growth in various cancers, such as breast and lung cancers, as well as leukemia and lymphoma.

CXCL12-Expressing Cells in Bone Marrow

As the CXCL12-CXCR4 signaling plays a key role in hematopoiesis, we were prompted to visualize cells, which expressed CXCL12 in bone marrow. For this, we generated mice with the green fluorescent protein (GFP) reporter gene knocked into the CXCL12 locus and found that CXCL12 as well as stem cell factor (SCF), which was essential for HSC proliferation, was preferentially expressed in a population of stromal cells with long processes, termed CXCL12-abundant reticular (CAR) cells (11–13). CAR cells are adipo-osteogenic progenitors, which express adipogenic and osteogenic genes, including peroxisome proliferator-activated receptor γ (PPARγ) and Osterix (Osx), and largely overlap with SCF-expressing cells predominantly expressing leptin receptor (Lepr) (13–15). Histological analysis showed that most HSCs and very early B cell progenitors were in contact with CAR cells (4, 11), and the experiments using diphtheria toxin-based system that allows the inducible, short-term ablation of CAR cells *in vivo* revealed that CAR cells were essential for maintenance of hematopoietic stem and progenitor cells (HSPCs) in bone marrow (**Figure 1**) (14). Recently, we found that the transcription factor Foxc1 was expressed preferentially in CAR cells and was essential for CAR cell development and maintenance of bone marrow niches for HSPCs up-regulating

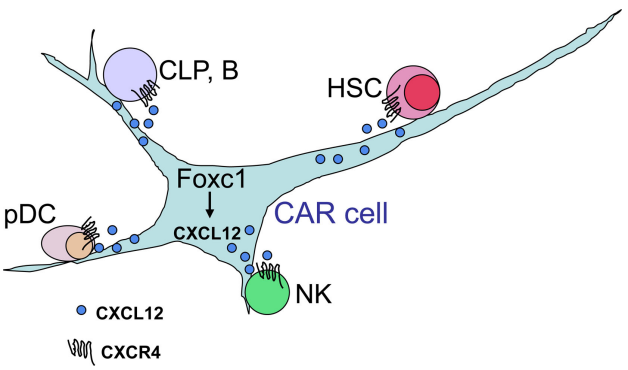

FIGURE 1 | CXCL12-abundant reticular (CAR) cells. In adult bone marrow, the transcription factor Foxc1 induces development of CAR cells and maintains bone marrow niches for HSPCs, up-regulating the expression of CXCL12, which is essential for the maintenance of HSCs, common lymphoid progenitors (CLPs), B cells, pDCs, and NK cells, in CAR cells.

SCF and CXCL12, which plays major roles in HSC maintenance and immune cell production (**Figure 1**) (15).

Taken together, CXCL12 and CXCR4 have been identified as key spatiotemporal regulators of migratory stem and progenitor cell behavior, and our studies provide considerable new insights into the biology and pathology of tissue stem cells as well as hematopoiesis, vasculogenesis, and neurogenesis, and in some cases, for clinical application in various diseases.

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CXCR5, the defining marker for follicular B helper T (T_{FH}) cells

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The discovery of follicular B helper T (T_{FH}) cells has its roots in the early 90s, the "childhood" of chemokine research that has since grown into an independent, global specialty within immunology. The class of chemoattractant proteins with shared structural features was named "chemokines," and early work with non-chemokine (FMLP, C5a) and chemokine (IL-8/CXCL8) receptors revealed that chemokine receptors belong to the large family of G-protein-coupled receptors (GPCRs) distinguished by their prototypical seven-transmembrane protein architecture. The search for novel chemokines and their receptors greatly intensified during that time, because it became increasingly clear that this novel cytokine system is essential for controlling immune cell mobilization and tissue localization and, hence, for controlling the entirety of immune processes in health and disease. Molecular identification of chemokine receptors led to the identification of immune cells that responded to the corresponding chemokine ligands and allowed their tracking during acute and chronic immune responses. At the last count, the inventory of chemokine receptors that, together with adhesion receptors, make up the address codes on human immune cells includes 18 individual members recognizing one or multiple of a total of 45 chemokines. Further underscoring the complexity of the chemokine system, we also know of six atypical chemokine receptors, some of which control chemokine positioning and degradation.

Since my earliest steps in research, I was fascinated by the cytokine network controlling the highly complex interactions between immune cells and their functions in immune defense. In fact, an ambitious project aimed at the molecular characterization of the elusive "antigen-specific T helper factors" tipped the balance in favor of carrying out my Ph.D. studies in the lab of Profs. D. G. Kilburn, R. C. Miller Jr., and R. A. J. Warren at the University of British Columbia in Vancouver. It was not the skiing in the Whistler Mountains nor the salmon fishing along the Sunshine Coast that did the job, as suggested by some of my colleagues at the Federal Institute of Technology in Zurich. Needless to say that the molecular identification of the T cell antigen receptor in 1984 brought our project to an immediate halt. Still, when the question about postdoc projects arose, I was fascinated by the new world of chemotactic cytokines (to be called "chemokines" a few years later) that I was introduced to by Prof. Marco Baggiolini, the director at the Theodor-Kocher Institute of the University of Bern. Therefore, upon arrival at the Theodor-Kocher Institute in 1989, I was determined to clone the receptor for NAF, the first chemokine with selectivity for neutrophils (now known as IL-8 or CXCL8 according to the systematic chemokine nomenclature). This young field of research turned out to be highly competitive, not least because of its translational potential. Unsurprisingly, we were beaten by two labs who reported the cloning of the CXCL8 receptors well before our own initiative had a chance to take off (1, 2). As a small consolation, we succeeded to be first in demonstrating that human neutrophils carried two types of CXCL8 receptors on their cell surface distinguished by their variable affinity for other CXCL8-related chemokines (3, 4). Still, our multipronged cloning efforts paid off and revealed numerous orphan GPCRs with similarity to the CXCL8 receptors. In a great team effort by many colleagues, including Marcel Loetscher, Daniel Legler, Patrick Schaerli, and Regula Stuber-Roos, together with the protein chemist Ian Clark-Lewis at the Biomedical Research Centre of the University of British Columbia (who sadly died in 2002), we were

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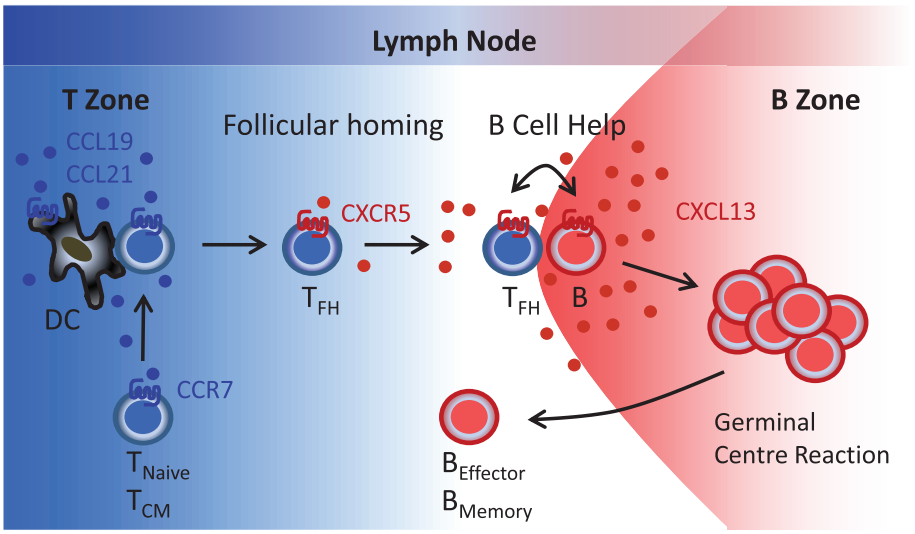

FIGURE 1 | Follicular B helper T (T_{FH}) cells. T cells reach the lymph node paracortical region (T zone) *via* high endothelial venules (blood) or afferent lymphatics (peripheral tissues) in response to the CCR7-specific chemokines CCL19 and CCL21. During contact with antigen-presenting DC, T cells become primed that includes induction of CXCR5 expression. Reduced CCR7 expression allows newly generated $CXCR5^+$ T (T_{FH}) cells to respond to follicular CXCL13 and to relocate to the B cell follicles where T_{FH}-B cell interactions involving a series of co-stimulatory receptors (CD40, ICOS, etc.) and cytokines (IL-10, IL-21, etc.) initiate the germinal center reaction and the formation of antibody-secreting plasma cells and memory B cells. T_{Naive}, naïve T cells; T_{CM}, central memory T cells; T_{FH}, follicular B helper T cells; B, B cells; $B_{Effector}$, plasma cells; B_{Memory}, memory B cells.

then able to "deorphanize" some of these novel GPCRs in the subsequent years.

As part of our chemokine receptor cloning initiatives, Luca Barella, who was a Ph.D. student in my lab, identified and characterized an orphan GPCR, termed monocyte-derived receptor 15 (MDR15) (5), which turned out to be a structural variant of Burkitt's lymphoma receptor 1 (BLR1) published several years ahead of us by Martin Lipp's group in Berlin (6). In fact, we first heard about BLR1 during a conversation with Martin Lipp whom we met in July 1992 at the fifth International Congress on Cell Biology in Madrid. Based on the structural similarities to the chemokine receptors that were known at that time (CXCR1, CXCR2, CCR1, and CCR2), it was clear to us that MDR15/BLR1 must be a novel chemokine receptor. However, none of the known chemokines bound to it.

Intriguingly, MDR15/BLR1 transcripts were primarily found to be present in the lymphocyte fraction of peripheral blood mononuclear cells, and most notably in chronic B leukemia cell lines, but not in cells characterized by the other known chemokine receptors. It took another 3 years to "deorphanize" MDR15/BLR1. While searching expressed sequence tag (EST) cDNA databanks, Daniel Legler, a Ph.D. student at that time, identified a novel chemokine, which we termed B cell-attracting chemokine 1 (BCA-1; now officially known as CXCL13) because of its efficacious chemoattractant activity for B cells (7). The mouse ortholog of BCA-1/CXCL13 was published by the group of Michael Gunn at UCSF within the same month (8). Importantly, Michael Gunn and our group found that BCA-1/CXCL13 was the selective chemokine ligand for mouse and human MDR15/BLR1 (now officially known as CXCR5), respectively. Of note, the highly selective expression of CXCL13 in secondary lymphoid tissues matched perfectly well the findings of Martin Lipp's group about the importance of BLR1 in the localization of B cells within murine secondary lymphoid tissues (9).

In addition to B cells, we noticed that a large fraction of $CD4^+$ memory T cells present in tonsils expressed CXCR5. By contrast, $CXCR5^+$ T cells were relatively scarce in peripheral blood of healthy individuals. We also found that its single ligand CXCL13 was discretely expressed in the follicular mantle zone but not in the paracortical T cell zone or high endothelial venules where the CCR7-specific chemokines CCL19 and CCL21 are present. Unlike CCL19/CCL21, it appeared that CXCL13 did not play a role in the recruitment of T cells (and B cells) into lymph nodes but instead controlled the segregation of lymphocytes between T cell and B cell compartments, as Michael Gunn's group has shown for BLC in mice (10). Could it be that tonsillar $CXCR5^+$ T cells corresponded to the elusive T helper cell subset postulated to control B cell responses to protein antigens? Indeed, tonsillar B cells produced large amounts of isotype-switched antibodies during co-culture with $CXCR5^+$ T cells but not $CXCR5^-$ T cells. During a subsequent discussion with Martin Lipp, who contributed his CCR7-specific antibodies to our study, we found out that both of our groups had similar results and, therefore, we agreed to submit our findings as back-to-back manuscripts to the *Journal of Experimental Medicine* (11, 12). Together with the journal editors, we then decided to designate this novel T helper subset as T_{FH} cells, which today is also known as "follicular helper T cells" and "B helper T cells" (**Figure 1**).

The separation of T helper cells into Th1 and Th2 cells was instrumental in delineating immune responses to distinct classes of pathogens, such as viruses and intracellular bacteria for Th1 cells and extracellular pathogens and allergens for Th2 cells.

Following the fundamental dogma underscoring the inseparable relationship between tissue localization and immune cell function, we and Martin Lipp's group jointly discovered T_{FH} cells as a third distinct T helper cell subset, which was highlighted in a commentary by Charles Mackay (13). An avalanche of murine studies by numerous outstanding labs worldwide confirmed and extended our initial findings about the role played by T_{FH} cells in humoral immunity. Thanks to their efforts, it is now clear that defects in their generation and/or function have a profound effect on antibody dependent immune responses. In fact, increased numbers of T_{FH} cells are now known to be associated with B cell autoimmunity and lymphomas whereas defects in T_{FH} cell generation cause severe humoral immunodeficiency [reviewed in Ref. (14)]. Finally, since the presence of $CXCR5^+$ T cells in peripheral blood reflects ongoing humoral immune responses (15), CXCR5 may even serve as a unique and convenient biomarker for the evaluation of ongoing vaccination responses. Today, T_{FH} cells have a firm place among an increasing number of T helper cell subsets distinguished by their characteristic migration and functional properties.

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The Duffy antigen receptor for chemokines DARC/ACKR1

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The discovery that the Duffy antigen was a promiscuous chemokine binding protein was entirely serendipitous and resulted from research begun at Genentech in 1991. The company had a strong interest in chemokines because of their role in disease and this was furthered by the recent cloning of two chemokine receptors CXCR1 and CXCR2. Both were IL-8 (CXCL8) receptors expressed on immune cells, primarily neutrophils, and belonged to the G-protein coupled receptor (GPCR) subfamily, which were highly druggable targets. Numerous reports linked CXCL8 to respiratory diseases like COPD, and Genentech had developed a CXCL8 neutralizing antibody that it thought might be useful therapeutically to treat respiratory diseases like emphysema, bronchitis, and COPD.

Because a number of signal-transmitting polypeptides such as human growth hormone and somatomedin are bound to plasma binding proteins, it was of interest to determine whether blood contained a CXCL8-binding protein. To determine this Walter Darbonne and Caroline Hèbert working in Joffre Bakers lab at Genentech set up a CXCL8 whole blood assay in which increasing amounts of radiolabeled ^{125}I-CXCL8 were added to whole blood (1). Interestingly, at low CXCL8 concentrations, the recovery of the chemokine in the plasma was very low but it increased as the CXCL8 concentration was increased. This appeared to be a saturable process and could result from CXCL8 binding to a blood protein. Since erythrocytes are the major cell type in blood, an experiment was set up to examine the effect of adding increasing amounts of radiolabeled ^{125}I-CXCL8 to these cells. Using this assay, it was determined that CXCL8 was specifically absorbed by erythrocytes in a saturable manner (1). Furthermore, the erythrocyte-bound CXCL8 was not able to activate the neutrophils by engaging CXCR2 but this process could be reversed by the addition of excess unlabeled CXCL8. The molecule binding the CXCL8 on erythrocytes was a protein because it was sensitive to chymotrypsin treatment; however, trypsin treatment had no effect on binding of CXCL8 (1). Scatchard binding experiments revealed that the binding was saturable and defined by a high affinity receptor, with a binding K_D of 5 nM and around 2,000 binding sites per red blood cell. Interestingly, it was not always possible to successfully detect CXCL8 binding to blood samples and it appeared that the blood from these CXCL8 non-responders was always from African American donors.

Taking all of this information together, a search of the literature revealed that the CXCL8-binding protein that was discovered at Genentech had molecular properties consistent with those described for a human red blood cell antigen called the Duffy antigen. The Duffy antigen is a human erythrocyte blood group antigen that was shown to be a portal of entry for the malarial parasite *P. vivax* (2). A promoter mutation in the GATA box of the Duffy gene prevents its expression on erythrocytes (3) and most West Africans are resistant to *P. vivax*-induced malaria because they are homozygous for this mutation (4). To test the hypothesis that the CXCL8-binding protein was the Duffy antigen, we obtained whole blood from Duffy-positive and Duffy-negative donors and showed that there was an absolute correlation of CXCL8 binding to Duffy-positive but not to Duffy-negative blood (5). Further, we demonstrated that the Duffy antigen was a promiscuous chemokine binding protein binding both CXC and CC chemokines (5). Based on these observations, we renamed the Duffy antigen DARC (Duffy antigen receptor for chemokines) (6). Recently, a new nomenclature for atypical non-signaling chemokine receptors such as DARC was adopted and approved and DARC is now known by the acronym ACKR1 (atypical chemokine receptor 1) (7) However, for the purpose of this review, we will stick to the old nomenclature of DARC.

Further work showed that DARC was expressed in other tissues in the body including kidney and brain (8, 9). Around this time, the protein had also just been sequenced (10) and was shown to be a member of the GPCR family. We transfected the newly cloned receptor into K562 cells and were able to recapitulate all of the molecular and functional properties of the protein (11). Furthermore, chemokine binding to DARC blocked both the binding and the infection of human erythrocytes by the malarial parasite *P. vivax* (12).

There is a variety of evidence in support of the idea that DARC on erythrocytes can act as a depot for chemokines reducing their concentration in the circulation (1). In line with this notion, cancer patients undergoing IL-1 immunotherapy were shown to have high erythrocyte CXCL8 binding compared to plasma levels perhaps indicating a potential protective role to prevent chemokine activation of neutrophils and inflammation. In addition, a recent study examining the influence of DARC in kidney transplant rejection in African Americans found that DARC-negative patients had lower allograft survival than DARC-positive patients (13) suggesting to the investigators that perhaps DARC may attenuate the inflammatory

effects of chemokines by inactivating them. Further evidence for the protective nature of DARC comes from transfusion experiments in which either DARC wild-type erythrocytes or DARC-negative erythrocytes were transfused into DARC wild-type endotoxemic mice. The mice receiving DARC-negative erythrocytes had increased neutrophil migration into the lungs, increases in inflammatory cytokine concentrations, and increases in lung microvascular permeability compared with mice receiving DARC-wild-type erythrocytes (14). The authors speculated that the pulmonary inflammation that appeared to be induced by a reduction in erythrocyte chemokine scavenging in these experiments could translate to an increase in existing lung inflammation in susceptible Duffy-negative patients.

The idea that DARC can signal in direct response to ligand binding is highly unlikely because although it is a seven-transmembrane-spanning receptor, most members of which are GPCRs, DARC lacks the entire DRYLAIV sequence, a highly conserved determinant of G-protein coupling found in GPCRs at the boundary between the third transmembrane domain and the second intracellular loop. This sequence plays a crucial role in mediating GPCR action and its absence in DARC leads to a failure in its coupling to G-proteins and thus DARC does not mediate a biological signal upon direct chemokine binding.

Of course, there is still a formal possibility that DARC can provide an indirect biological signal in response to chemokine binding. Some support for this idea is provided by the observation that DARC is highly expressed on endothelial cells lining post-capilliary venules (8). The authors speculate that its cellular location could be consistent with a role for DARC in leukocyte trafficking. Arguing against this idea is the fact that individuals lacking expression of DARC on erythrocytes appear to have normal immune function. However, the finding that DARC is expressed on the endothelial cells of both Duffy-negative and -positive individuals (6) suggests that perhaps DARC expression on endothelial cells may be more important for its role in leukocyte trafficking than DARC expression on erythrocytes. The authors speculate that the retention of DARC expression on endothelial cells in Duffy-negative individuals hints at the retention of a possible physiological function in these cells. One feature that characterizes both signaling receptors and chemokine transporters is the internalization of bound ligands. Interestingly, K562 cells transfected with DARC were shown to be able to induce the internalization of radiolabeled chemokines (11). Taken together, these studies suggest that DARC may have a signaling and/or transporter-like role in endothelial cells but that it lacks features associated with direct ligand-activated G-protein receptor signaling.

Evidence suggesting a role for DARC in endothelial cells as a chemokine transporter that can influence leukocyte transmigration comes from studies by Rot and coworkers (15). They have shown that DARC is involved in the transport of chemokines across endothelial cells. This transcytosis of chemokines led to their apical retention but unlike other decoy receptors such as D6 (ACKR2) ligand internalization by DARC did not lead to chemokine degradation. Thus DARC appears to function as an endothelial transporter for chemokine ligands and, leads to chemokine immobilization on apical cell surfaces. It thus appears to play a critical role in leukocyte trafficking. The authors also speculate that DARC on endothelial cells might function as a chemokine rheostat on the blood–tissue interface by supporting the placement and function of suboptimal concentrations of chemokines, but eliminating their excess (16).

The fact that DARC belongs to a family of seven transmembrane domain proteins and that almost 40% of all marketed medicines interact with this class of proteins strongly suggests that DARC is an excellent target for successfully developing therapeutics to treat malaria. A similar approach has already shown benefit in treating AIDS. AIDS is a lethal infectious disease of the immune system caused by the human immunodeficiency virus (HIV), which can be viewed as a paradigm for *P. vivax*-induced-malaria since like its protozoal counterpart, it requires an interaction between an HIV protein, the viral glycoprotein gp120, and either one of the human chemokine receptors CCR5 or CXCR4.

The discovery that chemokine receptors were major coreceptors for HIV entry into the cell prompted a number of pharmaceutical companies to screen for CCR5 inhibitors. The most successful of these is a small molecule inhibitor of the HIV gp120–CCR5 interaction, maraviroc, which is now a registered drug to treat AIDS. An approach to identify small molecule inhibitors of DARC should prove to be of similar benefit in drastically reducing *P. vivax* malaria (**Figure 1**). Such inhibitors

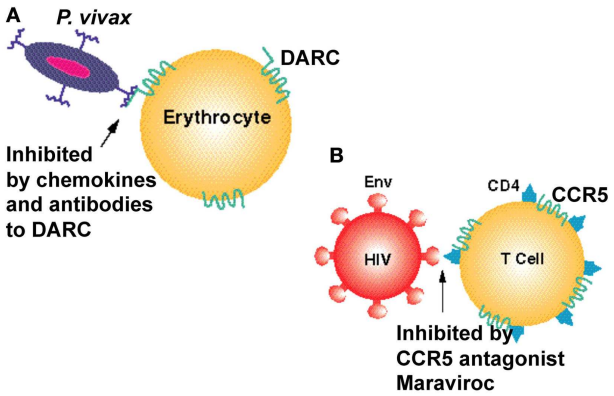

FIGURE 1 | Seven transmembrane domain receptors are used as vehicles of entry by infectious agents. **(A)** The human chemokine binding protein DARC is expressed on erythrocytes and is used by *P. vivax* to gain entry to the cell. It can be inhibited by a monoclonal antibody to DARC, Fy6, and by chemokines such as CXCL1 and CXCL8. **(B)** The chemokine receptor CCR5 is used as a co-receptor by the HIV-1 virus to enter human T cells and monocytes. It can be inhibited by chemokines and by the CCR5 inhibitor maraviroc. Cluster of differentiation 4 (CD4) is a glycoprotein expressed on the surface of some immune cells including T lymphocytes. HIV-1 envelope glycoprotein (Env) is responsible for binding to the receptor (CD4) and the chemokine coreceptors (CCR5 or CXCR4) on the host cell, for subsequent fusion of the viral and cellular membranes.

could be a valuable addition to drug combinations that target both *P. falciparum* and *P. vivax* in regions of the world that are endemic for these parasites.

Another interesting parallel between HIV infection and *P. vivax*-induced malaria is that individuals have been identified who are resistant to infection by one or the other of these agents. In the case of HIV, humans with a frameshift mutation in the coding region of the HIV receptor have been identified. This mutation, called delta32, results in the premature truncation of the receptor such that it is no longer expressed. As a consequence, homozygous CCR5 delta32 individuals are resistant to infection by CCR5-tropic HIV strains. These people, who are essentially CCR5 knockouts, do not exhibit any obvious deleterious effects from the lack of this receptor (except for some reports that they are more susceptible to brain infection with West Nile Virus), which may be rationalized by the functional redundancy that seems to be built into the chemokine system where several other chemokine receptors can compensate for a lack of CCR5.

Similarly, individuals in West Africa do not express DARC on their erythrocytes, and they are resistant to *P. vivax*-induced malaria (4). The parallels to the CCR5 delta32 mutation as a protective factor in infection by HIV and the successful development of a CCR5 inhibitor to treat AIDS are striking and we envisage that development of a DARC inhibitor might also work for *P. vivax-i*nduced malaria.

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D6/ACKR2

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At the outset, it is worth noting that, for historical reasons, I have referred to D6/ACKR2 as "D6" throughout the majority of this essay. Issues relating to the complexity of D6 nomenclature are discussed below.

IN THE BEGINNING . . .

Very much in keeping with the name of the chemokine receptor subfamily to which D6 belongs, the majority of my research in the field of chemokine biology has been "atypical" in the sense that it has rarely focused on classical immunological roles for these molecules. Indeed, the story behind the discovery of D6 starts from an unusual research perspective! In 1988, I was employed as a postdoctoral researcher in the laboratory of Prof. Ian Pragnell who became a close friend, and with whom I enjoyed many international adventures. Ian, at the time, was interested in trying to identify inhibitors of hematopoietic stem cell proliferation with the idea that these might be used as myelo protective agents during cancer chemotherapy. He had identified an "activity" in the conditioned media of J774 cells, which was capable of inducing quiescence in primary murine and human hematopoietic stem cells and my job was to purify and characterizes this factor. The protein responsible for this activity proved reasonably easy to purify and turned out to be CCL3 although, at the time, we called it stem cell inhibitor, or SCI. This work was published in Nature in 1990 (1) and represented the first demonstration of a role for chemokines in regulating stem cell function and this, of course, has now become a prominent sub specialty in the chemokine field. The next objective was to clone the receptor for this stem cell inhibitor. I spent a frustrating period of time trying to "expression clone" this CCL3 receptor from "stem cell like" cell lines but these approaches met with little success. Alternative approaches were needed.

THE CLONING OF D6

In 1993, I was lucky to be able to recruit, to the group, a very talented young post doctoral researcher named Rob Nibbs who was (and is!) a highly gifted molecular biologist. Rob then set about developing new strategies for the cloning of CCL3 receptors. At this stage, only CCR1 had been identified and we had shown that this was not involved in mediating the stem cell inhibitory effects of CCL3 suggesting that an, as yet unidentified receptor, was key. Rob set his mind on using a degenerate genomic PCR cloning strategy based on the emerging indications that the majority of the coding regions for chemokine receptors were incorporated within a single genomic exon. This strategy led to the identification of a number of novel murine chemokine receptors. Frustratingly, as is the way in competitive science, a number of these receptors were published by other groups just as we were drafting out our publications reporting their cloning. However, one receptor that we had identified was not published by other groups and was reported by us under the name of "D6" in 1997 (2). Notably, Steiner and colleagues also reported the cloning of D6 around the same time (3) but they did not pursue further biological studies of this molecule. Shortly after the cloning of murine D6, we reported the cloning of the human homolog (4). One of the curious features of both murine and human D6 was that they lacked the canonical DRYLAIV motif, which had been found in all other cloned chemokine receptors and which was regarded as being important for cellular signaling. This suggested an unusual aspect to the biology of D6 function.

THE EXPRESSION OF D6

In collaboration with Paul Ponath, and his colleagues at Leukosite (a former Biotech company in the United States), we generated monoclonal antibodies to human D6 and used these to demonstrate that the predominant cells expressing D6 in adult tissues were lymphatic endothelial cells (5). In addition, strong D6 expression was seen throughout the syncytiotrophoblast layer in the placenta and expression was also noted on some leukocyte subtype (6). Therefore, again in keeping with the atypical nature of this molecule, D6 expression patterns were markedly different from the other chemokine receptors further suggesting unusual aspects to D6 biology.

INSIGHTS INTO D6 FUNCTION

Exhaustive ligand binding studies demonstrated that D6 was a highly promiscuous receptor capable of binding the majority of (if not all) inflammatory CC-chemokines. It did not bind homeostatic CC-chemokines not did it bind CXC, XC, or CX3C chemokines. We therefore characterized it as a promiscuous receptor with a specificity for inflammatory CC-chemokines. Binding affinities for the ligands were generally in the high pM and low nM range and therefore equivalent to those seen with the other chemokine receptors. In keeping with the altered DRYLAIV motif, and contrary to data reported in our initial cloning paper (2) (which we presume was a consequence of a mutation introduced into the receptor clone used), we

were never able to demonstrate signaling through D6 or chemotactic responses in cells expressing this receptor. This led us to the tentative assumption that D6 was a non-signaling chemokine receptor [more recent observations from our Milan colleagues have suggested "atypical" signaling pathways downstream of ligand binding by D6 (7)] but quite what this meant for function was not immediately apparent.

The breakthrough was provided when Alberto Mantovani and Massimo Locati and their group in Milan demonstrated that D6 was capable of internalizing and effectively scavenging its ligands (8). Shortly after, we showed that D6 spontaneously internalized and recycled to the cell membrane in any cell type, which it was expressed (9). Together, these observations led to a model of D6 function, which proposed that D6 does not support cellular migration but that, following binding, it internalizes ligand and deposits it in lysosomes for intracellular degradation. The great advantage of D6 is its promiscuity and all the analyses that we, and our Milan colleagues, have performed have demonstrated that it is an exquisitely efficient scavenger of inflammatory CC-chemokines. Notably, all these data were generated using *in vitro* approaches and so the next challenge was to demonstrate a role for D6 *in vivo* and to see if such a role was compatible with *in vivo* chemokine scavenging activity.

D6 *IN VIVO*

Our next target was to generate D6-deficient mice to allow us to study their responses in a range of inflammatory models. At the time, this was not an area of expertise that we possessed and so I initiated a collaboration with our friends Don Cook and Sergio Lira who were expert in this area and who were both, at the time, employed by the Schering-Plough Research Institute in Kenilworth New Jersey. Don quickly generated the D6-deficient mice and sent them to us for analysis. Import of these mice into Scotland, however, did not go quite as smoothly as initially planned! During the flight from the United States to Scotland, the mice managed to gnaw through the wall of the container in which they had been kept. Once the authorities discovered this they were concerned that mice might have escaped into the electrics of the aeroplane and might therefore cause serious problems with the plane's function. We therefore had to prove, without doubt, that no mice had escaped from the cage. Fortunately, the mice were not sufficiently interested in exploring the plane and we were able to demonstrate that all mice that had been sent remained in the cage. This was a massive relief as the cost of stripping down, and rebuilding, a Jumbo Jet to find a lost mouse would have bankrupted the Institute in which Rob and I were employed at the time! Anyway, the mice arrived safely and we proceeded to examine their responses in a relatively simple model of cutaneous inflammation involving the topical application of the phorbol ester TPA. What we found, and very much in keeping with a role for D6 as a scavenger of inflammatory chemokines, was that these mice displayed an inability to effectively resolve this cutaneous inflammatory response. Indeed, the mice developed a pathology that displayed remarkable similarities to human psoriasis. This work was published in Nature Immunology in 2005 (10) and was followed by numerous other studies in different tissue systems both from our own group and from the Milan group (11). Together these studies unequivocally demonstrated a role for D6 in the resolution of inflammatory response. The importance of D6 for scavenging inflammatory CC-chemokines was also reflected in other pathological phenotypes in D6-deficient mice. For example, and as mentioned above, a major site of D6 expression is the syncytiotrophoblast layer of the placenta and D6-deficient mice display enhanced susceptibility to miscarriage in response to maternal systemic inflammation (12). In addition, D6-deficient mice display exaggerated tumorigenic programs in a variety of inflammation-dependent cancer models. D6 is therefore a scavenger of inflammatory chemokines with important roles to play in a range of tissue and pathological, contexts. Notably, we have recently published evidence indicating a developmental role for D6 in regulating the density of lymphatic vessel networks in embryonic skin (13). Together these studies implicate the D6 in the regulation of pro-lymphangionenic macrophage proximity to developing lymphatic vessel networks and provide the first evidence of a role for inflammatory chemokines, and their regulators, in developmental processes.

THE NOMENCLATURE PROBLEM!

The name "D6" refers to nothing more complicated than the coordinates, on a multiwell plate, of the clone encoding this receptor. As mentioned above, we erroneously initially believed that D6 was a classical signaling molecule and therefore contacted the chemokine receptor nomenclature committee to register it. It was initially designated as CCR9. However, the Steiner group also requested a systematic nomenclature for their D6 clone around the same time and was provided with CCR10 as a designation. Therefore, for some time, this receptor was variously known as D6, CCR9, and CCR10! To confuse things even further the GenBank accepted name was "ccbp2" standing for chemokine binding protein-2. Eventually, both the CCR9 and CCR10 nomenclatures were assigned to other receptors and D6 became the accepted name for this molecule. However, most recently, we have developed a systematic nomenclature system for the entire atypical chemokine receptor family to which D6 belongs and refer to these as ACKRs. Within this IUPHAR approved nomenclature system D6 is now known as ACKR2, which is now its settled nomenclature (14).

IN SUMMARY

Starting from an unusual standpoint, and with essential input and insights from our Milan colleagues, we have cloned and characterized D6/ACKR2 as a scavenger of inflammatory CC-chemokines and have demonstrated its importance for the resolution of inflammatory response in a variety of contexts. D6/ACKR2 provides a paradigm for the function of other members of the atypical chemokine receptor family and similarities with the function of ACKR3 and ACKR4 have already become apparent (15). We believe that this molecule has both diagnostic and therapeutic value although this potential has yet to be realized.

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Chemokines and glycosaminoglycans

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Although it was not known at the time, the chemokine field started with the identification of a protein by Heparin Sepharose affinity chromatography, platelet factor 4 (PF4) (1) now called CXCL4 in the systemic nomenclature introduced in 2000. As with many pathways in scientific research, our entry into the field of chemokines and glycosaminoglycans (GAGs) was a series of fortuitous co-incidences. Christine Power, working on the chemokine project in the Glaxo Institute of Molecular Biology (GIMB) under the leadership of Tim Wells, had hired a postdoctoral scientist, Arlene Hoogewerf to clone murine CCR4, and to generate the KO mouse. Arlene had previously studied the role of proteoglycans in regulating the function of other molecules and had also previously published a paper on the enzymatic activity of CTAP-III, and NAP-2, truncated forms of platelet basic protein/CXCL7, to degrade heparin. Since our group was currently working on these chemokines derived from the β-thromboglobulin precursor, and moreover we were all biochemists, our interest was piqued by the relationship between chemokines and GAGs. This interest was of course inspired by the paper recently published by Antal Rot demonstrating the evidence for haptotaxis as opposed to chemotaxis (2), earning him the title of Godfather of Chemokine–GAG biology. Arlene then followed a dual Post-doc pathway, partially hijacked by Tim to collaborate with him and his Ph.D. student, Gaby Kuschert (now Gaby Campanella) to lay much of the biochemical groundwork of chemokine–GAG interactions, and also fulfilling her goal of creating the $CCR4^{-/-}$ mouse. They were able to show that chemokines demonstrated selectivity in their interaction with GAGs, beyond the obvious electrostatic interactions between basic and acidic molecules, and importantly made the observation that this interaction could trigger oligomerization of chemokines (3, 4). In addition, they were able to define the pharmacophore responsible for GAG binding of the chemokine IL-8/CXCL8 (5).

My lab became more directly involved in this research direction through the serendipitous encounter of a Ph.D. student, Sarah Fritchley, working in Simi Ali's lab in Newcastle, UK, who was interested in expressing the putative GAG binding mutant of RANTES/CCL5 in *E. coli*, but who did not have a viable expression system. Sarah spent a couple of months in the lab under the tuition of our expert chemokine protein chemist, Fred Borlat, successfully producing the 40's mutant, as we colloquially called it. We published it in JBC with its correct biochemical nomenclature, ^{44}AANA^{47}-RANTES (6). Perhaps an omen as to the importance of this mutant for us was that it was an exception among most papers we had submitted as it was accepted overnight!

We were heavily involved in screening for chemokine receptor antagonists at this time, and Marie Kosco-Vilbois had set up a simple cell recruitment assay to test putative inhibitors *in vivo* – chemokine-induced peritoneal recruitment in mice. Therefore, to investigate the effect of the abrogation of GAG binding *in vivo*, we asked her technician, Suzanne Herren, to test it for us. Being very rigorous, and accustomed to testing compounds for their ability to inhibit chemokine induced recruitment, in this instance RANTES, Suzanne tested it both for its agonist and antagonist activity. I will never forget my amazement in seeing that the mutant was not only unable to recruit cells but actually inhibited the recruitment induced by RANTES.

Again serendipity stepped in. I gave a talk at a BALR meeting in the UK, and after dinner at the speaker's table, joined the youngsters at the adjacent table for a post-prandial "relaxation". . . (which

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would never happen in this day and age). There I met Zoë Johnson, whom I learned the next day, was toying with the idea of doing a Ph.D., after having worked for a few years as an *in vivo* pharmacology laboratory assistant in *in vivo* pharmacology in industry. Since I was responsible for the Student program at the Institute, now the Serono Pharmaceutical Research Institute, she emailed me soon after expressing her interest. Tim was by now Director of the Institute, but had kept his interest in the chemokine project close to his heart, and having the flair and ability to make quick opportunistic decisions, immediately approved her appointment to investigate this phenomenon further.

We then embarked on a marvelous and exciting 3 years of research during Zoë's thesis as the importance of the chemokine–GAG interaction unveiled itself. This period was also the beginning of my collaboration with Tracy Handel, one of the most enjoyable and mutually fruitful collaborations between two laboratories that I have had the pleasure to be part of, where we shared everything without any trace of competiveness, leading to several "duo" presentations of our joint discoveries at chemokine meetings. The result of these 3 years, during which we had access to other GAG binding mutants, notably those of MCP-1/CCL2 produced by Tracy's lab in Berkeley, CA, USA, and that of MIB-1β from Patti Liwang in Texas, was the demonstration that the immobilization of chemokines on GAGs was essential for their *in vivo* activity, and that moreover, certain chemokines needed to form oligomers in order to exert their property of cellular recruitment *in vivo* (7). The inter-relationship between these two properties was shown by the failure to include another chemokine–GAG mutant, that of murine MIP-1α, sent to us by Gerry Graham in Glasgow. He included of course the WT control, which in our hands was inactive in recruiting cells *in vivo*, despite his assurance that it was fully active *in vitro*, so we saw no use in testing his GAG binding mutant in our in *in vivo* assay. What he neglected to tell us was that in line with the work carried out by Lloyd Czapeklski at British Biotech some years previously the WT chemokine had been mutated to no longer oligomerize – obviously an obligate monomer that was inactive *in vivo*, in accordance with our results with the three obligate monomers described above!

We then exploited the inter-relationship of GAG binding and oligomerization using our RANTES mutant, now commonly called 004, in our lab, an abbreviation of its company nomenclature used for all biologicals, AS900004, and AANA-RANTES to our collaborators, as a very effective anti-inflammatory tool (8–10). However, the fact that it retained agonist activity precluded its development as a biological therapeutic. However, we felt that we were on the right path to discovering a novel set of molecules that would interfere with the chemokine–GAG interaction and would give us a superior niche to differentiate from our competitors who were all targeting the chemokine:receptor interaction. To achieve this, we used two approaches. The first lead by our talented head of chemistry, Matthias Schwarz, was to carry out an approach coined "SAR by NMR" to identify protein binders. Our target protein RANTES/CCL5 had the advantage that (a) it was small and therefore amenable to NMR technology, and (b) its three dimensional structure in complex with a GAG – a disaccharide – had been solved by our X-ray crystallographer, Jeffrey Shaw (11). The aim was to screen a small library of about 200 sulfated compounds by NMR to identify RANTES/CCL5 binders. The first screen yielded a hit, which prevented binding to heparin, and inhibited RANTES-induced peritoneal cell recruitment, despite only having micromolar affinity. The aim was to then identify a second molecule in a second round of screening, this time in the presence of the first compound, and then using the data obtained from the structures of the complexes solved by Jeff, to design a linker to form a dimer, which would have considerably higher affinity. The screening and structural biology arms worked beautifully, and the dimer was synthesized by the chemists – but the product no longer inhibited cell recruitment *in vivo* – and much to our chagrin and despair, it even enhanced it.

However, we were still believers and decided to follow another lead. Zoë had shown that the minimal repeating unit of heparin that could inhibit RANTES-induced peritoneal recruitment was a tetrasaccharide. We therefore hired another postdoctoral scientist, India Severin, a chemist whose objective was to identify and then synthesize GAG-based mimetics. Despite a very assiduous program in collaboration with a glycobiology group in Australia, led by Deidre Coombe, we had to admit defeat. Although we identified moieties that inhibited GAG binding to RANTES as well as RANTES binding to the receptor CCR1, we did not achieve our aim of identifying a lead candidate for an anti-inflammatory program (12).

To my delight, several years later, Deidre contacted me with the explanation as to why the design of our dimer resulting from our screen by NMR was incorrect. We had performed our crystallization studies at an acidic pH in order to maintain the monomeric form of RANTES, which would crystallize without aggregating. Modeling studies at physiological pH values revealed that our compounds had bound to the protein at acidic pH in a manner different from that predicted by the docking studies at physiological pH, presumably due to their different protonation states (13). And even more consoling was the publication of a GAG moiety that had anti-inflammatory properties in a model of lung inflammation by preventing T-cell recruitment (14).

However, we still have a long way to go to fully understand the inter-relationship between the two interactions that chemokines have, especially *in vivo*. Chemokine biologists have always talked about gradients, but without defining whether these gradients are in the fluid phase or caused by immobilized chemokines through their interaction with GAGs. Our work showed that chemokines needed to be immobilized but did not address the question of a gradient. This has recently been beautifully demonstrated by Michael Sixt, where he visualized gradients of CCL21 leading to lymphatic vessels (15). We believed that the active form of the chemokine must be that, which is immobilized on the extracellular surface. However, our recent work at Novimmune, with Nicolas Fischer and Marie Kosco-Vilbois and another very talented Ph.D. student, Pauline Bonvin, characterizing two anti-murine CXCL10 antibodies, has led to revisiting this hypothesis. The mAb that is active in *in vivo* models of disease does not recognize GAG bound chemokine, whereas the mAb that is ineffective does, a result, which contradicts the notion that it is the GAG bound form of the chemokine that is active *in vivo* (Bonvin et al., manuscript in preparation). However, the active mAb inhibits the binding

of the chemokine to GAGs, indicating that this interaction does indeed play a role, but the point of intervention appears more subtle that initially thought. Hopefully, more detailed studies of these two antibodies will provide a greater in depth understanding of the role of GAG binding in chemokine-induced cell migration *in vivo*.

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Chemokines and HIV: the first close encounter

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The first "close encounter" between the fields of chemokines and HIV occurred in the late spring of 1995, when my group at NCI's Laboratory of Tumor Cell Biology (LTCB) received from Harvard Microchemistry and Proteomics the amino acid sequences of three peptide fragments from an HPLC-purified fraction that we had submitted a few weeks earlier. The sequences showed a perfect match with the human chemokine RANTES/CCL5. The fraction had been isolated from the culture supernatant of an immortalized T-cell clone (FC36.22) producing the elusive "CD8-derived HIV-suppressive factor" whose existence had been postulated by Jay Levy's group at UCSF since 1986 (1), but whose identity had remained enigmatic for nearly a decade.

Two years earlier, when Fiorenza Cocchi, at that time a post-doctoral fellow from Milan, Italy, had approached me with the proposal to embark on the quest for the "Levy factor," my first reaction had been anything but enthusiastic. Not that I had any doubt about the significance of identifying an endogenous factor that was believed to help HIV-infected individuals remain asymptomatic by suppressing the virus in a non-cytolytic fashion (2). On several occasions, we had heard Levy illustrate his model, and Bob Gallo, our inspiring lab chief at the LTCB, had often remarked on the importance of resolving this long-standing riddle. However, all attempts to identify the factor until then had failed in the midst of confusion among different designations, experimental models, and mechanistic hypotheses (3), and skepticism about the very existence of such factor was on the rise. Furthermore, my laboratory's focus was on pathogenesis, with very limited expertise in protein chemistry. And so, we concurred to give it a try, but I made it clear that we would soon drop the project unless we could come up with a robust and reproducible experimental system to justify a long-term commitment.

The original phenomenological observation on the non-cytolytic HIV-suppressive activity of $CD8^+$ T cells was made by Chris Walker and Jay Levy in the mid-80s while they were attempting to increase the rate of HIV-1 isolation from asymptomatic seropositive individuals. They found that removal of $CD8^+$ T cells from the cultures greatly enhanced their odds of success; when autologous $CD8^+$ T cells were added back to the cultures, virus replication was again suppressed but the number of $CD4^+$ T cells remained constant, thus ruling out a classic "cytotoxic T-lymphocyte" effect (1). In subsequent years, the same group went on to show that the activity was not bound to MHC restriction and was at least in part mediated by a soluble factor – initially nicknamed "CAF" – that was diffusible through a semi-permeable membrane (4). Moreover, they established a correlation between the levels of CAF production and the asymptomatic state of HIV-1 infection (2), corroborating the clinical significance of this unconventional $CD8^+$ T-cell activity. Despite intensive efforts, however, no progress toward the identification of the factor was made over the following years. One of the key challenges was the extremely low level of factor that could be rescued from primary $CD8^+$ T-lymphocyte cultures, further complicated by marked donor–donor variability. Yet, Levy insisted that the activity was an exclusive product of primary $CD8^+$ T cells and a specific attribute of HIV-seropositive individuals (4), which posed major challenges for production scale-up.

We reasoned that the first critical step to tackle this project was to devise a high-yield and reproducible cellular source for the factor, and we began testing primary and immortalized $CD8^+$ T cells from both seropositive and seronegative donors under diverse conditions of activation and culture. The LTCB was an ideal site in this respect because a major focus for over two decades had been the optimization of T-cell growth conditions, culminating in the discovery of "T-cell growth factor," subsequently named interleukin-2, by Doris Morgan, Frank Ruscetti, and Gallo in 1976 (5), and the isolation of the first human retrovirus, HTLV-1, which can immortalize both $CD4^+$ and $CD8^+$ T cells *ex vivo*, by Bernie Poiesz and Gallo in 1980 (6). Thus, besides testing primary $CD8^+$ T cells, we derived $CD8^+$ T-cell lines immortalized with HTLV-1 or its little brother, HTLV-2, and dug deep into the freezers in search for every vintage $CD8^+$ T-cell line that we could test. Among the many cells that we screened was 67-I, an HTLV-I-immortalized clone obtained a few years earlier by Anita DeRossi at the LTCB (7), which eventually turned out to be the key to our success. Derived from the peripheral blood of a healthy blood donor, 67-I retained many features of primary $CD8^+$ T cells, but unlike the latter it provided a stable and scalable source of soluble factors and was adaptable to grow under serum-free culture conditions, which would eventually simplify purification of the factor.

In parallel to developing an efficient "factor factory," a second critical need was to establish a highly standardized read-out system for the quantitative determination of antiviral activity. Again, it was essential to overcome the inconsistencies of primary cells and, even worse,

of poorly characterized endogenous viral strains harbored by patient-derived $CD4^+$ T cells (1, 2). Thus, we embarked in the screening of a wide panel of target cells and viral strains. We eventually opted for PM1, a $CD4^+$ clone that we had recently derived from the leukemic T-cell line Hut78, which featured an uncommon susceptibility to diverse HIV-1 variants, including laboratory-adapted and primary isolates with both T-cell and macrophage tropism (8). Taking advantage of this unique quality of PM1, we enhanced our chances of success by entering two divergent HIV-1 variants into our default testing protocol: a typical laboratory strain, IIIB, adapted to grow in continuous T-cell lines, and a macrophage-tropic strain, BaL, passaged exclusively in primary cells, which shared many properties with primary HIV-1 isolates. The system was highly standardized and suitable for high-throughput screening. But when Fiorenza showed up one afternoon with the results of the first experiment, we could hardly believe our eyes: the culture supernatant of 67-I had completely suppressed the BaL strain, while the IIIB strain had continued to replicate impassively. At first, we thought it could only be a technical error, and we decided to independently repeat the experiment in separate laboratories. The results came out a few days later and again they were stunning: in both repeats, BaL was completely inhibited, while IIIB was untouched! Not only did we have in our hands a powerful and reproducible source of HIV-suppressive factor but also the unequivocal bias in favor of the primary-like viral isolate gave us confidence in the specificity of the suppressive effect.

As we had finally pulled together the right experimental tools for the biological side of the project, we set out to identify a skilled protein chemist who could plunge into the backbreaking process of biochemical purification. Thus, we made contact with Tony DeVico, at that time a young research associate at ABL, an NIH-contractor laboratory in Rockville, who had the necessary know-how and enthusiasm to dive into this high-risk/high-reward endeavor. After discussing multiple strategies, we established a basic purification protocol, leaving the option open to modifying it at any time based on the progress of the project.

Looking backwards, although "serendipity" is a term commonly used to describe discoveries in which a "mystery object" remains mysterious until the epilog of the story, never was in my scientific career an experimental design so meticulously and systematically planned ahead in its finest details. This notwithstanding, we were bound for a string of false leads and dead ends that put our trust and determination to serious trial.

Over the next several months, the cycle was repeated over and over: large volumes of serum-free culture fluid conditioned by a high-producer 67-I subclone, FC36.22, were collected, clarified, concentrated by size fractionation, and subjected to HPLC purification using different matrices. Purified fractions were then individually tested against HIV-1 BaL in PM1 cells. Many a cycle was to go "dry," with no fractions retaining sufficient activity to justify further analysis. Then, a few months down the road, a first intriguing lead: a bioactive fraction containing a single peak was sent for proteomics analysis. Our excitement was sky-high when the results came back a few weeks later showing that the fraction contained human insulin-like growth factor-1 (IGF-1), an immunomodulatory hormone. We immediately attempted to validate the lead, but commercial IGF-1 preparations did not show antiviral activity in our system, and neutralizing antibodies to IGF-1 did not abrogate the activity in crude FC36.22 supernatants. Thus, the lead was abandoned even though IGF-1 was later reported to inhibit HIV-1 (9). After several other "dry" cycles, we stumbled upon another candidate factor, which opened a fascinating new perspective: a bioactive fraction yielded a fragment identical to an HTLV-1 protein, suggesting an intriguing scenario of virus–virus interference. Though captivating as this hypothesis was, the results were not reproducible using concentrated HTLV-1 fractions and, besides, the model was incompatible with the bulk of previous observations made with HTLV-1-negative patient $CD8^+$ T cells. We had to move on, and the cycles resumed.

It was a bright and hot late-spring afternoon in Milan. One of those days that give your senses a first savor of the imminent summer: my first one back in Italy after nine and a half years in Bethesda. The phone rang in my temporary office in the new DIBIT building at the San Raffaele Institute where I was creating my own Laboratory of Human Virology. When I picked up, Fiorenza's voice on the other side could hardly conceal her excitement: "We've got new sequences – she said right off the bat – It's RANTES!". While the call was still on, I jumped on MEDLINE and crossed the two keywords: "RANTES" and "HIV." I hit "return" and, to my astonishment, the result was . . . zero! Even just by chance, almost any two keywords yield at least a half dozen citations. "Zero" was not only really amazing but also somewhat frightening. It was like in those science-fiction movies when they open a door in a dark empty hallway to find themselves into the dazzling light of a totally new dimension: there we were, all of a sudden projected into the fantastic world of the chemoattractants!

Of course, having chased several false leads in the previous months made us temper our enthusiasm and keep our feet on the ground. But a few weeks later, when we received the sequences from a second bioactive fraction matching 100% the "sister" chemokine MIP-1α/CCL3, our adrenalin level had a dramatic jolt. We knew that this time we were on the right track. We swiftly ordered a set of recombinant proteins and neutralizing antibodies, including those specific for the third "sister" chemokine, MIP-1β/CCL4, even though we had not yet received proteomics confirmation for this last member of the trio. In early September, we submitted an article to *Science* that, in spite of a single hopeless negativist referee who raised all sorts of questions about the past, present, and future relevance of our findings, was rapidly accepted and appeared in print on December 15, 1995 (10). With an unorthodox move, *Science* heralded our paper with a Commentary by Michael Balter in the December 8 issue (11), concomitant with the publication in *Nature* of another candidate CD8-derived antiviral factor, interleukin-16 (12). In the paper, we presented conclusive evidence that: (i) RANTES, MIP-1α, and MIP-1β are three potent and specific endogenous HIV-1 inhibitors; (ii) they are abundantly produced by both immortalized and primary $CD8^+$ T cells; and (iii) altogether they constitute a major component of the soluble HIV-suppressive

activity produced by these cells (10). For the first time, we were exposing the "double life" of certain chemokines, turned overnight from aseptic cellular-traffic policemen into specific endogenous virus-busters. One of the greatest surprises in this saga was the realization that the long-sought-after CD8 anti-HIV factor in fact, comprises multiple factors, breaking a central dogma of the original model (4). Indeed, evidence continues to accumulate on the existence of a wide range of endogenous HIV-suppressive factors, as illustrated by our recent identification of XCL1/lymphotactin as a novel anti-HIV chemokine produced by $CD8^+$ T cells (13).

The rest of the story is well known. Almost fictional was the extraordinary time coincidence whereby <6 months after the publication of our paper Ed Berger and his colleagues at the NIAID reported in *Science* the first HIV-1 coreceptor, fusin/CXCR4 (14) – another "serendipitous" discovery? – which happened to be an orphan "chemokine" receptor originally identified by Bernhard Moser's group in Bern (15). Likewise, virtually at the same time, Phil Murphy's group at the NIAID (16) and Marc Parmentier's group in Brussels (17) were both characterizing the same novel "chemokine" receptor (CCR5) specific for RANTES, MIP-1α, and MIP-1β, which was almost immediately shown by Berger's and four other groups to be the second, physiologically most relevant, HIV-1 coreceptor (3). Curiously, despite having worked on the same campus for years, I had never previously met Ed or Phil, who later became good friends of mine and, in the case of Ed, a close collaborator. The extraordinary convergence and synergy among these independent discoveries, collectively saluted by *Science* as one of the "breakthroughs-of-the-year" at the end of 1996 (18), inaugurated a new era of AIDS research, triggering a chain-reaction of additional breakthroughs which altogether dramatically accelerated our understanding of HIV physiology and pathogenesis, and posed the foundations for new therapeutic and preventive strategies with far-reaching consequences for the ultimate control of the HIV/AIDS pandemic.

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Finding fusin/CXCR4, the first "2nd receptor" for HIV entry

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Shortly after the pioneering Montagnier/Gallo discoveries of HIV as the etiologic agent of AIDS, the CD4 antigen was identified as the primary receptor for HIV entry. The focus of the present story begins with 1986 report from Richard Axel's group that recombinant human CD4 conferred permissiveness to HIV-1 infection when expressed on diverse human cell types, but not on mouse cells. The block was at an early replication step after virion binding, perhaps virus internalization (1).

My entry into the HIV/AIDS field came at a particularly opportune time (1987) and place (Laboratory of Viral Diseases, NIAID, NIH, headed by Bernie Moss). I was interested in learning the vaccinia virus-based system for recombinant protein expression, and applying it to study HIV entry. Bernie's group had generated a vaccinia recombinant encoding HIV-1 Env that induced robust CD4-dependent cell fusion as measured by syncytia (2). The strictly cytoplasmic nature of the vaccinia replication cycle turned out to provide a fortuitous advantage for studying Env, since it obviated the as-yet unrecognized need for co-expression of HIV rev to export unspliced Env RNA out of the nucleus; moreover, the extremely broad host range of vaccinia enabled studies of Env-mediated fusion with a variety of cell types from diverse species. In a reductionist system using the corresponding vaccinia recombinants, we showed that cells expressing HIV-1 Env formed syncytia when mixed with cells (lymphoid and non-lymphoid) expressing human CD4, provided the latter were of human origin (3). Parallel results were obtained by other groups (4, 5).

Was this phenomenon due to a requirement for an additional human-specific factor, or to a dominant restrictive feature of the non-human cells? In collaboration with Robert Blumenthal's group at NCI, NIH, we demonstrated that CD4-expressing transient hybrids between human and murine cells were fusion-permissive, arguing against the non-human restriction model (6). These findings in the reductionist cell fusion system were consistent with studies by others examining HIV infection of transient or stable or cell hybrids (4, 5). Thus, by the early 1990s, it was evident that the CD4-human cell requirement was manifest at the level of Env-CD4-mediated fusion/entry, apparently reflecting target cell expression of an essential human-specific cofactor (perhaps a 2nd receptor, or "coreceptor").

Further adding to my good fortune was my partnering with postdoc Tom Fuerst in the Moss lab, who had led their development of the vaccinia/T7 hybrid expression system. They had shown that a target gene linked to the phage T7 promoter is activated by the vaccinia-encoded T7 RNA polymerase expressed in the same cell; the presence of all components in the cytoplasm leads to robust transient expression of the target gene (7). I realized that this system could be adapted to study Env-receptor-mediated cell fusion by expressing the vaccinia-encoded T7 polymerase in one cell partner and introducing a reporter gene (e.g., the *E. coli LacZ* gene) linked to the T7 promoter in the other; reporter expression would be triggered in the cytoplasm of fused cells. Postdocs Ofer Nussbaum and Chris Broder in my group demonstrated the highly sensitive and specific nature of this reporter assay and its superiority over the subjective and laborious semi-quantitative syncytium-counting assay (8). Specific fusion was observed when Env-expressing "effector cells" were mixed with CD4-expressing "target cells"; a robust β-galactosidase signal was detected at 2–3 h, either by *in situ* staining or colorimetric assay of detergent cell lysates (**Figure 1A**). Importantly, the reporter assay corroborated the requirement that CD4 be expressed on a human cell, whereas Env could be on a human or non-human cell. Membrane vesicle transfer experiments demonstrated that the fusion deficiency of CD4-expressing non-human cells was not due to their detrimental modification of CD4.

The specificity of Env-mediated fusion/entry took on an additional layer of complexity beginning in the late 1980s with the growing awareness that different HIV-1 isolates displayed markedly distinct *in vitro* tropisms for infection of different CD4-positive target cell types (10). Some isolates infected $CD4^+$ continuous T cell lines (and non-lymphoid human cell lines such as HeLa-CD4 transformants) but not primary macrophages; others displayed the reverse tropism, infecting primary macrophages but not $CD4^+$ T cell lines. The terms "T cell line-tropic" (TCL-tropic) and "macrophage-tropic" (M-tropic) were used to distinguish these variants. Both phenotypes replicated in primary $CD4^+$ T cells. This phenotypic distinction was more than simply a laboratory curiosity; in the real world of human HIV infection, the isolates obtained shortly after transmission and throughout the asymptomatic phase invariably displayed the M-tropic phenotype; TCL-tropic variants emerged only (years) later, during the transition to the symptomatic phase and progression to AIDS (and not in all cases). Studies from many groups in the early-mid 1990s pinpointed Env as the principle viral determinant mediating this

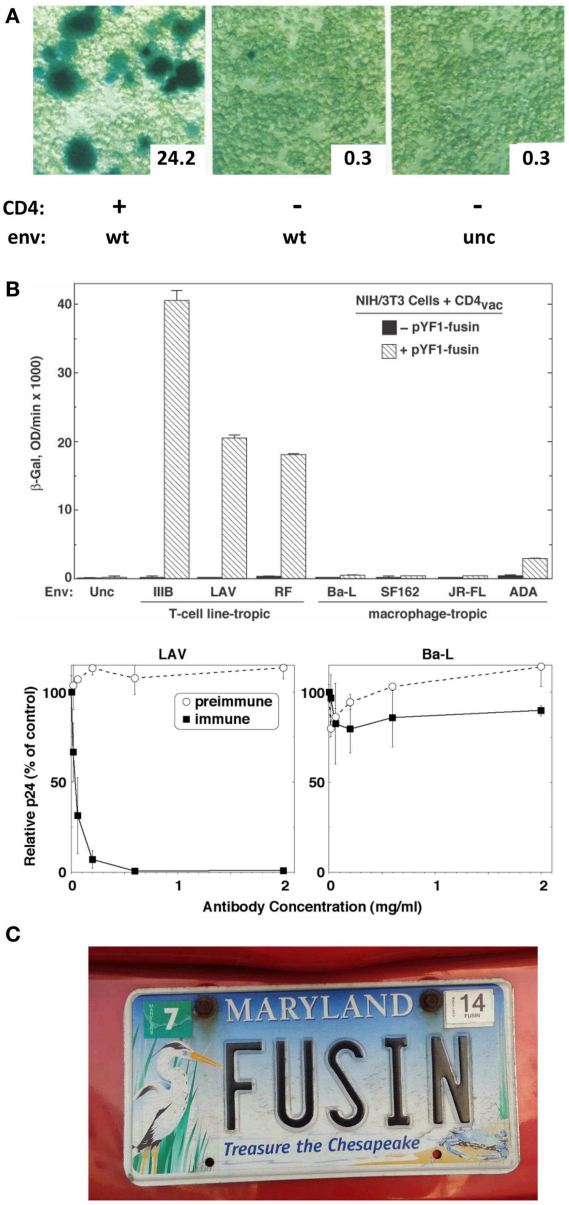

FIGURE 1 | (A) Reporter gene assay for HIV-1 Env/CD4-mediated cell fusion. Effector HeLa cells expressed vaccinia-encoded HIV Env wild type (WT) or a non-functional uncleaved mutant (unc) and were transfected with a plasmid containing the *LacZ* gene linked to the T7 promoter. Target HeLa cells expressed vaccinia-encoded T7 RNA polymerase with (+) or without (–) CD4. Duplicate cell mixtures were incubated at 37°C for 2.5 h and β-galactosidase was measured in one set by *in situ* staining (photomicrographs) and in the other by colorimetric assay of detergent cell lysates (insets, arbitrary units). Adapted from Ref. (8). **(B)** Demonstration of fusin's function as an entry receptor for TCL-tropic HIV-1. *Top panel*. Cell fusion assay. Effector NIH 3T3 cells expressed vaccinia-encoded Env from the indicated TCL-tropic or M-tropic HIV-1 isolate as well as T7 RNA polymerase. Target NIH 3T3 cells were co-transfected with the plasmid containing the *LacZ* gene linked to the T7 promoter plus either a control plasmid (filled bars) or a plasmid encoding fusin (cross-hatched bars). Cell mixtures were incubated at 37°C for 3 hr, and β-galactosidase was measured by the colorimetric assay of detergent cell lysates. *Bottom panel*. HIV-1 infection assay. PBMCs were pre-incubated with the indicated concentrations of purified rabbit antibodies [preimmune, and immune against the fusin N-terminus], then infected with HIV-1 LAV (left, TCL-tropic) or Ba-L (right, M-tropic). Culture supernatants were assayed by ELISA for p24 content at day 7. Results for each isolate are expressed as the percentage of p24 produced at each antibody concentration compared to the control value with no antibody. From Ref. (9). **(C)**. Rare detection of the name "fusin." From the Maryland Department of Motor Vehicles.

tropism phenotype (4, 5). Using the reductionist cell fusion assay, Chris Broder in my group demonstrated a marked correlation between the fusion specificities of vaccinia-encoded HIV-1 Env glycoproteins and the infection tropisms of the strains from which they were derived (11). Subsequently, postdocs Ghalib Alkhatib and Chris performed fusion assays with transient hybrids between continuous cell lines and macrophages; the results suggested that the fusion specificities were attributable to distinct cellular cofactors (coreceptors?) mediating TCL- vs. M-tropism rather than to cell type-specific fusion restriction factors (12). Identification of these cofactors thus became the focal point of extensive searches by many groups worldwide; numerous candidate molecules were proposed (specific proteins, glycolipids), but these did not withstand detailed experimental scrutiny (4, 5).

Our initial identification efforts focused on the TCL-tropic cofactor, for the simple reason that it appeared to be expressed in diverse human cell lines (e.g., HeLa), thereby providing a technical advantage compared to primary macrophages. Yu Feng, a new postdoc in the group, initiated a strategy based on mRNA microinjection. At the outset, we committed to an unbiased approach with no preconceived notions about what type of protein we were seeking; our only criterion was gain-of-function in a fusion assay with CD4-expressing non-human host cells; microinjection of mRNA from a permissive human cell type (e.g., HeLa) should confer fusion-permissiveness. But what host cells to use? We knew that an NIAID investigator in a nearby lab, Phil Murphy, was doing microinjection experiments in *Xenopus* oocytes. Hearing that Phil was a highly congenial colleague, we approached him with the idea even though his research interests centered on a subject that had nothing to do with HIV, i.e., receptors for chemokines (small proteins that function as chemoattractants guiding leukocyte migration). Phil expressed enthusiasm, but we soon realized that the experimental features of the *Xenopus* oocyte system were incompatible with Env/CD4-mediated cell fusion. A more expeditious approach employing mammalian cells was required.

We then turned to the idea of transfecting a cDNA library from a fusion-permissive human cell type into a CD4-expressing non-human cell and testing for fusion gain-of-function. We knew that CD4-expressing HeLa cells were highly permissive fusion targets (presumably because of high cofactor expression) whereas CD4-expressing murine NIH 3T3 cells were consistently refractory (presumably cofactor-negative); moreover, a HeLa cDNA library was commercially available. We devised a functional screening assay involving transfection of the HeLa cDNA library into 3T3 target cells expressing vaccinia-encoded human CD4 (and T7 RNA polymerase); a small fraction of these cells would become fusion-permissive due to expression from the rare cDNA encoding the cofactor, and would fuse with added effector cells expressing a vaccinia-encoded TCL-tropic Env (and containing a transfected plasmid with the T7 promoter/*Lac Z* reporter). Staining *in situ* for β-galactosidase would reveal cell fusion. In the very first experiments (May 1995), the library-transfected target cells yielded decisively more β-galactosidase-positive cells compared to controls. After several rounds of library sub-fractionation and screening, a single cDNA clone was isolated that conferred robust fusion-permissiveness to the CD4-expressing murine cells.

DNA sequencing results obtained at the end of July 1995 indicated that the ~1.7 kb cDNA insert encoded a 352 amino acid protein with 7 putative transmembrane domains, i.e., a likely member of the G protein-coupled receptor superfamily. The nucleotide sequence had been reported by several groups during the previous 2–3 years, but the normal function of the protein was unknown. Since the only observed activity was in rendering CD4-expressing non-human cells permissive for HIV-1 fusion, we gave it the name "fusin." During the following months, we accumulated critical experimental evidence proving fusin's role as the sought-after entry cofactor for TCL-tropic HIV, including (a) gain-of-function experiments showing that fusin rendered CD4-expressing non-human cells permissive for HIV-1 Env-mediated cell fusion and virus infection, (b) specificity assays demonstrating fusion gain for TCL-tropic but not M-tropic Envs (**Figure 1B**, top), (c) loss-of-function experiments demonstrating the fusion-blocking and infection-neutralizing activity of rabbit antibodies against the putative N-terminal domain of fusin, and specificity based on selective antibody blocking for TCL-tropic but not M-tropic HIV-1 (**Figure 1B**, bottom), and (d) Northern blots demonstrating the presence of fusin mRNA in permissive human target cells and its absence from unusual non-permissive human targets (and, of course, from non-human cells). Taken together, these results convincingly established fusin as the critical entry cofactor for TCL-tropic HIV-1.

Some intriguing implications became apparent during the course of our work. First, the previous cDNA cloning papers indicated that the closest amino acid sequence homology with a protein of known function was with the human receptor(s) for interleukin 8, a CXC chemokine. How ironic, since one of the two back-to-back 1991 papers describing that first cloning of a human chemokine receptor was from none other than our nigh-collaborator Phil Murphy! Second, the possibility that fusin might be a chemokine receptor took on greatly added significance with a December 1995 paper from Paolo Lusso and Fiorenza Cocchi in Bob Gallo's lab at the NCI, NIH; these investigators demonstrated that three CC chemokines, RANTES, MIP-1α, and MIP1-β accounted for the HIV-1 soluble suppressive activity released by CD8 T cells (13), a phenomenon first described by Jay Levy's group during the preceding decade. Most interestingly, these CC chemokines suppressed a M-tropic much more than a TCL-tropic strain. Thus, the fusin discovery, together with the Lusso suppressive chemokines, provided a possible clue to the identity of the M-tropic cofactor: perhaps it was a chemokine receptor, in this case for RANTES, MIP-1α and MIP1-β.

I presented our fusin findings at a Keystone meeting in Santa Fe NM in February 1996, well before we were ready to submit the manuscript. Perhaps naively, I disclosed not only the evidence supporting fusin as the TCL entry cofactor but also the full amino acid sequence of the protein. The brush fire was now ignited, in both the HIV and chemokine research communities. But just in time for my group came the next irony. In late January 1996, we attended a seminar by Phil in which he revealed his lab's cloning of a new chemokine receptor called CCR5, with precisely the specificity for the Lusso chemokines. Surely, there must be some connection with HIV, but what could that be? There we sat, with our knowledge of fusin, and our fledgling struggles to find the M-tropic cofactor by a similar functional cloning strategy using a cDNA library from primary macrophages. After some urgent pleas from the postdocs, I relinquished my stubborn adherence to the intellectual purity of the unbiased library screening approach and agreed instead to go for the direct kill. I contacted Phil in early March 1996, at last beginning a most productive collaboration. While attending another Keystone meeting at Hilton Head SC later that month, I phoned the lab and got the great news from Ghalib – he had the first data indicating a role for CCR5 as the M-tropic entry cofactor. The definitive experiments were completed over the next couple of months.

By the time, our fusin paper came out in May 1996 (9), the firestorms were raging in full. I give here only brief summaries, since there are fascinating stories to be told by other investigators who made major contributions to these developments [see reviews in Ref. (4, 5, 14, 15)]. On the HIV front, five independent papers (including ours) describing CCR5 as the essential entry cofactor for M-tropic HIV-1 were published within a week in June 1996. August–September 1996 saw the discovery of the CCR5 delta32 mutation, encoding a truncated non-functional protein; because of the high prevalence of this allele in Caucasian populations coupled with its simple Mendelian inheritance, CCR5 delta32 homozygosity provided the first and only molecularly understood mechanism for resistance to HIV infection. Moreover, this genotype was the basis for the first, and still only, documented cure of HIV infection. By October–November of 1996, both fusin and CCR5 were upgraded from cofactors to true "coreceptors," based on demonstrations of their physical interactions with Env. The findings that coreceptor engagement occurs only after CD4 binding means that designation of CD4 as the primary receptor refers not only to its chronology of discovery but also to its obligate mechanism of action. In the

ensuing nearly two decades, the coreceptor discoveries have engendered entirely new paradigms for understanding HIV transmission and pathogenesis, and have provided novel targets for antiretroviral drug development and gene therapy strategies aimed at curing HIV. In the chemokine field, our fusin paper was quickly followed (August 1996) by two back-to-back papers identifying the CXC chemokine stromal cell-derived factor 1 (SDF-1) as the natural ligand for fusin; SDF-1 was shown to inhibit TCL-tropic but not M-tropic HIV-1. Fusin was immediately renamed CXCR4 in keeping with chemokine receptor nomenclature. Thus, the impact of finding fusin/CXCR4, the first "2nd receptor" for HIV entry, endures to this day and likely well into the future. The fusin name, however, persists only in rare places (**Figure 1C**).

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SDF-1/CXCL12: a chemokine in the life cycle of HIV

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In the Arena of HIV-1 Research

Since 1986, the beginning of our involvement in HIV research, our major interest was focused on the regulation of HIV-1 replication by the transcriptional host cell machinery. Thus, we successively investigated the consequences of inflammatory and specific responses in HIV replication in primary monocytes and memory $CD4^+$ T cells, and explored with special emphasis the role played by the transcriptional factors NF-kB and the viral trans-activator Tat in the induction and maintaining of the activity of HIV-1 promoter region, while in parallel we elucidated some of the critical mechanisms leading to the activation of NF-kB factors.

Our initial interest in chemokines was based on our hypothesis that they could act both as chemoattractants for HIV-1 target cells and inducers of HIV-1 transcription and replication. During our early chemokine studies, the group of Paolo Lusso reported in 1995 that the CC chemokines CCL5/RANTES, CCL3/MIP-1α, and CCL4/Mip-1β, isolated from an immortalized $CD8^+$ T lymphocyte clone, blocked infection of a $CD4^+$ T cell line susceptible to primary HIV-1 isolates and some HIV-2 and SIV isolates (1). Based on the tight relationship between T cell activation and HIV replication, the blockade of HIV infection by these factors was in an apparent contradiction with the strong and recently reported potent antigen-independent activation in T lymphocytes by RANTES.

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The Converging Paths of HIV-1 Entry and Chemokine Research

The HIV inhibitory effect of the chemokines identified by Paolo Lusso's group was associated to the previously known, although poorly characterized, suppressive effect of $CD8^+$ T lymphocytes culture supernatants (1, 2). However, the hypothesis of a possible interference of this mechanism on HIV entry was not raised in the report. HIV entry in CD4 T lymphocytes was known to critically rely on the interaction of the HIV envelope glycoproteins (surface subunit gp120) with CD4, a viral receptor and a critical determinant of viral tropism, in that gp120 binding to CD4 eventually leads to viral/target cell membrane fusion and entry of viral replication machinery. Importantly, this early research clearly established that an essential cofactor for HIV entry was missing as CD4 alone did not support HIV infection. While many teams all over the world were trying unsuccessfully to identify such CD4 cofactor(s) enabling productive infection by HIV, scientist working in the field of chemokines were making tremendous progress identifying new chemokines and receptors and elucidating their biological roles.

Among them, Bernhard Moser at the Theodor-Kocher Institute in Bern, directed by Marco Baggiolini, had isolated the cDNA for the orphan receptor LESTR, which shared typical characteristics of G protein-coupled receptors (GPCRs) (2). Although the ligand for LESTR could not be identified among a large number of identified chemotactic cytokines, the high expression in white blood cells and the marked sequence relation to CXCR1/IL-8R1 and CXCR2/IL-8R2 suggested that LESTR may be a novel receptor for an unknown chemokine. In a collaboration with Conrad Bleul, a post-doctoral scientist in Tim Springer's laboratory at Harvard University, Bernhard obtained

preliminary evidence indicating that LESTR is the selective chemokine receptor for the so-called Pre-B-cell growth-stimulating factor/stromal cell-derived factor 1 (PBSF/SDF-1/CXCL12), a member of the C–X–C subfamily, which was originally cloned by two independent groups (3, 4). This highly conserved chemokine turned out to have an essential (non-redundant) role in B cell lymphopoiesis as well as the normal development of the heart, the vasculature, and the brain. The characterization of tissue expression of CXCL12/SDF-1 was first shown in mice and we provided the first tissue expression mapping in humans (5). In keeping with the high degree of homology, the mouse sample of active CXCL12/SDF-1 that Conrad Bleul brought to the Theodor-Kocher Institute behaved as an agonist on cells expressing human LESTR.

Another breakthrough in HIV research was announced in early 1996 at a Keystone Symposium by Edward Berger from the National Institute of Health in Bethesda who reported unpublished findings about a novel cell fusion coreceptor allowing the infection by T cell line-tropic (TCL-tropic or T-tropic) but not macrophage-tropic (M-tropic) HIV-1 isolates when co-expressed on $CD4^+$ target cells. In the subsequent paper by Ed Berger's group (6), this fusion coreceptor was called "fusin" and turned out to be identical to LESTR that Bernhard's group was working on. The ground-breaking discovery by Ed Berger's group revealed the definitive link between the interaction of HIV-1 and its target cells and the rapidly expanding field of chemokine receptors.

CXCL12/CXCR4 and HIV-1 Infection: The Meeting Point

In early 1996, scientists from the Theodor-Kocher Institute have already been engaged in collaborations with groups at the Pasteur Institute in Paris who were investigating host immune cell responses to parasites and the "fusin" discovery led to joint efforts of our laboratories in the new arena of HIV research. This was the beginning of a very fruitful and effective collaboration between the scientists at the Theodor-Kocher Institute in Bern and our department at the Pasteur Institute in Paris.

Thus, we immediately confirmed Bernhard's findings about LESTR being the specific receptor for CXCL12/SDF-1 using both CHO cell lines transduced to express LESTR and human leukocytes, including neutrophils, monocytes, and T lymphocytes. In all these cell types, CXCL12/SDF-1-induced robust chemotaxis and intracellular Ca^{2+} responses, which are typical responses seen with chemokines binding of cognate receptors. Simultaneously, our findings were confirmed by Conrad Bleul at Harvard University and, consequently, LESTR was renamed as CXCR4, the fourth CXC chemokine receptors to be identified. Also, both groups obtained simultaneously evidence that CXCL12/SDF-1 prevented selectively the infection by T-tropic HIV isolates (laboratory adapted or primary) but was unable to prevent infection of activated T lymphocytes by M-tropic viral isolates (7, 8). However, as we demonstrated, CC chemokines CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES were unable to block infection by T-tropic isolates thus proving the specific and selective inhibition by CXCL12/SDF-1 for this type of viruses (**Figure 1**). Moreover, Conrad Bleul's work demonstrated that only CXCL12/SDF-1 including an intact amino-terminal domain was active as suppressor factor for infection by T-tropic viruses, indicating that the amino-terminal domain in CXCL12/SDF-1 was required for both activation of CXCR4 and inhibition of T-tropic HIV-1 species. This experiment suggested that CXCL12/SDF-1 could inhibit HIV infection by steric hindrance of viral pg120 binding to CXCR4. We also showed that CXCL12/SDF-1 blocked T-tropic HIV-1 infection at an early step without affecting the rest of the HIV-1 life cycle by setting up two complementary experiments (7). First, we demonstrated that CXCL12/SDF-1 potently prevented the accumulation newly reverse-transcribed HIV proviral DNA from the genomic viral RNA, a mandatory process required for productive infection. Second, using HIV-1 particles whose envelope glycoprotein was replaced by the one from vesicular stomatitis virus, which enables infection in a CD4- and coreceptor-independent manner, we proved that CXCL12/SDF-1 failed to inhibit the viral replication (occurring after viral entry). Both sets of findings described above were published as back-to-back papers in August 1996.

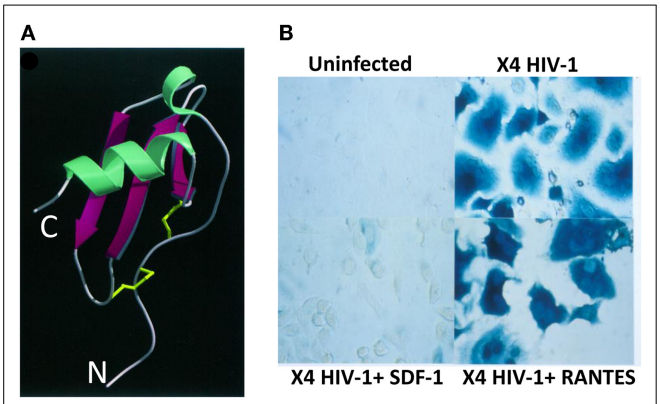

FIGURE 1 | *Structure and mode of action of CXCL12/SDF-1 in HIV-1 infection*. (A) Ribbon structure of human CXCL12/SDF-1α. The two disulfur bonds are indicated in yellow (9). N, amino-terminus; C, carboxy-terminus. **(B)** Human cells expressing CD4 and CXCR4 infected or not with an X4 HIV-1 isolate in the presence of SDF-1/CXCL12 or RANTES/CCL5. Infected cells are visualized by β-galactosidase staining.

Obviously, CXCL12/SDF-1 was an excellent new target for the development of novel inhibitors of infection by T-tropic HIV-1 species. Based on a collaboration that included Ian Clark-Lewis from the University of British Columbia, we carried out detailed structure–function studies and came up with a two-site model for binding of CXCL12/SDF-1 to CXCR4, which became a general a model for chemokine binding to their cognate receptors (9). Similar work with CCL5/RANTES led to the identification of CCR5 derivatives that failed to induce chemokine responses in leukocytes yet retained the ability to block HIV-1 entry of M-tropic but not T-tropic viruses (10). Simultaneously, we reported that the anti-HIV-1 blocking activity of CXCL12/CXCL12 critically involved the cell surface depletion of CXCR4, a phenomenon that was mediated by the intracellular carboxy-terminal region of CXCR4 in a G protein-independent fashion (11).

Following the Track: Chemokines and HIV-1 Some Years Later

The accumulation of native chemokines that bind CCR5 with high affinity (R5-CHKs) into the anatomical sites of HIV replication suggests that they could act as a natural barrier against HIV infection, both by displacing the viral envelope glycoprotein gp120 from binding to CCR5 and by promoting CCR5 endocytosis. However, the concentration of R5-CHKs required to inhibit R5 HIV-1 primary strains in primary CD4 cell targets exceed by several orders of magnitude the receptor K_d^s value and chemotaxis-induction, a G-protein-dependent mechanism. A possible explanation for this intriguing phenomenon is now provided by recent work from our laboratory (12). We showed that different CCR5 conformations at the cell surface are differentially engaged by R5-CHKs and gp120, making R5-CHKs weaker inhibitors of HIV infection than would be expected from their binding affinity constants for CCR5. These distinct CCR5 conformations rely on CCR5 coupling to G-proteins. While R5-CHKs bind with high affinity ($K_d < 1$ nM) to active conformations of CCR5 coupled to nucleotide-free G-proteins (NFG), gp120/HIV-1 does not discriminate between NFG-protein coupled an uncoupled CCR5. Interestingly, the antiviral activity of R5-CHKs is G-protein independent, suggesting that inactive CCR5, which are of low affinity for R5-CHKs, represent a portal for viral entry. This is reminiscent of infection by R5 HIV-1, which occurs also in a G-protein-independent fashion (13). Furthermore, R5-CHKs are weak inducers of CCR5 endocytosis, as is revealed by their potencies in the submicromolar range for inducing endocytosis reflecting their low-affinity constant value for NFG-protein-uncoupled receptors. Abolishing CCR5 interaction with NFG-proteins eliminates high-affinity binding of R5-CHKs but preserves receptor endocytosis, indicating that R5-CHKs preferentially endocytose low-affinity receptors. These data are consistent with HIV-1 evading R5-CHK inhibition by exploiting CCR5 conformations that are weakly recognized by native chemokines, named "spare receptors" that are unlikely to take part in R5-CHKs-mediated functional responses. Importantly, and in contrast to native chemokines, some RANTES/CCR5 antagonists and agonist analogs displaying improved anti-HIV-1 activity recognize this fraction of CCR5 receptors, thus proving the importance of blocking "spare receptors" for preventing HIV-1 infection (14).

By sharp contrast, the affinity of CXCL12/SDF-1 for CXCR4 correlates well with its HIV-1 inhibitory activity and its ability to induce CXCR4 internalization. This property could explain the selective CXCR4 down-modulation on intestinal lymphocytes in response to local CXCL12 constitutively produced by gut epithelia (15). Mucosal epithelia are a site of prominent HIV-1 replication and local CXCL12/SDF-1 could in part explain the observed predominance of M-tropic HIV-1 variants, which are not affected by CXCL12/SDF-1.

Conclusion

The seminal work reported by the laboratories of Paolo Lusso and Ed Berger initiated an unprecedented storm of collaborative activities across the fields of chemokine and HIV research. It is now firmly established that CCR5 and CXCR4 are the principal coreceptors for M-tropic and T-tropic HIV-1 variants (also referred to as R5 and X4 HIV variants), respectively. Maraviroc, a CCR5-specific antagonist, is currently used in the treatment of HIV infected individuals. Still, many questions remain. For instance, R5 HIV-1 viruses are transmitted and propagated preferentially during the early and asymptomatic stages of infection while viruses showing CXCR4 tropism (X4 HIV-1 and, mainly, dual tropic X4R5 HIV-1) emerge progressively and become detectable in roughly 40–50% of infected people at later stages of the infection or during the AIDS phase. This apparent paradox is still unresolved, as CXCR4 expression is constitutive and ubiquitous, including most nucleated cells and, most notably, $CD4^+$ T cells. By clear contrast, expression of CCR5 is restricted to activated effector T cells, which are a minor subset of T cells in peripheral blood, and dendritic cells indicating that target cells for R5 HIV-1 are much more limited. The causes underlying this phenomenon are likely multifactorial and a number of possible mechanisms had been proposed. The fact that X4 HIV-1 viruses rapidly emerge in a significant proportion of HIV-1-infected patients treated by the CCR5-specific antagonist maraviroc and spontaneously regress as the administration of this drug is interrupted, suggests that a certain degree of competition between R5 and X4 HIV-1 viruses exists.

The hectic research activities carried out during the first half of 1996 was due to intense collaborations set up by research teams working in, *a priori,* separated fields such as molecular virology, chemokine biology, or GPCR pharmacology. Within this setting, the real contribution of chemokine and chemokine receptor research to the new field was that it progressively implemented and transformed our basic knowledge of HIV cell tropism into a detailed view and understanding of the complex molecular mechanisms of HIV entry leading to novel therapeutic strategies for blocking HIV infection.

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CCR5 and HIV infection, a view from Brussels

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HIV co-receptors were quite far from our main interests at the end of 1995, and we got involved in this field in a totally unexpected way. Our interest in chemokines was even relatively new at that moment, as we were mostly dealing with the characterization of new G protein-coupled receptors (GPCRs) in various areas such as endocrinology, neuroscience, and olfaction. Candidate receptors for leukocyte chemoattractant factors were part of an expanding repertoire of "orphan" receptors under study. I will essentially describe here a relatively short period of years 1995–1996, which has been one of the most hectic in my scientific career. This period is viewed from our perspective in Brussels, as I do not know for sure what was going on in other laboratories around the world, besides the results of these activities in terms of publications, communications in meetings, or personal contacts. I apologize in advance for the unavoidable bias in this sort of "historical" review.

A few years earlier, in the end of 1980s, our Institute had a strong focus on thyroid research. The most dynamic part of this activity was the cloning of some of the main actors of thyroid hormone biosynthesis, the hormone precursor thyroglobulin and the iodinating enzyme thyroperoxidase. A primary objective at that time was the cloning of the main regulator of thyroid function, the thyrotropin receptor, which was known as coupled to the stimulation of adenylate cyclase through the G_s protein. It is the search for the thyrotropin receptor that ultimately led, amongst many other unexpected findings, to our contribution to the characterization of CCR5 and its role in HIV infection.

G protein-coupled receptors constitute the largest family of membrane receptors and collectively play a major role in all physiological and pathophysiological processes. GPCRs share a common structural organization with seven transmembrane segments, and a common way of modulating cell functions by regulating effector systems through heterotrimeric G proteins and arrestins. The first GPCR sequences (rhodopsin, β-adrenergic, and M1 muscarinic receptors) were obtained in 1986–1988, following protein purification and peptide sequencing approaches. As a result, the common transmembrane organization and structural relatedness of GPCRs became obvious. Gilbert Vassart, leading the molecular biology group of the Institute, suggested applying the newly developed PCR method to the search of new members of the GPCR family, by using degenerate primers corresponding to the most conserved motifs among the small number of available GPCR sequences. A Ph.D. student in the Institute, Frédérick Libert, set up the procedure very successfully, and cloned within a few weeks, four new members of the GPCR family, that were referred to as "orphan" receptors (1). These were later characterized as CXCR7, serotonin 5HT1Dα, and adenosine A1 and A2a receptors. In the aftermath, a bunch of other orphan receptors were cloned, and we characterized the target of this new cloning strategy, the thyrotropin receptor (2). This PCR cloning approach, used first in Brussels, was applied broadly by other labs afterwards, and contributed significantly to the vigorous reporting of new GPCRs in the early 1990s.

In our hands, the first CCR5 sequences originated from a screen performed by Catherine Mollereau in early 1993 with the aim of identifying subtypes of opioid receptors. This screen led among others to the cloning of ORL1, a fourth member of the opiate receptor family, and the identification of its peptidic ligand nociceptin (3). A number of partial sequences were also similar to the first chemokine receptors, CXCR1, CXCR2, and CCR1, reported by the groups of Phil Murphy and Tom Schall (4, 5). We thus decided to engage into the functional characterization of these candidate chemokine receptors. The cDNA encoding CCR5 was expressed in CHO-K1 cells and tested in a microphysiometer, an ancestor of the "label free" instruments, which measured changes in cell metabolism by monitoring the acidification rate of the culture medium. MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5 were identified by a French post-doc, Michel Samson, as three chemokines able to activate the receptor. The manuscript was first submitted to JBC in early September 1995, but was rejected after a 3-month reviewing process. It was resubmitted to Biochemistry in December (6).

In the meantime, a paper was published in December 1995 by the group of Paolo Lusso and Robert Gallo (7), describing that three chemokines, MIP-1α, MIP-1β, and RANTES, were able to inhibit infection of cells by macrophage-tropic HIV-1 strains. The link between the pharmacology of CCR5 and the profile of HIV inhibitory factors was of course striking. With no tools at hand for studying HIV, we first mailed Robert Gallo in January 1996 to propose some kind of collaboration to study the role of CCR5 in HIV infection. We never got an answer to this letter. It was quite clear at that time that we were not the only group to have CCR5 on hands. There were a bunch of very active groups in the chemokine receptor field, such as those of Philip Murphy, Craig Gerard, and Tom Schall. CCR3 and CCR4 had been published in late 1995 and Phil Murphy had

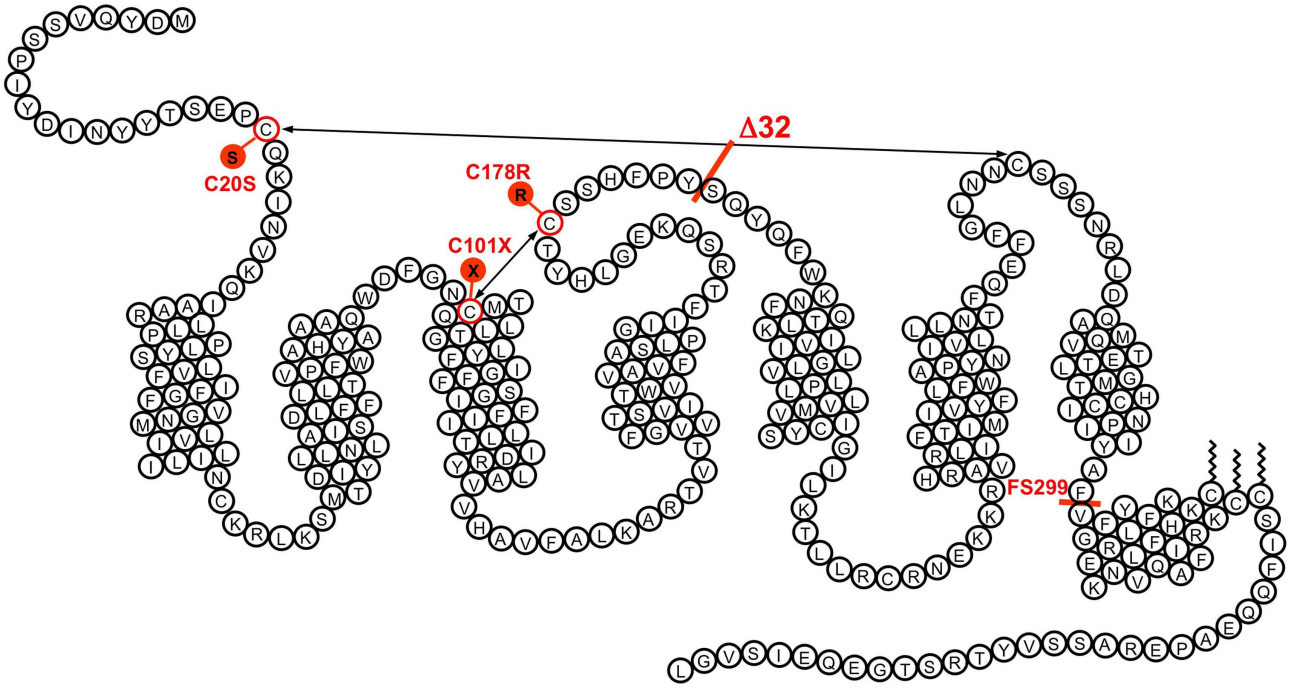

FIGURE 1 | The transmembrane organization of CCR5 is represented with its seven transmembrane segments, the eighth α-helix parallel to the plasma membrane, the two disulfide bonds, and the palmitoylated cysteines. The position of some of the variants demonstrated to affect CCR5 function is indicated. The Δ32 mutant, with an average allele frequency of about 10% in European populations is the most frequent. Two missense mutations (C20S and C178R) affect one of the disulfide bonds necessary for the correct folding of the receptor. Two other mutations (C101X and the frame shift mutant FS299) result in early termination of translation. None of these mutant receptors is properly expressed at the surface of cells. Besides Δ32, the most frequent mutation is FS299, with an allele frequency of 2.9% in Chinese subpopulations. The allele frequencies of other mutations are well below 1%.

reported the CCR3 sequence with MIP-1α, MIP-1β, and RANTES as agonists. This was later retracted as a result of a clone handling mistake, but it was quite clear that CCR5 and its pharmacology were in other hands as well.

While considering other potential collaborators, our manuscript dealing with CCR5 pharmacology became available, and very rapidly afterwards, I got a mail from Bob Doms in Philadelphia, proposing to join efforts on this topic. We sent to Bob plasmids encoding CCR5 and a set of related receptors we had at that time. Bob was obviously not alone in this game. In the HIV community, the existence of an HIV co-receptor, the orphan GPCR LESTR (and future CXCR4), for T-tropic HIV strains was already well known. The data would appear 1 month later in an April issue of Science (8). Many HIV groups were therefore looking for other GPCRs that would mediate the entry of HIV in macrophages and got in touch with teams involved in the chemokine receptor field. The race was fierce, and five papers reporting CCR5 as HIV co-receptor were published within a week in Nature, Cell, and Science in June 1996 (9–13). As a measure of the rush that took place in editorial offices and printing houses, our common paper with Bob Doms submitted on June 10 was published by Cell on June 28 with several pages printed upside down.

CCR5 seemed to play a key role in the entry of HIV strains involved in disease transmission. Soon after the first feedback by Bob Doms of the experiments performed in Philadelphia, Gilbert Vassart suggested to check whether variants of CCR5 could be responsible for the variable susceptibility to HIV infection. We first obtained from a clinician of the nearby hospital, Claire Farber, DNA samples from three patients with slow disease progression and a few uninfected controls. Unexpectedly, Frédérick Libert and Michel Samson identified in this small series one slow progressor but also two control individuals as heterozygous for the same mutation of CCR5, a 32-base pair deletion in a region corresponding to the second extracellular loop of the receptor, and resulting in a frame shift and early termination (**Figure 1**). This mutant form of the CCR5 gene did not explain the slow progression of the patients tested. It was clear however that the resulting CCR5 mutant could not act as a functional receptor, and that the mutant allele was quite frequent. Within days, we sent a plasmid encoding this CCR5 mutant to Bob Doms for testing its function as HIV co-receptor, initiated experiments to demonstrate its deficiency as a chemokine receptor, and started collecting samples to study the frequency of the mutation at a larger scale. There were well-established cohorts of uninfected but multiply exposed individuals, but a few phone calls suggested to us that obtaining the genomic DNA from these cohorts would take ages compared to the pace at which this field was developing. We opted therefore for a more accessible approach. Starting from

our local contacts in the campus hospital, were gathered within a week from various hospitals in Belgium and France, collections of DNA samples from cohorts of HIV-infected patients and uninfected controls, reasoning that the frequency of the mutant CCR5 allele should be different between these two groups if this allele was protective against HIV infection. We also collected DNA samples from about a hundred volunteers in the Institute's staff. Testing these samples as they arrived builded progressively what is now known as the allele frequency of the Δ32 allele, around 10% in Western Europe. More importantly, while the number of homozygotes was in the expected range for Mendelian distribution in the uninfected group, there was a lack of homozygotes in the HIV-infected group. When each group reached over 700 individuals, the *p* value was below 0.0005. In the meantime, we had also found three Δ32 homozygotes within the institute personnel. We could rush blood cells to our Philadelphia collaborators to check whether these cells were indeed resistant to macrophage-tropic, but not T-tropic HIV-1 strains. This was indeed the case.

The manuscript was submitted to Nature in mid-July 1996. Although there was a strong interest of the Editor, one of the referees opposed us the fact that our cohorts were not constructed according to the rules. While we quite agreed on this, we had to fight to convince the editor that the data were clear enough to overcome weaknesses in cohort structure, and that there was no time to be spent on theoretical considerations. The final argument came when we could state that a concurrent manuscript had been submitted to Cell by the Ned Landau group and that it was being reviewed positively. As a result, we were requested to respond to the latest referee comments by correcting the text at the proof stage, and the two papers appeared in August 1996 (14, 15).

It was shown later on by various groups that protection by the Δ32 allele was not complete, and a few infected Δ32 homozygotes have been reported within the following years. In the following months and years, we have studied the structure–function relationships of CCR5 in relation to its role of chemokine receptor and HIV co-receptor, analyzed the distribution of the Δ32 mutation in various populations of the world, and tested the functional consequences of other, less frequent, variants and mutants of CCR5 (**Figure 1**). But somehow, the excitement was over, and subsequent research became more "routine." The characterization of the CCR5 Δ32 mutation and its consequences on infection rate by HIV had validated CCR5 as an obvious target for the development of drugs targeting CCR5 and the entry of macrophage-tropic HIV strains. Many pharmaceutical companies, including Takeda, Pfizer, GSK, and Schering Plough, started immediately screening programs that resulted a few years later into CCR5 antagonists. While Takeda's TAK779, GSK's aplaviroc, and Schering Plough's vicriviroc failed in clinical trials for toxicity reasons, Pfizer's maraviroc went successfully through clinical testing and was approved in 2007 as the first-in-class CCR5 antagonist and HIV entry inhibitor. Overall, this has been a very short path (11 years altogether) between the discovery of a target and the launch of a small molecule in the clinics. With the present availability of fast and efficient mutagenesis techniques such as the CRISPR/Cas9 system, gene therapy approaches for inactivating CCR5 in the hematopoietic system are also being considered actively for the treatment of HIV infection.

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Viral chemokine receptors

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When my lab sequenced the first chemokine receptors CXCR2 and CCR1 in 1991, the top BLAST hit for CCR1 was open reading frame (ORF) *US28* of human cytomegalovirus (HCMV), indicating an obvious common ancestor and a possible example of gene piracy. Pox virologists had already identified virally encoded TNF and IFN-γ binding proteins, copied from the host and redeployed as cytokine scavengers and immune evasion factors; however, there were no precedents for G protein-coupled receptors in viruses or signaling viral immunoreceptor homologs. Tom Schall, then at Genentech, who had candidate CC chemokine receptor clones, learned about our CCR1 discovery and proposed a collaboration. I told him about the key features of CCR1: its specificity for CCL3 and CCL5, as well as monocytes and lymphocytes, and its sequence homology to US28 (1); however, he eventually wanted to pursue CCR1 independently, and published it in *Cell* in fall, 1992 along with data that US28 could bind to CCL2, CCL3, CCL4, and CCL5. A month earlier, our paper had been rejected by *Cell* for lack of sufficient binding data; however, we eventually published our paper in early 1993 in the *Journal of Experimental Medicine* (1), and the NIH received the patent for cloning CCR1.

Meanwhile, the complete sequence of the non-human primate herpesvirus *Herpesvirus saimiri* appeared in 1992, revealing a CXCR2 homolog known as ECRF3. Sunil Ahuja, then a post-doc in my lab and now a Professor at the University of Texas at San Antonio, used our *Xenopus* oocyte expression system to show that ECRF3, like CXCR2, mobilized calcium in response to the ELR$^+$ CXC chemokines CXCL1, CXCL7, and CXCL8, the first example of a virally encoded chemokine receptor that signaled (2). My long-time colleague Ji-Liang Gao, who was a post-doc in my lab at the time, then showed that US28 was also a calcium flux signaling receptor for the same chemokines that Schall's group had found bound to US28 (3). Together these papers pioneered a new field of virally encoded chemokine receptors that has expanded as more herpesvirus and poxvirus genomes have been sequenced. In addition, many virally encoded chemokines and secreted chemokine binding proteins were later identified, along with information about structure, signaling pathways, biological functions, and potential disease connections.

Together, this work demonstrated unequivocally that the chemokine system has been selectively and preferentially expropriated by these types of viruses; however, exactly why remains unresolved. At an evolutionary level, it is remarkable that the viral chemokine receptors could be so distantly related to mammalian receptors with which they share ligands, particularly since this was not the case for the human chemokine receptors known at the time, CXCR1, CXCR2, CCR1, and CCR2.

We also began cloning mouse counterparts of the human receptors we were finding and noticed that the mouse–human orthologs were more distantly related than expected. I decided to investigate this systematically by doing an *in silico* study of the mouse–human orthologs then in the data base, and found that for the ~500 available sequence pairs, the distribution of divergence was highly heterogenous. Most orthologs had high homology, but the ones that did not were mostly immunoregulatory factors. I published a paper in *Cell* describing this exceptionalism and proposed that it might relate to evolutionary pressure imposed by the predilection of viruses for this type of host gene (4).

We continued to use homology cross-hybridization to clone additional chemokine receptors, including, in 1994, one we first named CC CKR5 that was later renamed CCR5. Christophe Combadiere, a post-doc in my lab now with his own lab in Paris, actually cloned CCR3 and CCR5 cDNAs from the same screen. He determined their sequences and leukocyte specificities in parallel, then investigated their chemokine specificities sequentially, starting with the eosinophil-selective CCR3. We reported that CCL3, CCL4, and CCL5 were agonists for CCR3 in May, 1995, accepted by *JBC* 1 day after submission with no revisions (5). However, colleagues in the field, and ultimately we were skeptical since CCL3 and CCL4 lacked eosinophil activity. As a check, Christophe sequenced transfected cDNA from the original "CCR3" cell lines and, to our chagrin, found the CCR5 sequence, not CCR3. Apparently, the plasmid tube labeled "CCR5" had been mistaken for "CCR3". We wrote a correction published in December, 1995 in *JBC* indicating that CCL3, CCL4, and CCL5 were agonists for a new receptor named CC CKR5. We then submitted a new paper with the CC CKR5 sequence, its RNA distribution, and ligands to *JBC*, which after an ~6 month review was rejected in part because the reviewers regarded it as partly duplicative of the CCR3 paper.

The same month that our correction appeared in *JBC*, Paolo Lusso and colleagues in Bob Gallo's lab at the NCI of NIH reported in *Science* that CCL3, CCL4, and CCL5, the signature ligands for CCR5, were able to suppress replication of macrophage

(M)-tropic but not T cell line (T)-tropic strains of HIV (6). Taken together, the most obvious and parsimonious hypothesis was that CCR5 was used for M-tropic HIV infection, but how was unclear to me. On January 31, 1996, I gave a seminar at NIH about our new chemokine receptors, concluding with Paolo's new finding about HIV-suppressing chemokines and how CCR5 was an ideal candidate to mediate their action. My colleague Ed Berger from NIAID and his staff were at the talk, and Ed emailed me a few days later about his recent unpublished work identifying fusin (later identified as a chemokine receptor for CXCL12 and renamed CXCR4) as the first HIV coreceptor, acting with CD4 at the level of cell entry, and its specificity for T-tropic strains of HIV (7). He said he was still looking for a specific HIV coreceptor for the disease-transmitting M-tropic HIV and agreed that CCR5 was the logical candidate. This is how I realized that CCR5 might work at the level of cell entry, from Ed, and of course his lab had the assay to test the idea. From me, he learned about the CCR5 sequence and its leukocyte and chemokine specificities that matched Paolo's chemokine suppressor signature. We provided the plasmid to Ghalib Alkhatib, a post-doc in Ed's lab, who validated the hypothesis on the first attempt and highlighted the result by writing "BINGO!" in his lab notebook.

Meanwhile, Marc Parmentier from Brussels had beaten us to press with his own independent cloning and functional characterization of CCR5, in *Biochemistry* on March 19, 1996 (8). This was the key piece needed to allow four other labs to join the CCR5-HIV connection frenzy that year, that started with Paulo's *Science* paper about three CC chemokine suppressors of M-tropic HIV, our *JBC* correction reassigning these same chemokines from CCR3 to the unpublished CCR5 sequence, followed in February, 1996 by Ed's prepublication announcement of fusin's T-tropic HIV coreceptor activity at a Keystone meeting. To the astute observer, the only missing piece to the puzzle was the CCR5 sequence, provided first by Parmentier. Within 2 weeks of each other in late June, 1996, all five groups in the hunt published papers in *Science*, *Nature*, and *Cell* that used complementary approaches to draw the same basic conclusion that CCR5 was an M-tropic HIV coreceptor (9, 10). Two weeks after that, we published the sequence of CCR5 with its leukocyte and chemokine specificities, at last, in the *Journal of Leukocyte Biology* (11). The foundational discovery, the first HIV coreceptor fusin/CXCR4, was published by the Berger lab 1 month earlier in *Science* (7). The pace of discovery and publication had become breathlessly exciting, and the pages of scientific journals as well as the lay press were ablaze with stories of the HIV-chemokine receptor connection, for the new insights as well as for the potential for new drugs targeting a host factor in HIV/AIDS.

Ironically, Ed and I had first met several years earlier when he came to my lab to ask about using my oocyte system to expression clone a putative HIV coreceptor from his cDNA library. We never actually did any experiments then, and instead ended up with the converse collaboration: using my lab's CCR5 cDNA clone in Ed's system to identify the M-tropic HIV coreceptor. Ultimately, my lab's contribution was to accelerate the discovery of CCR5 as the M-tropic HIV coreceptor, since Ed's expression cloning system used to find fusin/CXCR4 would probably have succeeded in also discovering CCR5.

But what role did these coreceptors actually play in pathogenesis? My lab took a lead in answering this next key question, through the discovery of *CCR5Δ32*, the deletion mutant of *CCR5*, which provided strong evidence that CCR5 was critical for HIV transmission at the population level. I thought that if a common inactivating CCR5 mutation existed, homozygotes should be rare among HIV-infected individuals, but overrepresented among highly exposed but persistently uninfected individuals. I proposed the idea of looking for such a mutation poolside at our kids' swim meet to my neighbor, good friend, and colleague Pete Zimmerman, a human geneticist working as a post-doc at the time with Tom Nutman in the Laboratory of Parasitic Diseases of NIAID and now a Professor at Case Western Reserve University School of Medicine. Pete agreed to collaborate, and using a heteroduplex DNA mobility shift assay for polymorphism detection he found among 100 blood donors from the NIH Clinical Center Blood Bank, 21 individuals with a massive shift: 20 heterozygotes and one homozygote for what was eventually named *CCR5Δ32*, which we later nicknamed "the mother of all mutations in the molecule of the year." All of our criteria had been met: it was common (but restricted mainly to Caucasians), and the 32 base pair deletion caused a massive truncation incompatible with expression and function. Next we received approval from NIAID's Division of AIDS to analyze several thousand DNA samples from participants in the Multicenter AIDS Cohort Study (MACS), and we collaborated with HIV/AIDS expert Tony Fauci, the Director of NIAID, whose laboratory was right around the corner from mine, to obtain DNA from two cohorts of long-term non-progressors and one group of HIV exposed-uninfected (EU) individuals. As predicted, compared to the frequency in the general population, *CCR5Δ32* homozygosity was markedly increased by about fivefold in the EU population. However, our analysis of the critical MACS samples was delayed by 2 months during which Steven O'Brien from the NCI of NIH, who had custody of the MACS samples and had been directed to send them to us as well as to Rick Koup at NYU for analysis, conducted his own study of *CCR5Δ32* in HIV. We finally received the samples a few weeks before his paper was published in *Science*, and completed our study validating the second and third parts of our hypothesis that homozygotes should be underrepresented from the HIV-infected population and that heterozygotes would have a delayed time from infection to the diagnosis of AIDS. Importantly, homozygotes in the general population appeared to be healthy. Together, our paper published in *Molecular Medicine,* O'Brien's *Science* paper, and papers reporting independent discoveries of *CCR5Δ32* by the groups of Marc Parmentier in Brussels in *Nature*, and Rick Koup at the Aaron Diamond AIDS Research Center in *Cell* provided strong proof of principle for targeting CCR5 in the treatment of patients with HIV/AIDS (10, 12). Thirteen years later, this discovery culminated in FDA approval of the small molecule CCR5 antagonist Selzentry (maraviroc, from Pfizer) for the treatment of CCR5-tropic HIV. In addition, the "Berlin patient," an HIV^+ individual who developed leukemia and was functionally cured of HIV by a transplant with bone marrow from a *CCR5Δ32* homozygote given after

leukemia chemotherapy, provided proof-of-principle that targeting CCR5 might be a cure strategy in HIV/AIDS. The hope for the future is that cure strategies will be available for every HIV$^+$ individual through deliberate genome editing of CCR5.

Overall, we were pleased that our NIAID collaborative group contributed the four main arms for the underlying proof-of-principle discoveries about CCR5 and HIV: the independent cloning of CCR5, demonstration that CCR5 was an M-tropic HIV coreceptor, discovery of *CCR5Δ32*, and demonstration that *CCR5Δ32* is an HIV genetic restriction factor at the population level. The foundational discoveries on which the CCR5 work rested also came from NIH: Paolo's discovery at the NCI of HIV suppressive activity for CC chemokines (6) and Ed's discovery at NIAID of fusin/CXCR4 as the first HIV coreceptor (7). After this work, my lab went on to discover the first beneficial role for CCR5 as a host defense factor in West Nile virus infection (13, 14).

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The structure of a CXCR4:chemokine complex

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CXCR4 was the first chemokine receptor to be identified as an HIV coreceptor in 1996 (1). Along with the importance of CXCR4 in development, it was also discovered as a key chemokine receptor in the metastasis of breast (2) and numerous other cancers (3). These were the main reasons that motivated us to pursue structural studies of CXCR4 with synthetic inhibitors and chemokines. However, my laboratory took a rather circuitous route to this goal, and I did a lot of reinventing myself as a scientist along the way.

As background, I got my Ph.D. in chemistry/membrane biophysics at the California Institute of Technology, and then in 1989 accepted a postdoctoral position to do "protein design" with Bill DeGrado at E. I. Du Pont de Nemours (we called it Du Pont University back then, given the amazing freedom we had to do truly basic research). Du Pont (primarily a chemical company) formed a Joint Venture and became Du Pont Merck Pharmaceuticals; and when I was transitioning to a full time employee in 1992, we were tasked with coming up with new therapeutic targets for the expanded pharmaceutical side of the business. I was hired as part of the macromolecular NMR group headed by Peter Domaille, and thus my target choices were biased by some of the exciting work emerging from the structural biology community. This included the first structure of a chemokine, interleukin-8 (IL-8, now CXCL8), which was published in 1990 by Angela Gronenborn's NMR group at NIH (4). I remember being intrigued by the dimeric structure and thinking (as they described in their paper) that the dimeric architecture of two alpha helices on top of a beta sheet platform might provide a perfect binding site for the IL-8 receptor, as it was reminiscent of the human class I histocompatibility antigen HLA-A2 binding pocket for antigenic peptides. In 1989, two separate groups had cloned the gene for the related CC chemokine, monocyte chemotactic protein-1 (MCP-1, also called MCAF, now CCL2) (5, 6), and although the MCP-1 receptor (CCR2) had not yet been cloned, it looked like this system might be a good target for inflammation. It was consequently adopted as a focus of the Du Pont Merck inflammatory disease group, with the goal of inhibiting the receptor. Inspired by the IL-8 structure and the expectation that MCP-1 would also be a tractable target for NMR, Peter Domaille and I began working on its structure around 1992. We were hoping to obtain the structure of the first CC chemokine, but not surprisingly, the powerhouse NIH group beat us by a long shot and solved MIP-1β in 1994 (7), as did Nick Skelton and Tom Schall at Genentech, who solved the structure of RANTES in 1995 (8). Nevertheless, we persisted, and although I left Du Pont Merck for a faculty position at the University of California Berkeley in 1994, we published the structure of MCP-1 in 1996 (9).

At Berkeley, I continued working on MCP-1 in collaboration with a group at Roche led by Kurt Jarnagin. A major question that arose from the prevalence of dimeric chemokine structures that had been solved was whether they bound receptors as dimers (the prevailing hypothesis) or as monomers. By identifying a mutant that was incapable of dimerizing but was as potent as WT MCP-1 in migration and receptor binding assays, we demonstrated that it bound CCR2 as a monomer (10). This conclusion was consistent with a prior study by Ian Clark-Lewis who had shown that IL-8 was also a functional monomer (11). We also did a fairly comprehensive mutagenesis study of the residues involved in binding and signaling and came up with a model, which was published in 1999 (**Figure 1A**) (12). Although we never properly docked MCP-1 to the rhodopsin-based model of the receptor, we were qualitatively on the right track of what the structure might look like. However, it was just a model based on mutagenesis data, and I really wanted to determine high-resolution structures of intact receptors with chemokines and/or small molecule antagonists.

Because membrane receptors are so challenging, there was *no way* I was going to even consider working on intact chemokine receptor structures until/unless I got tenure at Berkeley, and fortunately that occurred in 2000. In 2002, I managed to hire a talented postdoc, Samantha Allen, from University of Bristol. She had a background in protein folding studies of bacteriorhodopsin, was interested in moving onto studies of eukaryotic membrane receptors and had the bravery (or perhaps naivety) to join me in the pursuit of chemokine receptor structures. Not having a track record in the expression, biochemistry or structural biology of membrane receptors, it was very difficult to get funding. Fortunately, Richard Horuk managed to convince his company, Berlex, to provide matching funds for a UC Discovery grant to pursue CCR1. It was not a lot of money, but that money along with fellowships that Samantha managed to garner, enabled us

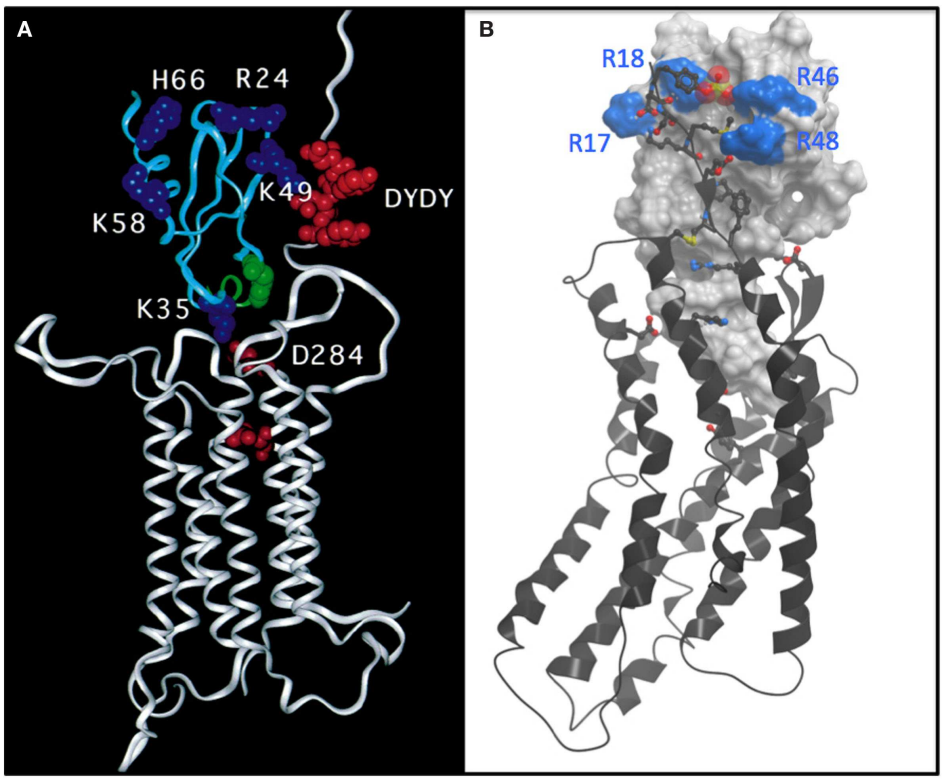

FIGURE 1 | (A) Undocked model of the complex between CCR2 (white ribbon) and MCP-1 (cyan ribbon) based on mutagenesis and reproduced directly from Hemmerich et al. (12). The model features a number of basic residues (blue CPK) on MCP-1 that are important for binding. Acidic residues, particularly a DYDY tyrosine sulfation motif (red CPK) are highlighted on the N-terminus of CCR2. The model suggests how the DYDY motif might bind to a pocket on MCP-1 that is flanked by the basic residue cluster, similar to that shown **(B)**. **(B)** Structure of a CXCR4:vMIP-II complex where the N-terminus of CXCR4 is extended by two residues to include the sulfated tyrosine sTyr21 (13). vMIP-II is shown as a white mesh surface with basic residues colored blue. CXCR4 is shown as a black ribbon with acidic and basic side chains that make important interactions with vMIP-II shown as sticks with oxygens colored red and nitrogens colored blue. The sulfate group on Tyr21 is shown as a cluster of red and yellow spheres.

to hobble along. Samantha was ultimately able to express decent levels of CCR1 and to demonstrate reasonably high-affinity chemokine binding to purified receptor, and eventually we received a small NIH grant. However, CCR1 turned out to be a poor choice of receptor to pursue for structural studies. Chemokine receptors, like other GPCRs, are challenging, not only because they are membrane proteins but also because they are unstable and tend to fluctuate between multiple active and inactive conformations. As a consequence, they tend to aggregate when extracted from cell membranes unless heavily engineered and stabilized by ligands. CCR1 was on the wrong end of the challenge spectrum because it had an exceptionally high level of constitutive activity, which we discovered later, clued in by its poor biophysical behavior.

In 2005, I moved to University of California San Diego, to be with Peter Domaille, whom I married in 2004 (MCP-1 was definitely a chemoattractant!). Around 2008, I reconnected with Ray Stevens who had been at Berkeley when I started, but had moved to The Scripps Research Institute (TSRI). At TSRI, he had managed to build a rather large NIH-funded center, which later became the GPCR Network, with the goal of determining the structures of as many GPCRs as possible. We began working together and contributed to the first structure of CXCR4 with a cyclic peptide and small molecule antagonist, work that was spearheaded by his postdoc Beili Wu (14). This collaboration led to more substantial funding for my lab and our computational collaborators in the Abagyan group through an NIH funding mechanism called PSI:Biology. We were specifically paired with the GPCR Network as a "biological partner" to focus on determining structures of chemokine receptor complexes. People in my laboratory received training from the collective expertise of the GPCR Network team. We were then able to establish key elements of infrastructure (equipment, insect cell expression, biophysical assays) in our laboratory so that we could operate fairly independently, and we set our sights on determining the structure of CXCR4 with chemokine.

Compared to small molecule complexes with chemokine receptors or other GPCRs, which are challenging, complexes of CXCR4 with chemokine turned out to be even more difficult. The problem was that the detergent solubilized complexes were not sufficiently stable to survive crystallization conditions. We came to this

conclusion after spending ~2 years using a strategy in which we made on the order of 100 mg of chemokine every 2 weeks to extract CXCR4 from membranes and to keep it stable during the purification process. This may make some people cry if they do the math, but recall that Peprotech was selling 50 μg of chemokine for ~$650 USD, and we were basically pouring it down the drain. Undeterred, but realizing that just adding chemokine to receptor was not the answer, we tried making fusions of chemokine to receptor; this strategy gave us sufficiently positive results to make us waste yet another year before giving up. Finally, I thought about the disulfide trap approach that Brian Kobilka had used to make a covalent complex of the β2-adrenergic receptor with a small molecule agonist (15); this seemed like an ideal approach for a receptor with a protein ligand because of the possibility of coexpressing single cysteine mutants of the receptor with cysteine mutants of the ligand. Moreover, because of my background in NMR, I thought it might provide a way of getting structural information in the form of disulfide-based distance restraints, even in the absence of a crystal structure. However, after all of these failures, imagine trying to convince your lab that the disulfide trap approach is a good idea, particularly when you do not know where to start! Fortunately, the lead post doc, Ling Qin accepted the challenge, although I am sure with considerable reluctance at first. Irina Kufareva, a computational chemist in the Abagyan lab was also on board and helped us identify an optimal disulfide pair through an iterative process of predicting potential disulfide pairs, experimentally testing coexpressed cysteine mutants of CXCR4 and chemokine for the presence and abundance of disulfide trapped complex, and evaluating the quality of the covalent complexes by various biophysical metrics. We pursued complexes of CXCR4 with both antagonist variants of the endogenous ligand CXCL12 (SDF-1) and the viral antagonist vMIP-II; antagonist ligands were chosen because we knew that WT CXCL12, an agonist, required G protein for high affinity, which would have added yet another enormous degree of complexity. Fortunately, in the first round of experiments with 11 different pairs, we identified one disulfide trap "hit" – just enough to be encouraging. Irina Kufareva was then able to use that hit as an experimental restraint in computational docking experiments to predict additional potential disulfide pairs, and eventually we identified a well-behaved complex of CXCR4 with vMIP-II, which crystallized (13) (**Figure 1B**). This structure explained a lot of biochemical data, and gave us insight into several other complexes including CXCR4 with CXCL12; it also provided insight into the specificity of CC versus CXC chemokines for their respective receptors, and further illustrated the structural plasticity of chemokine receptors, which enables them to recognize very different types of ligands. However, many more structures including agonist complexes will be required to fully understand how chemokines activate (or inhibit) their receptors, how even single amino acid changes can lead to changes in pharmacology (agonist versus antagonist responses), and the full basis of receptor:ligand specificity. Moreover, ternary complexes with intracellular signaling partners will be needed to understand the structural basis of the signaling and trafficking fate of receptors after chemokines bind, and how one can exploit this knowledge to develop drugs with finely tuned pharmacological properties.

The total elapsed time from the identification of the first disulfide trap to publication of the structure in January 2015 was ~2.5 years, but that was only after several years of failed strategies. Moreover, about 12 years elapsed between when we embarked on trying to express chemokine receptors for structural studies, and when we published the structure. During this time, I often wondered if I was out of my mind to go down this road; it certainly was not favorable for my publication record. I also wondered whether I should have taken over my grandmother's ice cream business, "Handel's," instead of pursuing science. Hopefully, going forward, additional structures will yield to crystallization a little faster and with a little less sweat. Hopefully, the funding would not dry up before we complete at least a structure of MCP-1 with CCR2. And hopefully, these and other structures will aid in the development of drugs that target the chemokine receptor axis. Then it will all have been worth it.

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Maraviroc – a CCR5 antagonist for the treatment of HIV-1 infection

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Despite the dramatic decline in human immunodeficiency virus type 1 (HIV-1)-related morbidity and mortality following the discovery of the protease inhibitors and the advent of combination highly active antiretroviral (ARV) therapy in the mid-1990s, many patients were still failing therapy due to resistance and/or intolerability (1). It was clear that more ARVs acting on different steps in the virus lifecycle, active against resistant viruses, and better tolerated were needed. The demonstration of the key role of the chemokine receptors CCR5 and CXCR4 in HIV-1 entry sparked interest in this process as a new ARV target (2, 3). CCR5 is the co-receptor for the majority of HIV-1 strains, and these viruses are termed CCR5 tropic (R5). Virus strains that use CXCR4 are called CXCR4-tropic (X4), while strains that can use both receptors are dual-tropic (4). Virus from a patient can often contain mixtures of R5, X4, and dual-tropic strains, collectively called CXCR4-using.

The key role of CCR5 in HIV-1 entry, coupled with the demonstration that individuals who were homozygous for a 32 base pair deletion in the CCR5 gene (CCR5Δ32), and subsequently do not express functional CCR5, were highly protected from infection with R5 HIV-1, focused attention on CCR5 as an attractive target (5). Although some studies have demonstrated subtle effects of the CCR5Δ32 mutation on immune function, such as decreased inflammatory scores in hepatitis C-infected individuals and recovery from hepatitis B in heterozygotes; while homozygotes are more susceptible to tick-borne encephalitis and severe West Nile virus disease, these individuals suffer little apparent adverse effects on their health (5, 6). This, together with the fact that members of the G protein-coupled receptor superfamily are often tractable to development of potent, selective, and orally bioavailable drugs (7), led to the initiation of CCR5 ligand discovery programs by multiple groups, including a team from Pfizer Global Research and Development based at the Sandwich laboratories in the United Kingdom.

Maraviroc (UK-427,857, MVC) was discovered through high-throughput screening of the Pfizer compound library using a chemokine radioligand-binding assay. The most promising compound from the screening process was optimized for potency against the receptor, antiviral activity, pharmacokinetic characteristics, and selectivity against human cellular targets through a large medicinal chemistry effort in which almost 1000 molecules were characterized (7). MVC binds in the transmembrane pocket of CCR5 and is a slow-offset functional antagonist that prevents internalization (7, 8). It has potent antiviral activity against a wide-range of HIV-1 isolates (7). Together with its excellent preclinical safety profile and acceptable pharmacokinetics, this resulted in it being nominated as a clinical candidate in December 2000 (7).

It was always clear that the clinical development of CCR5 antagonists would be challenging, as these would be the first host-targeted ARV drugs and we were therefore venturing into uncharted territory. In order to pre-empt key issues, a clinical development team was established very soon after the start of the discovery program and I was recruited to lead the early development team, joining Pfizer in February 1999. We identified several key challenges to address in the design of the clinical program, in addition to demonstrating safety and efficacy. The first of these was that no commercially available, clinically validated assay to identify patients infected with R5 HIV-1 existed. This was critical, as MVC is active only against R5 HIV-1 strains (7). Secondly, in spite of the apparently healthy phenotype of individuals with CCR5Δ32 (5, 6), concerns remained regarding the safety of long-term exposure to CCR5 antagonists, as blocking of CCR5 may be different from congenital absence of the CCR5 receptor, where the immune system has matured in the absence of CCR5 and compensatory mechanisms may have developed. Finally, in HIV-1 infected individuals, the incidence of CXCR4-using HIV-1 strains increases with disease progression and decrease in CD4 cell counts (9), although no causal link between CXCR4-using virus and CD4-cell depletion has been demonstrated. This has led to concerns that selective pressure from a CCR5 antagonist may drive the virus population to use CXCR4 and result in CD4 cell decline.

Phase 1 single and multiple dose studies in healthy volunteers, conducted in 2001 and the first half of 2002, demonstrated that MVC was safe and well-tolerated in multiple doses up to 300 mg twice a day (BID), had a pharmacokinetic profile compatible with once daily (QD) or BID oral dosing, could be combined with other ARVs, and that doses of ≥100 mg BID resulted in exposure above the geometric mean antiviral IC_{90} *in vitro* (7, 10). To demonstrate proof of pharmacology, CCR5 receptor saturation was measured using a bespoke *ex vivo* MIP-1β internalization assay. Dose-dependent

saturation was demonstrated, with doses of ≥25 mg QD resulting in near maximum saturation levels, raising the interesting possibility that MVC could be efficacious in doses as low as 25 mg QD. Receptor saturation remained high for several days after dosing was discontinued, reflecting slow offset from the receptor *in vivo* (11).

We were both excited and encouraged by the phase 1 data and rapidly moved on to a phase 2a proof of concept program. HIV-1 infected patients were screened for the presence of R5 virus only, using a novel phenotypic tropism assay (Trofile®, Monogram Biosciences, South San Francisco, CA, USA), and received MVC as monotherapy for 10 days (12). CCR5 receptor saturation was measured in this study to evaluate the possibility of using this as a biomarker for efficacy and in therapeutic monitoring. The keenly awaited data lived up to our expectations and demonstrated that doses of ≥100 mg BID resulted in mean maximum HIV-1 RNA reductions of >1.5$\log_{10}$ (**Figure 1A**), with all patients, excluding one patient with X4 virus who has been erroneously included, achieving an HIV-1 RNA reduction of at least 1$\log_{10}$ (12). This gave us confidence that the assay correctly identified patients likely to respond to MVC. HIV-1 RNA nadir occurred 1–5 days after the last dose of MVC, consistent with prolonged receptor saturation as demonstrated in the phase 1 studies (12). For all doses except 25 mg QD receptor saturation of >80% was observed throughout the dosing period. However, there was no correlation between viral load reduction and degree of receptor saturation. The most likely explanation for this is that very high levels of receptor saturation is required for antiviral efficacy and the inherent variability of the assay does not allow differentiation to that degree (11, 12).

The phase 2a data generated excitement throughout the company and we were keen to progress the clinical development program as quickly as possible as there was a high medical need for new ARVs to treat patients with no or limited treatment options. The extensive phase 1 program (including multiple drug-drug interaction studies) and wide dose range evaluated in the phase 2a proof of concept studies, together with modeling and simulation, gave us a very good understanding of the likely efficacious dose of MVC in combination with other ARVs. We were therefore able to move straight to phase 3 efficacy studies evaluating MVC at 300 mg (or equivalent, depending on co-administered drugs) QD and BID, without the need to do stand-alone phase 2b dose-ranging studies, thereby significantly shortening the development timeline. In late 2004, we initiated four large studies; MOTIVATE 1 and 2 in treatment-experienced patients with R5 HIV-1 (13, 14), MERIT (a phase 3 study with a phase 2b roll-in) in treatment-naïve patients with R5 HIV-1 (15), and study A4001029, a phase 2b safety study in treatment-experienced patients with non-CCR5 tropic virus (CXCR4-using or non-phenotypable virus) (16).

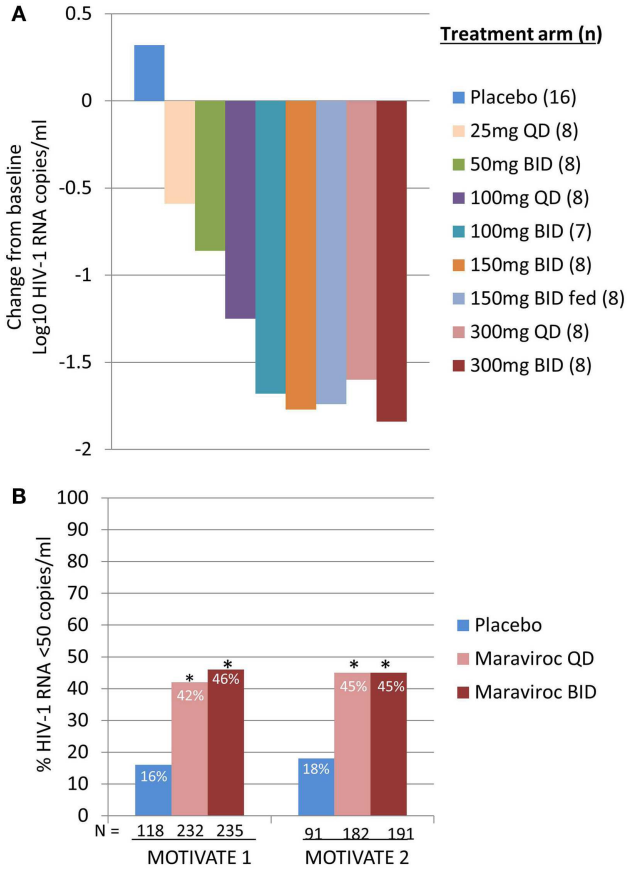

FIGURE 1 | Maraviroc proof of concept and phase 3 efficacy results. **(A)** Mean maximum change from baseline in HIV-1 RNA in patients receiving MVC monotherapy. Based on phase 1 data and modeling and simulation, doses ranging from 25 mg QD to 300 mg BID (including 150 mg BID fed and fasted) were selected. HIV-1 RNA, safety, and MVC pharmacokinetics were evaluated (12). **(B)** MOTIVATE 1 and 2 – proportion of patients achieving HIV-1 RNA <50 copies/mL at week 48. HIV-1 infected patients with R5 HIV-1 and triple class experience and/or resistance were randomized to receive MVC QD or BID, or placebo, in combination with an optimized background antiretroviral regimen (OBT). $P < 0.001$ (13, 14).

This was a massive undertaking, with 4794 patients screened at more than 200 sites in the USA, Canada, Europe, Australia, South Africa, Mexico, and Argentina. Two other small molecule CCR5 antagonists (aplaviroc and vicriviroc) were also being evaluated in phase 2b studies at this time (17, 18). In addition to the usual challenges of managing large clinical studies, we were thrown two curveballs, the first of these were the discontinuation of aplaviroc due to idiosyncratic hepatotoxicity. There was speculation that this could be a class effect of CCR5 antagonists as CCR5 knockout mice are more susceptible concanavalin-A mediated hepatoxicity (17). Additionally, a patient in the MERIT study developed severe hepatotoxicity. The

data implied that it was likely related to isoniazid or cotrimoxazole, but a contributory role for MVC could not be excluded (15). An in-depth review of all data for evidence of hepatotoxicity for MVC and a high level of vigilance for any signals, did not find any evidence for a systematic increase in hepatic enzymes or other markers for hepatotoxicity. Shortly afterwards concerns were raised regarding a potential increased risk for certain malignancies, following the occurrence of lymphoma in four patients receiving vicriviroc in study ACTG5211 (18). Initially there were concerns that this could be a class-effect based on the immune-modulatory potential of CCR5 antagonists, but review of data from other vicriviroc studies, as well as the ongoing MVC studies did not support this theory (18).

Data from MOTIVATE 1 and 2 and A4001029 were available ahead of that of MERIT, as study duration is typically shorter for studies in treatment-experienced patients. It was with great excitement that we awaited the week 24 interim analyses for the MOTIVATE studies in October 2006 and we were elated to see that significantly more patients receiving MVC had an HIV-1 RNA of <50 copies/mL (the key marker for efficacy) compared to those receiving placebo OBT. This was confirmed by the week 48 data, demonstrating durability of response (**Figure 1B**) (13, 14). In contrast, patients with non-CCR5 tropic HIV-1 receiving MVC in A4001029 did not appear to gain significant virologic benefit compared to placebo (16). Analysis of safety data raised no significant concerns. Specifically, there was no evidence of an adverse effect on immune function, with no increase in episodes of infection or malignancies in MVC treated patients. Assessment of virus tropism at failure demonstrated that >50% of patients failing MVC therapy had CXCR4-using virus at failure, but there was no evidence of a deleterious effect on CD4 cell count numbers (14). Virologic assessment demonstrated that the CXCR4-using virus that emerged under MVC selective pressure was from a pre-existing minority population and did not arise *de novo* (19). Altogether, these results clearly demonstrated the benefit of MVC in the management of treatment-experienced patients with R5 HIV-1. A supreme effort by the team resulted in submission of dossiers for registration in both the USA and Europe only 2 months after the interim data became available. MVC (300 mg BID) received approval for use (in combination with other ARVs) in the USA in August 2007, only 6.5 years after it was nominated as a candidate for clinical development. One month later, it was also approved for use in this population in the EU.

The week 48 analysis of the MERIT study was disappointing, as MVC plus zidovudine/lamivudine (HIV-1 RNA <50 copies/mL, 65.3%) did not meet the pre-set criteria for non-inferiority (lower bound of the 1-sided 97.5 confidence interval below −10%) to efavirenz plus zidovudine/lamivudine (HIV-1 RNA <50 copies/mL, 69.3%) (15). However, patients for this study were screened for R5 virus using the original Trofile assay. This assay has been improved in the meantime to be more sensitive for the detection of minority populations of CXCR4-using virus. All screening samples for patients in MERIT were subsequently retested using the enhanced assay and a *post hoc* analysis performed including only patients who had R5 virus only by the more sensitive assay. In this analysis the response rates for MVC and efavirenz were 68.3 and 68.5%, respectively, with the lower bound of the 97.5% confidence interval above −10% (15). Based on this data, MVC was also approved for use in treatment-naïve patients in November 2009 by the United States Food and Drug Administration.

MVC has not only proved to be a valuable addition to the ever growing ARV drug armamentarium, but data from these studies have improved our understanding of HIV tropism and the relationship between tropism and disease progression. For me, personally this represented a period of great excitement and satisfaction, both as a physician and scientist.

AUTHOR CONTRIBUTIONS

ER drafted this manuscript based on her personal experience as a member of the MVC development team. All the data have been published in full elsewhere. All studies were conducted in compliance with the principles of the Declaration of Helsinki and with all International Conference on Harmonization Good Clinical Practice Guidelines and local regulatory and legal requirements. All studies were approved by independent ethics committees and all patients gave written informed consent.

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AMD3100/CXCR4 inhibitor

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The original bicyclam, JM1657 (JM standing for Johnson Matthey) was discovered as a contaminant in a commercial preparation of monocyclams when evaluated for their anti-HIV activity. The original compound, in which the cyclam rings were tethered by a C–C linkage could not be re-synthesized but launched the synthesis of new bicyclams in which the cyclam moieties were linked through an aliphatic bridge: one of these derivatives, i.e., JM2763, exhibited an anti-HIV activity similar to that of JM1657 (1). The compound was postulated to interfere with the uncoating of HIV, a stage in the replicative cycle of HIV, which was (and still is) ill-defined. A quantum jump in anti-HIV potency was achieved with the synthesis of AMD3100 (AMD standing for AnorMeD) (which was originally called JM3100), where the two cyclam rings are tethered by an aromatic bridge (**Figure 1A**) (2). The compound was active against HIV in the low nanomolar concentration range and generated considerable commercial interest, although its precise mechanism of action remained enigmatic (3, 4). Finally, the viral glycoprotein gp120 was identified as the molecular target of AMD3100 (5). It appeared to be an indirect target. The direct target was CXCR4, with which gp120 has to interact for HIV to enter the cells. AMD3100 was shown to specifically antagonize CXCR4, and thus to block the entry of the T-lymphotropic HIV strains (6–8). AMD3100 appears to be a highly specific inhibitor of CXCR4 (9): it only blocks, as measured by the Ca^{++} flux, the signal pathway from CXCR4 (**Figure 1B**) and not that of any other receptor for either CXC- or

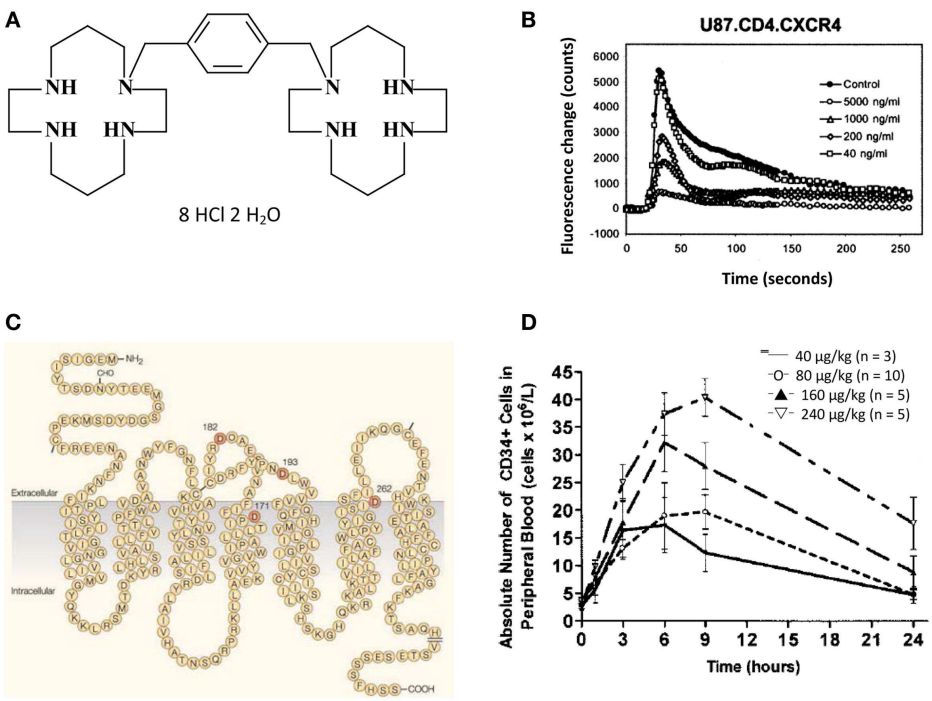

FIGURE 1 | (A) Structure of AMD3100. **(B)** Inhibitory effect of AMD3100 on Ca^{++} flux in CXCR4 transfected cells (9). **(C)** The CXCR4 receptor. Crucial aspartic acid residues at positions 171, 182, 193, and 262 in the interaction of CXCR4 with AMD3100 are indicated (11). **(D)** Mobilization of $CD34^{+}$ hematopoietic stem cells (HSCs) by AMD3100 (12).

C–C-chemokines (9). Certain aspartic acid residues play an essential role in the interaction of CXCR4 with AMD3100 (**Figure 1C**) (10, 11).

Within the scope of the potential clinical use of AMD3100 for the treatment of HIV infections, initial phase 1 clinical trials were initiated (13). These studies revealed an increase in the white blood cell (WBC) counts peaking at about 8–10 h after (subcutaneous) injection. These WBCs contained hematopoietic stem cells (HSCs) carrying the CD34 marker (12) (**Figure 1D**). In fact, the first proof-of-principle that AMD3100 could mobilize hematopoietic stem and progenitor cells was provided by Broxmeyer et al. (14). Thus, the concept was born that AMD3100 (now also called plerixafor or Mozobil®) could function as a mobilizer of HSCs. This mobilization is clearly based on the interaction of AMD3100 with CXCR4. CXCR4 is normally the receptor for the chemokine SDF-1 (now called CXCL12), which is responsible for the "homing" of the HSCs in the bone marrow. Under the influence of AMD3100, the HSCs leave the bone marrow to enter the bloodstream where they can be collected and subsequently used for autologous transplantation. In December 2008, Mozobil® was approved by the FDA for this indication in patients with non-Hodgkin's lymphoma or multiple myeloma. It is used in combination with granulocyte-colony stimulating factor (G-CSF) [for review, see Keating (15)]. For prescribing information, see Ref. (16).

AMD3100 was not further developed for the treatment of HIV infections essentially because of two reasons: (i) AMD3100 was not effective against the M-tropic CCR5 HIV strains, a problem that could be circumvented by the concomitant (oral) use of a CCR5 antagonist, maraviroc (Selzentry®), and (ii) it had to be injected subcutaneously, as it was not orally bioavailable. Subcutaneous injection is indeed a problem for long-term administration, and Fuzeon® (enfuvirtide) is the only anti-HIV drug out of more than 25, which has to be administered by injection, and, therefore, not widely used. Attempts to increase the spectrum of AMD3100 derivatives toward M-tropic HIV strains and, particularly, to increase their oral bioavailability led to the synthesis of AMD3465 (17), AMD11070 (18), and various other compounds (19–21), which, however, were not further developed as clinical candidates for treatment of HIV infections. Related CXCR4 antagonists such as KRH-1636 (22), KRH-3955 (23), and T140 analogs (24) were described by Naoki Yamamoto and his colleagues in Japan.

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