Ludwig-Maximilians-Universität of Munich
Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe

Role of regulatory T cells and associated chemokines in human gynecological tumors

Doctoral Dissertation of:
Christoph Paul Freier

2015

Aus der Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe
der Ludwig-Maximilians-Universität München
Direktor: **Prof. Dr. *med.* Sven Mahner**

Role of regulatory T cells and associated chemokines in human gynecological tumors

Dissertation

zum Erwerb des Doktorgrades der Naturwissenschaften an der Medizinischen Fakultät der Ludwig-Maximilians-Universität zu München

vorgelegt von
Christoph Paul Freier

aus
La Garenne-Colombes, Frankreich

2015

Mit Genehmigung der Medizinischen Fakultät
der Universität München

Betreuer: **Prof. Dr. *rer. nat.* Udo Jeschke**

Zweitgutachter: Prof. Dr. *rer. nat.* Vigo Heissmeyer

Drittgutachter: Prof. Dr. *rer. nat.* Roland Kappler

Viertgutachter: PD Dr. *rer. nat.* Reinhard Obst

Dekan: **Prof. Dr. *med. dent.* Reinhard Hickel**

Tag der mündlichen Prüfung: June 29, 2016

Wie man wird, was man ist.
F. W. Nietzsche (1844 - 1900)

Il faut imaginer Sisyphe heureux.
A. Camus (1913 - 1960)

Regrettably, open source is oftentimes relegated to being an afterthought or something for grad students to hack on while procrastinating on their Ph.D. theses.
Nonetheless, this document was handmade using the XeTeX typesetting system created by the Non-Roman Script Initiative and the memoir class created by Peter Wilson.
Analyses were designed and performed using R and occasionally powered by Python. The body text is set 12pt with Adobe Caslon Pro. Other fonts include Optima Regular and Envy Code R.

Printed in Münster • July 4, 2016
Contact me: christoph@freier.fr

Preface

This thesis embraces all the efforts I've made during the last four years as a Ph.D. student at the *Ludwig-Maximilians-Universität* of Munich. Or, actually, even since I eventually decided to study pharmacy, ten years ago. It has been quite a long journey since these warm days of July 2005.

I can remember this very first day of July were we went, my mother and I, for the first time to the faculty of pharmacy. I thought it would be to late or we couldn't make it. Indeed, alone, I certainly wouldn't have even reached the secretariat.

I can remember the first days in a pharmacy, luckily direct nearby Thomas' one. Also, every cup of tea we shared, me and Bogdann, and the innumerable and endless nights at Morgan's place. Guys, you made everything possible.

I can remember the first experience of what laboratory work means. I learned there everything I needed to successfully conduct this work and it has been the very beginning of my love for research. Without Marc, this thesis wouldn't exist today.

I can remember the first stays in Germany and the relaxing sailing trips with Jan. The laborious learning of German with Stefanie, Marie, and Eva. Looking back, it's easy to realize how much I learned and how important it was.

I can remember this summer evening at the *Königin 43* with my brother Niels. Only armed with our laptops and two *Weißbiere*, he taught me how to code and to run my very first R analyze. It certainly has been one of the most productive evening of this work.

I can remember that I also have two other brothers, Carl and Victor, both similarly important. I'm sorry I missed so much in France, while they grew up, became adults, studied, worked, lived and experienced so much. I'm so proud of them, too.

What I can't remember is my grandfather saying in front of me, "*Et dire qu'un jour, ça passera son bac.*" I was only a new born at that time. Now that I've grew up, his prediction became true, even outperformed. I'm sure he would have been proud, if he could have read this. This work is dedicated to his loving memory.

Christoph Paul Freier
München
July 12, 2015

Abstract

Purpose: To assess the clinical significance of different regulatory T cells (Treg) attracting chemokine intratumoral expressions in breast and ovarian cancer patients with available long-term follow-up (15 years). We investigated the prespecified hypothesis that the expression of Treg specific chemokines in cancer tissue predicts the overall survival (OS).
Patients and Methods: We screened all so far known chemokines of the CC-family for their capacity to selectively attract Treg *in vitro*. Three chemokines (CCL1, CCL22, and CCL27) with selective action on Treg migration were stained using extensively characterized antibodies. The numbers of positive cells in tissue microarray cores from ovarian cancer and invasive breast cancer were computer-aided determined.
Results: Within all analyzed chemokines, only CCL1, CCL22 and CCL27 selectively attracted Treg *in vitro*. All three chemokines were strongly expressed in most ovarian and breast cancer tissues. Moreover, there was a significant relationship between Treg infiltration in tumors and CCL1- and CCL27-expressing cell total numbers, whereas no association was seen for CCL22. High numbers of CCL1- or CCL27-positive cells identified patients with shorter OS.
Conclusion: Our findings indicate that quantification of intratumoral Treg attracting chemokines in ovarian and breast cancer is valuable for assessing disease prognosis. Unlike conventional clinicopathologic factors, high expression of certain chemokines can identify patients at risk of death over 15 years. CCL1 and CCL27 represent novel markers for identifying effect of immune response and tumor escape as well as patients who may benefit from immunotherapy. Such chemokines may gain to be considered together and could represent important therapeutic targets.

Key words: regulatory T cells, chemokine, CCL1, CCL27, ovarian cancer, breast cancer, clinical outcome, biomarker, therapeutic target, cancer immunotherapy.

Zusammenfassung

Zielsetzung: Untersuchung der klinischen Signifikanz von intratumoral exprimierten Chemokinen bei Mamma- und Ovarialkarzinompatientinnen mit Langzeit-Follow-up (15 Jahre) und deren Einfluss auf die Infiltration regulatorischer T-Zellen (Treg). Untersucht wurde die Hypothese, dass die Expression Treg spezifischer Chemokine in Karzinomgeweben das Gesamtüberleben beeinflusst.
Patientenkollektiv und Methode: Überprüft wurden alle bisher bekannten Chemokine der CC-Familie auf die selektive Kapazität Treg Migration *in vitro* auszulösen. Drei Chemokine (CCL1, CCL22 und CCL27), die die Treg spezifisch anlocken, wurden mit getesteten Antikörpern gefärbt. Die Anzahl positiver Zellen in Microarray-Gewebsschnitten von Ovarial- und invasiven Mammakarzinomen wurden mit Hilfe eines Computerprogramms bestimmt.
Ergebnisse: Von allen untersuchten Chemokinen, lockten nur CCL1, CCL22 und CCL27 Treg *in vitro* an. Die meisten Ovarial- und Mammakarzinomgewebe zeigten eine starke Expression dieser drei Chemokine. Weiterhin bestand ein signifikanter Zusammenhang zwischen Treg-Infiltration ins Tumorgewebe und der Gesamtanzahl CCL1- sowohl als auch CCL27- exprimierender Zellen, während kein Zusammenhang mit CCL22 gefunden wurde. Eine hohe Anzahl an CCL1 oder CCL27 positiver Zellen ging einher mit verkürztem Gesamt-überleben.
Schlussfolgerung: Unsere Ergebnisse weisen darauf hin, dass Treg anlockende Chemokine in Ovarial- und Mammakarzinomen von prognostischer Bedeutung sind. Im Gegensatz zu konventionellen klinikopathologischen Faktoren, identifiziert eine hohe Expression bestimmter Chemokine Patientinnen mit erhöhtem Risiko innerhalb der nächsten 15 Jahre an dem Karzinom zu versterben. CCL1 und CCL27 stellen neue Marker zur Untersuchung der Immunantwort und der Reaktion des Tumors diese zu umgehen dar. Des Weiteren dienen diese beiden Chemokine zur Identifizierung der Patientinnen, die von einer Immuntherapie profitieren würden. Eine Kombination von CCL1 und CCL27 könnte in Zukunft ein wichtiges therapeutisches Target darstellen.

Contents

Introduction 1

— It is by no means inconceivable that small accumulations of tumour cells may develop and, because of their possession of new antigenic potentialities, provoke an effective immunological reaction with regression of the tumour and no clinical hint of its existence.

Macfarlane Burnet, immunologist, 1957

1.1 The immunology of tumors

1.1.1 A short story of the immune regulation

The balance between effector response and mechanisms of immune regulation is one of the fundamental features of the immune system: the ability to correctly respond to any external aggression or infectious microorganism avoiding destruction of self-tissues determines its role [1]. This original paradigm was claimed more than fifty years ago by Medawar *et al.* and derived nowadays to include the pharmacological induced state of tolerance against any deliberately introduced antigen, as it is the case for a transplanted organ. From an evolutionary point of view, the critical importance of the immune tolerance is illustrated by the multitude of non-redundant mechanisms [2].

It is currently well accepted that immunological tolerance is mediated by two categories of mechanism: central and peripheral. After a first cellular selection in the thymus, the functionality and efficiency of the immune system is highly dependent on complex series of cellular and cytokine mediated interactions between effector and regulatory cells. In the periphery, effector but also regulatory cells have to be recruited both at the right place and at the right time, via complex chemokine networks coordinating cell traffics.

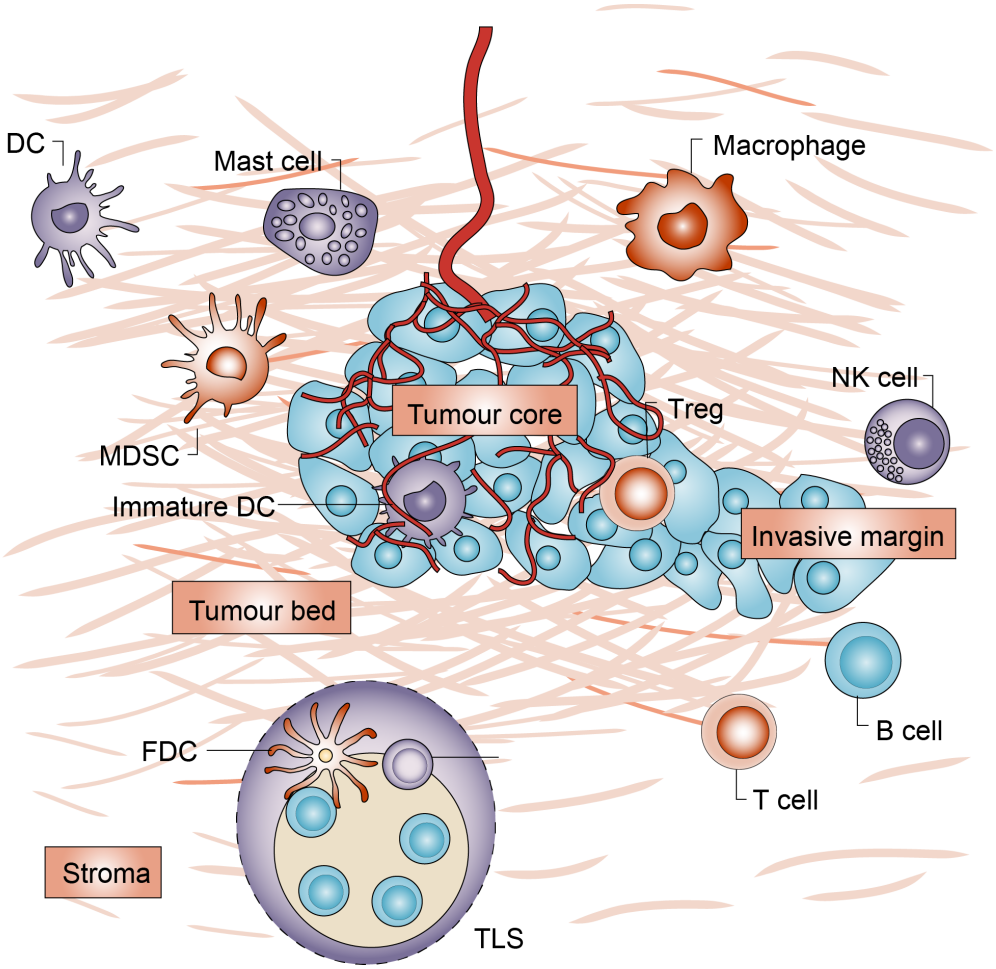

Figure 1.1 – *Tumor anatomy showing the immune contexture of cancer with features of the immune contexture and the distribution of different immune cells according to Fridman* et al.. *DC, dendritic cell; MDSC, myeloid-derived suppressor cell; Treg, regulatory T cell; NK cell, natural killer cell; FDC, follicular dendritic cell; TLS, tertiary lymphoid structure.*

These chemokines consist of small chemotactic cytokines characterized by critically positioned cysteine residues that are recruiting distinct cell types according to their corresponding receptors [3, 4]. Corresponding chemokine receptors are specifically regulated in regard of cell type, stage of activation and differentiation [5]. Given the central role of chemokines in the regulation of cell migrations, it was obvious that they also play a pivotal role in the regulation of the peripheral immune response. They allow the regulation of the adaptive immune response by recruiting effector cells, but also immunoregulatory cells. So far, over 50 chemokines and 22 chemokine receptors have been identified and their central function in the coordination of the immune system is now firmly established [6].

1.1.2 Role of the immune system in cancer

Many of the defenses against the appearance of cancerous growths are inherent in cells. The most obvious one lying in the different controls imposed by the apoptotic machinery. During the past decades, however, several authors linked human cancer progression with immune cell infiltration [7, 8, 9]. This followed the first observations realized in mice by Hanahan and Weinberg, which had led them to add "avoid immune destruction" as a hallmark of cancer [10]. In fact, tumor environments are an intricate network of epithelial cells, vascular and lymphatic vessels, cytokines, chemokines and infiltrating immune cells. All immune cells can be found in a tumor, including dendritic cells (DC), macrophages, natural killer cells, naive and memory lymphocytes, B cells and various subsets of T cells – **Figure 1.1, p. 2** [11]. Moreover, these different cell types seem to play a role which can vary according to the tumor nature and his localization: sometimes favorable, or in some other cases, paradoxically accelerating the tumor growth and reducing the OS of patients [12, 13].

The demonstration of the infiltrated cell influence on the clinical outcome of cancer conducted to the creation of the field of tumor immunology and already led to novel opportunities of prognostic marker identification and therapeutic options. Histopathological studies of human tumors have shown that variable numbers of infiltrating immune cells are found in different tumors of the same type, and are found at different localizations within and around a tumor; this distribution also varies between tumor types [14]. This is underlying the possibility of different roles for these immune cell populations, in respect to their localizations in tumors, but also the complexity of the role played by the immune system in cancer.

The observation of variable densities and localizations of these immune cells between tumors of the same cancer type in different individuals tends to prove the orchestrated fine migration of these cells. Considering the role of chemokines in cell migration, it was not surprising to find overexpressed chemokine synthesis in several tumor types, possibly responsible for the accumulation of immune cells [15, 16]. These issues are far from being completely answered, but analyses of the cytokine and chemokine milieu associated with a tumor immune contexture are accumulating. Moreover, this suggests that the immune system could indeed represent effective defenses that prevent the appearance of tumors.

1.1.3 Clinical impact of the immune contexture

One century ago Paul Ehrlich proposed that the immune system is programmed to avoid the generation of autoreactive immune responses and termed this aversion to autoreactivity "*horror autotoxicus*". Ehrlich's observations that goats could make antibodies against the blood components of other goats, but not against their own blood, represented the first evidence of immunological self-tolerance. However, nowadays, it clearly appears that an absence of immune response is as much deleterious for the organism.

Correlations between tumor levels of immune cell infiltration and clinical outcome have been investigated in many cancers and results are still a matter of debate since they present some contradictions. A strong lymphocytic infiltration has been reported to be associated with good clinical outcome in many different tumor types, including melanoma, head and neck, breast, bladder, urothelial, ovarian, colorectal, renal, prostatic and lung cancer [11]. Precisely, high densities of cluster of differentiation (CD)3$^+$ T cells, CD8$^+$ cytotoxic T cells and CD45RO$^+$ memory T cells were commonly associated with better prognosis [17, 18].

In contrast, analysis of the CD4$^+$ T cell population infiltration often shows an opposite effect on OS. Most conflicting are the different data for T helper cells (Th)2, Th17 and Treg, linked with difficult interpretations [11]. This is presumably due to the creation of an immunosuppressive micro-environment, inhibiting any chance of a correct immune response against tumor cells and thus leading to accelerated tumor growth and reduced OS. This immune contexture in tumor could serve to predict therapeutic responses, as shown in the colorectal cancer and the breast cancer. Considering the number of infiltrated cytotoxic CD8$^+$ cells and memory CD45RO$^+$ cells, Denkert *et al.* and Tosolini *et al.* could finely predict the risk of relapse when no other actual markers could do it [18, 19]. This of course, could be of paramount importance in patient clinical management, but also allows to target the accumulation of unfavorable immune cells; thus opening the way to interesting new therapeutic opportunities of treatment.

1.1.4 Emergence of the immunotherapy

During the last two decades, cancer treatments evolved from relatively nonspecific cytotoxic agents to selective, mechanism-based therapeutics. Although cancer chemotherapies initially were compounds that killed rapidly but unspecifically dividing cells, improved understanding of cancer pathogenesis has given rise to new treatment options, including targeted agents and cancer immunotherapy. Among these modern targeted approaches, immunotherapy aims to stimulate a host immune response that effectuates long-lived tumour destruction to improves clinical outcome, reduces toxicities and frequently acquired resistances. Significant advances in targeting the immune response were made and have led to cell-based vaccine composed of autologous antigen presenting cells (APC) that have been exposed to a recombinant protein consisting of granulocyte-macrophage colony-stimulating factor (GM-CSF) fused to a protein expressed by cancer cells. Upon administration, the vaccine may stimulate an antitumor T-cell response against tumor cells expressing this protein.

Secondly, tumour-specific T cells must differentiate into effector T cells, which requires a combination of signals from both the T cell receptor (TCR) and several co-stimulatory molecules [20]. Co-stimulatory signals are delivered through multiple transmembrane proteins of the B7 and tumour necrosis factor receptor (TNFR) families, as well as receptors for some cytokines, such as interleukin (IL)-12. Agonistic antibodies to these molecules can enhance co-stimulation to augment anti-tumour immunity [21, 22]. However, T cells must avoid negative regulatory signals (known

as immune checkpoints) that dampen their activation or that induce tolerance programmes such as anergy or exhaustion [23, 24]. Blockage of cytotoxic T lymphocyte-associated antigen 4 (CTLA4) and programmed cell death protein 1 (PD1), both major negative co-stimulatory molecules that are expressed on activated T cells, are now possible and has demonstrated encouraging anti-tumour effects in initial clinical testing [25, 26].

A major impediment to the therapeutic efficacy of anti-tumour T cells is the immunosuppressive tumour micro-environment [27]. Diverse mechanisms operate, including the production of inhibitory cytokines such as IL-10 and tumor growth factor (TGF)-β, the expression of negative co-stimulatory ligands such as programmed cell death ligand 1 (PDL1), and the presence of regulatory lymphocyte and myeloid cell populations. The identification of agents that attenuate these suppressive networks might substantially increase the efficacy of immunotherapies [28].

1.2 Regulatory T cells

1.2.1 About their role in the immunoediting process

Tumors are often infiltrated by various inflammatory immune cells creating a milieu that may function to either stimulate or inhibit cancer growth [29]. The chronic inflammation response found in many cancers creates an environment rich in substances that promote angiogenesis and cell proliferation, very similar to wound healing processes [30]. Evidence from murine studies strongly suggest, that adaptive immune cells are attracted and can directly eliminate tumor cells [31, 32].

Immunosurveillance, however, can fail to completely kill nascent tumors – due to poor immunogenicity of tumor cells or immune escape mechanisms – and therefore favorises the immunoediting process which eventually ends in tumor escape [8]. The analysis of the location, density, functional orientation of different immune cell populations, and their respective clinical impacts have been investigated in many cancers [11]. The ultimate goal is to allow the identification of components of the immune contexture that are beneficial, as well as those that are deleterious, to patients.

A strong lymphocytic infiltration, particularly $CD8^+$ cytotoxic T lymphocytes, has been reported to be associated with responses to neoadjuvant chemotherapy and good clinical outcome in breast cancer [18, 33]. $CD4^+$ $CD25^+$ $CD127^-$ Treg play a pivotal role in the control of immune responses [34, 35]. Treg normally function to protect tissues from autoimmune diseases by suppressing self-reactive cells, including $CD8^+$ cytotoxic T lymphocytes, B cells and natural killer cells [36, 37, 38]. Additionally, they have an important role in the immunoediting process, enabling the tumor to elude the host antitumor immune response, because of their inhibiting action on cytotoxic activity of $CD8^+$ and natural killer cells [36, 37].

1.2.2 Characteristics of regulatory T cells

Treg represent a subpopulation of $CD4^+$ lymphocytes that suppress the immune response at different levels. Human peripheral blood $CD4^+$ $CD25^+$ forkhead box P3 (FoxP3)$^+$ T cells, or Treg represent 4-8% of human $CD4^+$ T cells [39]. They have been characterized as suppressor T cells after the observation by Sakaguchi *et al.* of induced organ-specific autoimmune disease in mice depleted for this particular population [40].

The expression of CD25 (also known as IL-2 receptor-α) appears during the transition of the $CD4^+$ $CD8^+$ T cell to the $CD4^+$ $CD8^-$ T cell, therefore taking part in normal thymocyte differentiation [41]. This reflects the activation of cells during a process termed "altered negative selection": future Treg probably express a TCR with an intermediate affinity for self, to low for negative selection, but to high to allow them to pass through to the periphery [42].

Minor differences are observed in respect to Treg population of "naturally occurring", which are the thymus derived cells naturally isolated from peripheral blood and "induced", generated in the lymph node. However, it is widely accepted that they are both hyporesponsive and suppressive, as a result of the activation process, acting via an APC-dependent mechanism [43]. Thus in normal process, Treg modulate immune responses through selective migration and accumulation at different sites where regulation is required. Accumulative amount of data has demonstrated the role in controlling Treg migration of distinct signals from chemokines/chemokine receptors and integrins/integrin ligands in recruiting Treg in human pathologies and for the homing of these cells, indispensable for them to acquire their full activation state [44, 45]. In the mouse, direct evidences of roles for migration and functionality played by chemokines and integrins was also shown *in vitro* and *in vivo* [46, 47].

1.2.3 Regulatory T cell markers

Despite the immunologists' obsession to classify all immune cell types, the identification of discriminatory cell-surface markers for the characterization and isolation of Treg remains problematic. This could account for the sometime confusing results found in several Treg studies relative to their implication in cancer and is even more surprising considering that we already dispose of a large panel of Treg mouse markers. If traditionally, mouse and human Treg have been characterized as $CD4^+$ $CD25^+$, the purity of isolated human Treg remains an issue mainly because other human T cells also upregulate CD25 when activated [48].

One significant advance in the study of mouse and human Treg has been the identification of FoxP3 as key regulator of Treg development and function [49, 50]. Elegant mouse and human genetic studies demonstrated that mutation in the FoxP3 gene were linked to the autoimmune manifestations observed in the scurfy mouse that carry a spontaneous loss-of-function mutation or a deletion of FoxP3 and human immune dysregulations like polyendocrinopathy, enteropathy and X-linked syndrome disease. But again, if it appears useful in mice, many activated

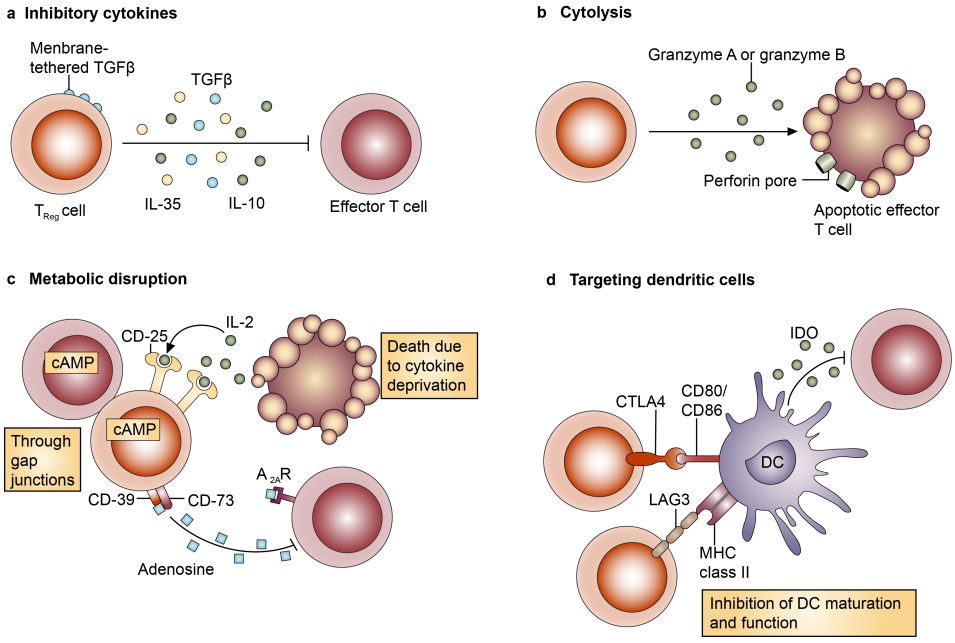

FIGURE 1.2 – *Regulatory T cell mechanisms of action according to Vignali* et al.. *Four basics mode of action are here illustrated (a) via inhibitory cytokines, (b) via the cytolysis of targeted cells, (c) via metabolic disruption and (d) via the inhibition of dendritic cells. TGF-β, transforming growth factor-β; IL, interleukin, A2R, adenosine receptor; MHC, major histocompatibility complex.*

non-regulatory human T cells transiently up-regulate FoxP3 under activation [48]. Moreover, FoxP3 is intracellular and could not serve routinely to Treg isolation so that the search for specific surface markers has continued in earnest with the discovery of the downregulation of CD127 (also known as IL-7 receptor) on Treg and the 90% correlation of the three membrane markers (*i. e.*, $CD4^+$, $CD25^+$ and $CD127^-$) and FoxP3 expression [35].

Finally, it is possible that Treg, like other T cell populations, are composed of heterogeneous subtypes, like suggested by Sakaguchi *et al.* [51]. They divided Treg into three populations based on the expression of FoxP3 and CD45RO and showed that these populations suppressed other cells via different mechanisms and not all with the same intensity. Thus it appears probable that the search for better membrane markers is not yet finished.

1.2.4 Regulatory T cell mechanisms of action

Defining how do these cells work is crucial to provide insight into the control processes of peripheral tolerance and could also indicate potentially important therapeutic targets. Treg require TCR triggering to become fully functional, but once activated they suppress T cells blindly, with no regard to the antigen specificity of targeted cells [43].

Suppressive mechanisms used by Treg have been regrouped into four modes

of action: (a) suppression by inhibitory cytokines, (b) suppression by cytolysis, (c) suppression by metabolic disruption and (d) suppression by modulation of DC maturation and/or function – **Figure 1.2, p. 7** [52]. To date, it is difficult to say if Treg functions are mediated by a single overriding suppressive mechanism or by multiple, redundant or not, mechanisms. It is, however, more likely that Treg suppressive functionality comes from multiple one, since none of the currently known mechanisms seems to be exclusive. Again, the choice for suppression mechanism(s) to use – dependent of the background, the context or the type of target cells – remains unclear, underlying the need of more research in this domain.

Several studies already suggested that Treg infiltrated in tumors may adversely affect prognosis via their immunosuppressive action. Increased tumoral Treg number have been correlated with poor OS in breast cancer, melanoma, lung carcinoma, and hepatocellular carcinoma [14, 53, 54, 55]. Importantly, the high number of Treg present in tumor environment raises the question of their recruitment.

1.3 Chemokines and their receptors

1.3.1 Discovery

The first description of directed, transendothelial leucocyte migration and accumulation into organs in case of inflammation was made more than one century ago by August Waller (1846) and Julius Cohnheim (1867). The first hypothesis, that leucocytes could phagocytose bacteria and thus should have to migrate to inflamed tissues, eventually in response to chemical agents, was postulated a couple of years later by Ilja Metchnikoff (1891). But it took almost one century to have the first *in vitro* demonstration of this phenomenon, when in the 60's and 70's the migration was observed via migration assays using the first chamber chemotaxis transwell plates.

The next step was done in the 80's, when several teams discovered and characterized a new chemoattractant polypeptide named monocyte-derived neutrophil chemotactic factor (MDNCF) or neutrophil-activating peptide (NAP). One year after, this chemokine was cloned, sequenced and renamed IL-8, first chemotactic cytokine [56]. With help from sequence homologies and hybridization methods other members of this new family were quickly identified. A few years later, on the "3rd International Symposium on Chemotactic Cytokines" it was decided to rename this cytokine subfamily after their CHEMOtactic-cytoKINE effects into CHEMOKINE.

The list of chemoattractant quickly grew from C5a, through platelet activating factor (PAF) to the more than 50 chemokines or chemoattractants known today [3]. All these different chemoattractants were observed to bind to specific surface membrane receptors, all members of the G protein-coupled receptors (GPCR) family. In the mean time, a lot of other signal polypeptides were discovered, with the result that it soon began urgent to order all these new cytokines in subfamily and to give

them an international nomenclature. Originally most of them were named with different names, according to their different effects on cells.

1.3.2 Nomenclature

In 2000, a system of nomenclature was introduced in which each ligand and receptor were respectively identified by its subfamily and given an identifying number. For example, CCR4 refers to the chemokine receptor of the CC-family number 4, and binds to CCL17 and CCL22 (chemokine ligand of the CC-family, number 17 and 22 respectively). Redundancy between different ligands and the same receptor or on the contrary, between different receptors and the same ligand is considerable. If very little is known about this lack of specificity, it may be an explanation for the quite normal phenotypes of knockout (KO) mice for most inflammatory chemokines or chemokine receptors unless challenged with inflammatory conditions or faced with pathogens [57]. On the other hand, this redundancy could allow fine tuning of immune responses, function of combination of ligands, receptors, but also other molecules, possible oligomerization, glycosaminoglycans, *etc*.

CCL#, CXCL#, CL# and CX3CL# refer to the four families of chemokine ligands. C stands for a cysteine and X for a non-cysteine amino acid, L for ligand, and # denotes the identifying number. The larger chemokine family, composed by the CCLs or β-chemokines, is characterized by two adjacent cysteines, near to the N-terminal end of the protein – **Table 1.1, p. 10**. 27 chemokines have been reported in this group, named CCL1 to CCL28 (CCL9 and CLL10 being the same one). Notably a small number of them possess six cysteines: CCL1, CCL15, CCL21, CCL23, CCL28.

In the second largest family, composed by the CXCLs or α-chemokines, the two first cysteines are separated by one amino acid, represented in the name with an "X" – **Table 1.2, p. 11**. 17 of them have been described, subdivided in two categories: those with a specific amino acid sequence ELR (glutamic acid-leucine-arginine) immediately before the first cysteine of the CXC motif, and those without.

XCL1 and XCL2 are the two chemokines characterized, in the third family, the XCLs or γ-chemokines, and are characterized by only two cysteines: one N-terminal cysteine and one cysteine downstream – **Table 1.2, p. 11**. These chemokines are presumed to attract T cell precursors into the thymus [5].

Finally, CX3CL1 is the only chemokine discovered in the fourth family, CX3CL or δ-chemokine – **Table 1.2, p. 11**. In the same fashion as for the CXC chemokines, the two first cysteines are separated, in this case indeed, by three amino acids. It is both secreted and tethered to the surface of the cell that expresses it, thereby serving both as chemoattractant and as an adhesion molecule [3].

It is of importance to mention, that not all of these chemokines are present both in mouse and human, underlying the difference between the two systems, but also the functional redundancy of chemokines.

Name		Old name	Produced	Binds on	Receptor(s)
CC (β)					
CCL1	*i*	I-309	M, T	M, act.Th2	CCR8
CCL2	*i*	MCP-1	M, Fib, En	M, T, Ba, Fib	CCR2,3
CCL3	*i*	MIP-1α	M, T, NK, N, DC, Fib, En	M, T, DC	CCR1,5
CCL4	*i*	MIP-1β	M, T, NK, N, DC, Fib, En	M, DC	CCR5
CCL5	*i*	RANTES	M, T, Fib, Epi, En, P, Eos	M, T, DC, Eo	CCR1,3,5
CCL7	*i*	MCP-3	M, Fib, P	M, T, NK, DC	CCR1,2,3,5
CCL8	*i*	MCP-2	N, Fib	M, T, NK, Eo	CCR2,3
CCL11	*i*	Eotaxin-1	M, Eos, Fib, resp. Epi	Eo, Ba, Th2	CCR3,5
CCL13	*i*	MCP-4	M, L, En, Epi / Lung, Gut	M, T, DC, Eo	CCR2,3,5
CCL14	*h*	HCC-1	S, G, Li, Mu	M, Eo	CCR1
CCL15	*h*	HCC-2	L, NK, M, DC / Liver, Gut	T, M, Eo	CCR1,3
CCL16	*h*	LEC	M / Liver	M, L	CCR1
CCL17	*m*	TARC	T, DC / Thymus, Lung, Gut	Thy, Th2	CCR4
CCL18	*h*	PARC	M, DC / Lung, LN, Thymus	T	CCR8
CCL19	*h*	ELC	DC / Thymus, LN	T, B, DC, Thy	CCR7
CCL20	*m*	LARC	Epi, M, DC, T / LN, Lung	T,DC	CCR6
CCL21	*m*	SLC	En / Thymus, Spleen, LN	T, B, DC, Thy	CCR7
CCL22	*m*	MDC	M, DC / Thymus, LN, Gut	Th2, NK, DC	CCR4
CCL23	*i*	MPIF-1	DC / Pancreas, Muscle	M, L	CCR1
CCL24	*i*	Eotaxin-2	M / Lung, Skin	Eo, Ba, Th2	CCR3
CCL25	*h*	TECK	Epi, DC / Thymus, Liver, Gut	Thy, T, M, DC	CCR9
CCL26	*i*	Eotaxin-3	Fib, En / Skin, Heart, Ovar	Eo, Ba	CCR3
CCL27	*h*	CTACK	Epi / Skin, Placenta	T, Fib, En	CCR10
CCL28	*i*	MEC	Epi / Brust, Colon	T, Eo	CCR3,10

i, pro-inflammatory; *h*, homeostatic; *m*, mixed function

Table 1.1 – Classification of human CC-chemokines. MCP-cluster in blue, MIP-cluster in red. B, lymphocyte B; Ba, basophil; DC, dendritic cell; Eo, eosinophil; En, endothelium; Epi, epithelium; Fib, fibroblast; Hep, hepatocyte; L, lymphocyte; M, monocyte/macrophage; N, neutrophil; NK, natural killer cell; P, platelet; T, lymphocyte T; Th1/2, type 1/2 helper T cell; Thy, thymocyte; LN, lymph node

1.3.3 Structure and classification of chemokine

Chemokines are small ligands, 70-130 amino acids and 8-12 kDalton (Da) proteins (with the notable exception of CXCL16 and CX3CL1) that contain 1-3 (mostly 2) disulfides, with critical roles in cell migration during immune surveillance, inflammation and development. They interact with GPCR on target cells and cause conformational changes that trigger intracellular signaling pathways involved in cell movement and activation and are classified as chemokine based on shared structural characteristics, small size and the presence of four cysteine residues (except for the γ family and a few exceptions in the β family) in conserved locations that interact with each other in pairs to create a Greek key shape that is a characteristic of chemokines.

Although the sequence homology is highly variable, ranging from less than 20% to over 90%, the tertiary structure is remarkably conserved – **Figure 1.3, p. 12**. This tertiary structure always presents a disordered N-terminal of 6-10 amino acids functioning as key signaling domain. The N-terminal has a critical role in receptor activation, and N-terminus truncations can render chemokines inactive or even able to act

Name		Old name	Produced	Binds on	Receptor(s)
CXC (α)					
CXCL1	*i*	GRO-α	M, Epi, En	N, L, M, En	CXCR2
CXCL2	*i*	GRO-β	M, Epi, En	N, L, M, En	CXCR2
CXCL3	*i*	GRO-γ	M, Epi, En	N, L, M, En	CXCR2
CXCL4	*h*	PF-4	P	Fib, En	CXCR3
CXCL5	*i*	ENA-78	M, N, Epi	N	CXCR1,2
CXCL6	*i*	GCP-2	M, Fib / Heart, Lung, Kidney	N	CXCR2
CXCL7	*i*	NAP-2	P, M, N	N	CXCR2
CXCL8	*i*	IL-8	M, L, N, Fib, Epi	N, T	CXCR1,2
CXCL9	*i*	Mig	M, Fib, Hep, IFN / Spleen, Liver	T, NK	CXCR3
CXCL10	*i*	IP-10	M, IFN / LN, Thy	T, NK	CXCR3
CXCL11	*i*	I-TAC	M, IFN / Spleen, Pancreas	T	CXCR3,7
CXCL12	*h*	SDF-1	Fib / Spleen, Gut	T, B, DC	CXCR4,7
CXCL13	*h*	BCA-1	DC, En / Spleen, LN, PeyerPatch	B	CXCR5
CXCL14	*i*	BRAK	all	M, DC	?
CXCL16	*i*	SR-PSOX	DC / LN, Spleen, Lung	T, NK, DC	CXCR6
CXCL17	*i*	DMC	Stomach, Trachea	D, M	?
C (γ)					
XCL1	*m*	Lympotactin	T / Thymus, Spleen	T, NK	XCR1
XCL2	*m*	MCP-1	T	L	XCR1
CX3C (δ)					
CX3CL1	*i*	Fractalkine	En / Brain, Lung, Heart	M, T, NK	CX3CR1

i, pro-inflammatory; *h*, homeostatic; *m*, mixed function

Table 1.2 – Classification of all other human chemokines. GRO-cluster in green and IP-10-cluster in yellow. B, lymphocyte B; DC, dendritic cell; En, endothelium; Epi, epithelium; Fib, fibroblast; Hep, hepatocyte; IFN, IFN-γ stimulated cell; L, lymphocyte; M, monocyte/macrophage; N, neutrophil; NK, natural killer cell; P, platelet; T, lymphocyte T; Thy, thymocyte; LN, lymph node

as antagonists. Most of them are secreted from the cell, with two notable exceptions: CX3CL1 and CXCL16, which also can stay on the extracellular cell membrane [3]. To produce directional migration signals for cell migration, concentration gradients of chemokines must be formed, since cells exposed to a uniform concentration of a chemokine move in random patterns rather than directionally. *In vivo*, the establishment of a gradient is thought to involve binding to glycosaminoglycans; conditions that could be mimiced *in vitro* by the chamber chemotaxis migration assay.

Chemokines can also be classified into three groups, according to their physiological roles. Inflammatory chemokines are inducible in organs in processes like immune response or inflammation (inflammation mediated by cytokines, bacterial toxins, *etc*). They attract specialized leukocyte populations or subpopulations (*e.g.*, neutrophil, monocyte, basophil, eosinophil, effector T cell, DC) to the inflamed localization and allow the immune system to respond various aggressions. They also regulate the expression of genes in those cells, acting as transcription factor or via stabilization of messenger RNA (mRNA). The multitude of inflammatory chemokines allows fine regulations of the immune response. This redundancy could be explained, from an evolutionary point of view, by multiple gene duplications. There are to date four clusters: MCP-cluster and MIP-cluster of the chromosome 17q11-17q12, GRO-cluster of the chromosome 4q13 and IP-10-cluster of the chromo-

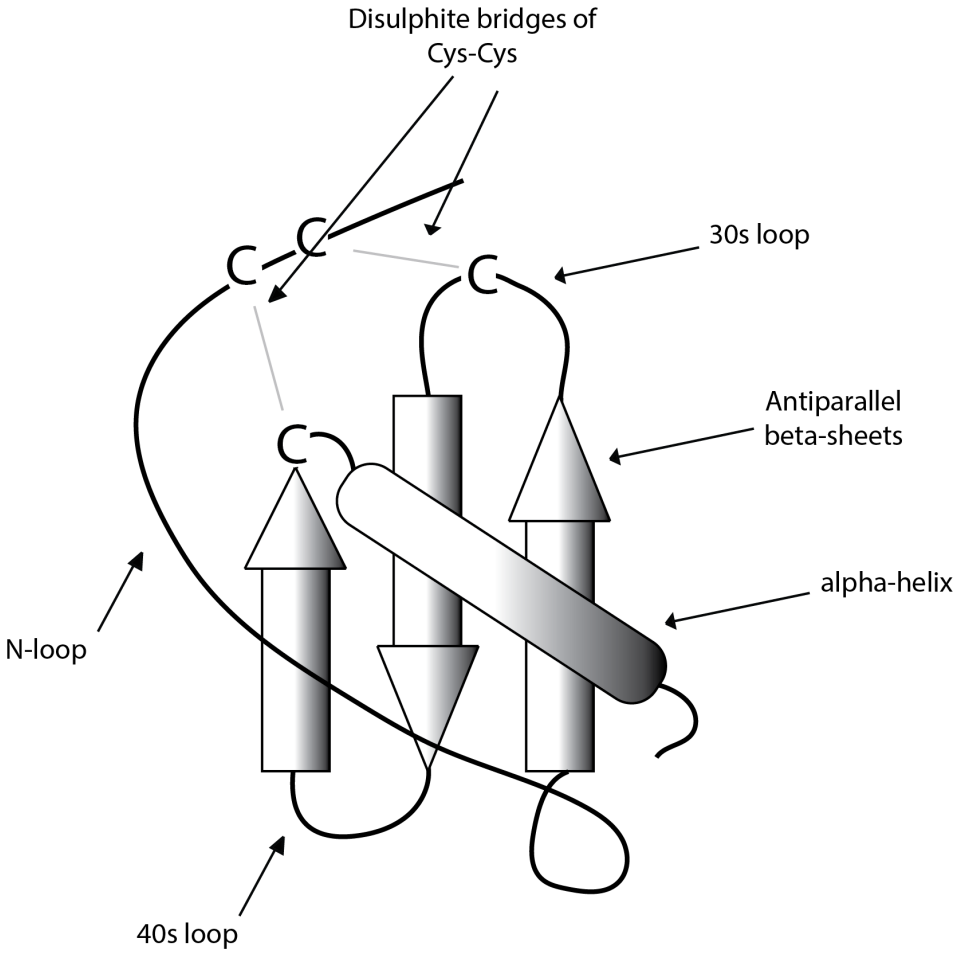

Figure 1.3 – *Schematic representation of the chemokine key functional regions. All chemokines have a similar tertiary structure comprising a disordered N-terminal of 6-10 amino acids followed by a long loop (known as the N loop), a 3_{10} helix, a three-stranded beta-sheet, and finally a C-terminal alpha helix.*

some 4q21. These chemokines present highly conserved tertiary structures and act mostly on the same receptor with a similar action. Homeostatic chemokines are constituently expressed in the primary and secondary lymphoid organs and control the migration and localization of T and B cells, as well as DC. This expression is particularly important for the maturation of these cells and for the adaptive immune response. Immune surveillance also involves migration of lymphocytes in response to homeostatic chemokines. Certain chemokines are continuously expressed in tissues such as the skin, intestinal mucosa, and lungs to promote constant monitoring of these at-risk areas. Lastly, some chemokines are included in a third group, reflecting their mixed function, depending on the biological context or pathological state. Expression of inducible chemokines is often triggered by inflammatory mediators such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, microbial products, or trauma. These chemokines have roles in both innate and adaptive immunity in

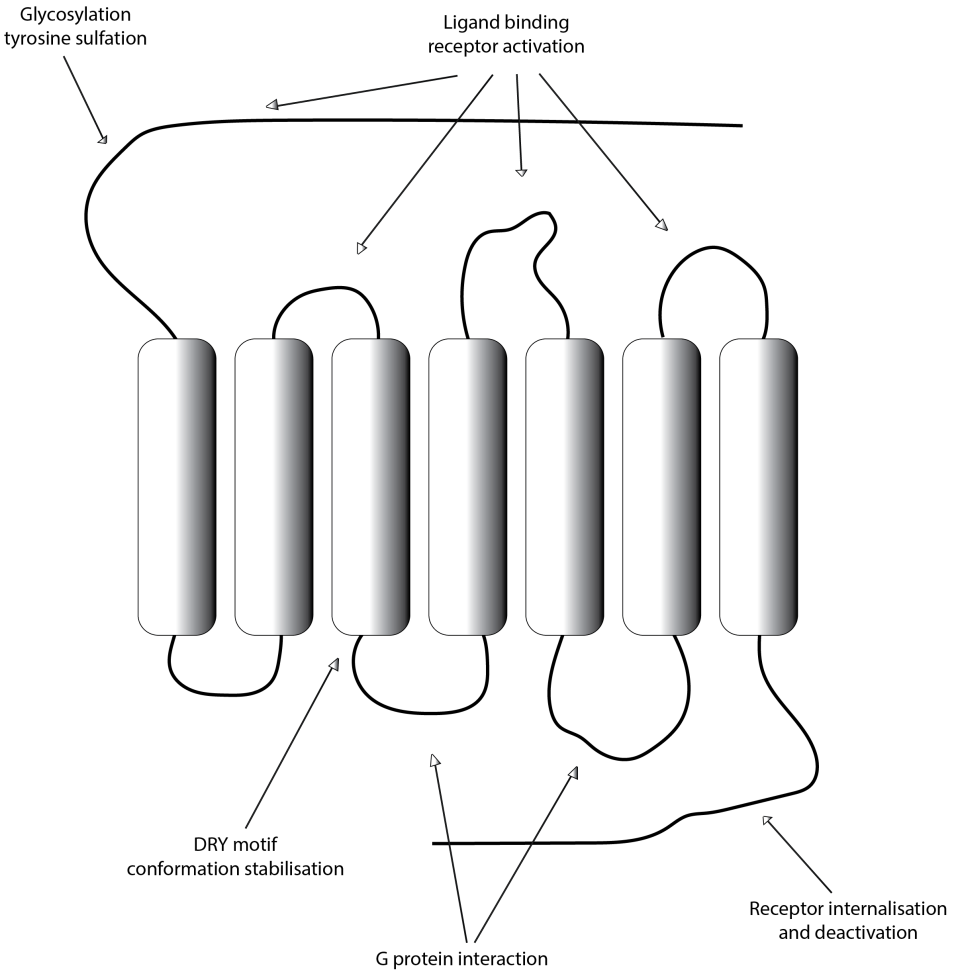

FIGURE 1.4 – *Schematic representation of the chemokine receptor key functional regions. Mutagenesis and chimeric receptor studies have identified regions of chemokine receptors important for ligand binding, receptor activation, and internalization, although specific sequences involved in signaling differ between different chemokine receptors.*

response to infections, tissue damages, and other physiological abnormalities. Their expression is temporary, until resolution of the situation.

1.3.4 Classification of chemokine receptors

The chemokine receptors are all class A GPCR, presenting three intracellular and three extracellular hydrophilic loops – **Figure 1.4, p. 13**. They are commonly 340-370 amino acids long and about 40 kDa proteins. The N-terminal domain is exposed outside the cell and binds to chemokine(s). The C-terminal domain, containing serine and threonine residues that act as phosphorylation sites, is coupled with the G protein. Conserved extracellular cysteine disulphide bridges stabilize the tertiary structure.

To date, 22 of them were described and since the "Gordon Research Conference

Name		Expressed on	Ligand(s)
CXC receptor			
CXCR1		N	CXCL6, 8
CXCR2	*alt*	N, En	CXCL1, 2, 3, 5, 6, 7, 8
CXCR3	*alt*	Th1	CXCL9, 10, 11
CXCR4		All	CXCL12
CXCR5		B, T	CXCL13
CXCR6		Th1, Tc1, NK	CXCL16
CC receptor			
CCR1		M, T, Eo, Ba	CCL3, 5, 7, 14, 15, 16, 23
CCR2	*alt*	M, Th1, DC, NK, Ba, Fib	CCL2, 7, 8, 13
CCR3		Eo, Ba, Th2, P	CCL2, 5, 7, 8, 11, 13, 15, 24, 26, 28
CCR4		M, Th2, DC, Ba	CCL17, 22
CCR5		M, Th1, Tc1, NK	CCL3, 4, 5, 7, 8, 11, 13
CCR6	*alt*	T, B, DC	CCL20
CCR7		T,DC	CCL19, 21
CCR8		Th2, M, NK, DC	CCL1
CCR9	*alt*	T, IgA-Pla	CCL25
CCR10		T	CCL27, 28
C receptor			
XCR1		T, NK, P, Thy	XCL1, 2
CX3C receptor			
CX3CR1		T, NK, M, En	CX3CL1
Silent receptor			
CXCR7			CXCL111, CXCL12, MIF
CCR11	*alt*		CCL19, 21, 25
Duffy		Ery, En	CXCL1, 2, 3, 5, 6, 7, 8, 11 CCL2, 5, 7, 11, 13, 15, 17, 18, 22
D6		Placenta	CCL2, 3, 4, 5, 7, 11, 13, 17, 22

alt, presence of alternative splicing(s)

Table 1.3 – Classification of all human chemokine receptors. B, lymphocyte B; Ba, basophil; DC, dendritic cell; Eo, eosinophil; En, endothelium; Ery, erythrocyt; Fib, fibroblast; IgA-Pla, IgA+ plasmacell; M, monocyt/macrophage; N, neutrophil; NK, natural killer cell; P, platelet T, lymphocyte T; Tc1, type 1 killer T cell; Th1/2, type 1/2 helper T cell; Thy, thymocyte

on Chemotactic Cytokines" in 1996, divided into four families: CC chemokine receptors, CXC chemokine receptors, one C chemokine receptor and one CX3C chemokine receptor that correspond to the four distinct subfamilies of chemokines they bind – **Table 1.3, p. 14**. In addition, chemokine receptors with structural features that are inconsistent with a signaling function have been described. When ligated, these "silent" (*i.e.*, non-signaling) chemokine receptors do not elicit migration or conventional signaling responses, but instead damped the immune response by binding, internalizing, and, in the case of D6, degrading chemokines – **Table 1.3, p. 14**. Chemokine decoy receptors recognize distinct and complementary sets of ligands and are strategically expressed in different cellular contexts. The ability of chemokine receptors to signal upon ligand binding is due, at least in part,

to the presence of a DRY (glutamic acid/aspartic acid-arginine-tyrosine) motif in the second intracellular loop, which is missing in the scavenger receptors [58]. No chemokine receptor structures have been solved to date, and models are generally based on bovine rhodopsin, the only seven-transmembrane receptor for which three-dimensional structures have been solved.

Chemokine binding on leukocytes leads to the rapid up-regulation of adhesion molecules: integrins like very late antigen-4 (VLA-4) (or $\alpha 4\beta 1$), which are needed to bind more strongly epithelial cells or intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) (both overexpressed in response to proinflammatory cytokines like IL-1, TNF-α or IFN-γ). Lastly, chemokine binding induced reorganization of the cytoskeletal, which finally leads to the cell polarization, migration and infiltration of activated leukocytes.

1.4 Goals of this work

1.4.1 Epidemiology of gynecological tumors

Breast cancer is a heterogeneous disease embracing a range of biologic behaviors and prognostic characteristics [59]. Worldwide, breast cancer is the most common invasive cancer in women, representing 23% of all cancers diagnosed in women in 2008 [60]. The same year, breast cancer resulted in 1.38 million diagnosed cases and 458,000 deaths [60]. During the last decades, early diagnosis and multiple therapies helped to improve survival rate. Survival rates in the developed world are high, between 80% and 90% of the women being alive 5 years after the first diagnosis [59, 60]. But since the 1970s, the number of case has significantly increased, possibly due to modern living style, better diagnostic or longer lifetime. In addition, the situation in the developing countries remains problematic [61].

Among gynecological tumors, ovarian cancer has the highest mortality rate. Worldwide, over 200,000 women are diagnosed with primary ovarian cancer every year [62]. These cancers are mostly diagnosed at a late stage, over 75% of patients are already presenting metastasis at first diagnosis. Overall five-year survival rate ranges between devastating 25% and 49% [63]. Although standard therapy initially leads to good response rates, the disease recurs in over 50% of the cases within the following five years [64]. The rate of ovarian cancer between 1993 and 2008 decreased in women of the 40-49 age cohort and in the 50-64 age cohort, possibly due to this group's widespread adoption of oral contraceptives [62].

1.4.2 Current problematic

Current therapeutics often fail to eliminate all tumor cells and tumors with reduced immunogenicity may escape after a period of equilibrium due to a process called immunoediting [31, 65]. This concept holds that the immune system not only protects the host against development of primary nonviral cancers but also sculpts tu-

mor immunogenicity [66]. Limitations of current therapies have led to increasing enthusiasm for defining new prognostic tools and highly targeted therapies.

1.4.3 Working hypothesis

During the last decade, three chemokine receptors (*i.e.*, CCR4, CCR8, and CCR10) were found to be specifically expressed on Treg. With their respective associated chemokines, they all play a pivotal role in Treg migration. CCR4 and CCR8 were the first receptors to be described on Treg [67]. Together with CCL22, the ligand for CCR4, they lead to Treg migration into tumors as described in ovarian cancer [15]. CXCR4 and CCR7 were also found expressed on Treg, although not specifically [44, 45]. Bone marrow strongly expresses CXCL12, the ligand for CXCR4, leading to human Treg traffic and accumulation. The CCR7 pathway is very important for the Treg migration into lymph nodes and the acquisition of their full suppressive functions. More recently CCR10 was found overexpressed on Treg [16]. In a tumors hypoxia context, it was shown that CCL28 promotes tumor tolerance in a Treg dependent mechanism.

1.4.4 Purpose of this work

Although previous studies associated increased intratumoral number of $CD4^+$ $CD25^+$ Treg with CCL22 and CCL28 and reported adversely affected prognosis in ovarian cancer, before our current work the clinical importance of other chemokines was unknown [15, 16]. Since several other chemokines were described to be potentially responsible for Treg accumulation in tumors and therefore, represent potential targets for cancer therapy, we aimed to investigate whether other chemokines could lead to specific migration of Treg. The purpose of this work was to assess the clinical significance of different Treg attracting chemokine intratumoral expressions in breast cancer and ovarian cancer patients with long-term follow-up. Thus, we here investigated the prespecified hypothesis that the expression of Treg specific chemokines in cancer tissue predicts the OS and could therefore be used as biomarkers or as targets for immunotherapy.

*
* *

Materials and Methods 2

— Anything found to be true of E. coli must also be true for elephants.

Jacques Monod, molecular biologist, 1954

2.1 Lymphocyte acquisition and manipulation

2.1.1 Peripheral blood mononuclear cell isolation

HUMAN peripheral blood mononuclear cells (PBMC) were purified from healthy donor peripheral blood by Biocoll (Merck, Darmstadt, Germany) density gradient centrifugation. All samples were collected in the laboratory and were extemporaneously performed. Briefly, peripheral blood was two times diluted in phosphate-buffered saline (PBS) (GE Healthcare, Munich, Germany) and 30 mL were then carefully set down on 15 mL Biocoll solution in a 50 mL tube. Tubes were centrifuged at 1000 g, 4°C for 20 minutes, without centrifugal brake. The formed PBMC ring was quickly removed in order to minimize interaction with Biocoll and cells were washed three times in PBS.

2.1.2 Lymphocyte maintenance and culture

Cells were then maintained in Roswell Park Memorial Institute medium (RPMI) 1640 (Merck, Darmstadt, Germany) with 10% heat-inactivated fetal calf serum, 2 mmolar (M) L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 100 international unit (IU)/mL penicillin, 100 mg/mL streptomycin and 0.05 mM β-mercaptoethanol supplemented with 10 ng/mL IL-2 (*i.e.*, 180 IU/mL) (all from Life Technologies, Paisley, United Kingdom). PBMC were set up for migration assays as soon as possible and were never kept in RPMI more than 12 hours.

2.1.3 Migration assay

Chemotaxis assays were performed using 3 μm pore polycarbonate filters in a 96-well transwell chamber (Corning, New York, USA). Total PBMC (10^6 cells/well), after being washed three times with PBS, were added in migration medium (RPMI 1640 supplemented with 0.5% bovine serum albumin and 10 ng/mL IL-2) on the top chamber. Different concentrations of chemokines (all from Peprotech, Hamburg, Germany) were added in the same medium supplemented on IL-2 to the lower well. All experiments were made at least in triplicate and migration occurred during three hours at 37°C and 5% CO_2. Cells migrated into the lower chamber were then washed and labeled with antibodies before being analyzed by flow cytometry.

Total migrated cells were counted using CountBright absolute counting beads (Life Technologies, Paisley, United Kingdom). Migration results are shown as chemotaxis index (CI), which represents the total number of migrated cells in response to a chemokine divided by the total number of migrated cells without chemokine. The enrichment factor (EF) was obtained dividing the initial percentage of cells by the final percentage of cells and adding 1.

2.1.4 Flow cytometry analysis

PBMC migration was analyzed using the BD FACS Canto II cytometer (BD Biosciences, Heidelberg, Germany). Antibodies (anti-CD4 PacBlue, anti-CD25 APC and anti-CD127 PE, all from Biolegend, San Diego, USA) were added to the different cell populations and incubated for 20 minutes at 4°C in the dark, before being washed with PBS and analyzed. Gates were set according to side scatter and forward scatter on the lymphocyte population before gating on $CD4^+$ $CD25^{high}$ $CD127^{low}$ cells – **Figure 2.1, p. 19**. $CD4^+$ $CD25^{high}$ $CD127^{low}$ labeling shows 90% correlation with FoxP3 expression [35]. The percentage of positive cells of each individual marker were calculated using FlowJo (FlowJo, Ashland, USA).

2.2 Chemokine receptor mRNA level expressions

2.2.1 Cell sorting

Isolation of the $CD4^+$ $CD25^{high}$ $CD127^{low}$ population was performed in a two-steps procedure directly from the freshly isolated PBMC using a $CD4^+$ $CD25^{high}$ $CD127^{low}$ Treg isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The non-$CD4^+$ $CD127^{high}$ cells were first magnetically labeled with a cocktail of biotin-conjugated antibodies and secondary anti-biotin monoclonal antibodies. Labeled cells were depleted from the total population by separation over a MACS LD column placed in the magnetic field of a MACS separator.

During the second step, the $CD4^+$ $CD25^{high}$ $CD127^{low}$ Treg were directly labeled among the $CD4^+$ $CD127^{low}$ enriched population with $CD25^{high}$ micro-beads

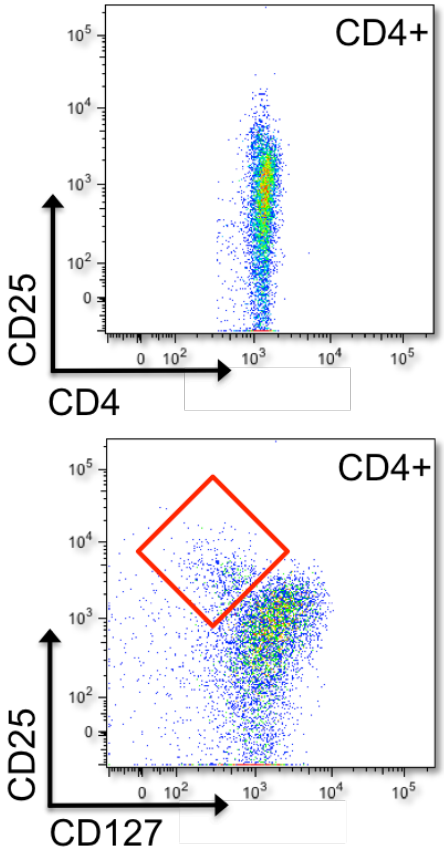

FIGURE 2.1 – *Gates were set according to side scatter and forward scatter on the lymphocyte population before gating on CD4⁺ CD25^{high} CD127^{low} cells (red square).*

and isolated by positive selection from the pre-enriched fraction by separation over a MACS MS column placed in the magnetic field of a MACS separator. During the separation, cells were kept cold, and all solutions were pre-cooled in order to prevent capping of antibodies on the cell surface and non-specific labeling. Cells were re-suspended in a buffer made from PBS, 0,5% bovine serum albumin (BSA) and 2mM ethylenediaminetetraacetic acid (EDTA). Antibodies were added as recommended by the fabricant and incubated 15 minutes at 4°C in the dark. Cells were three times washed before magnetic separation and then rapidly further performed.

2.2.2 mRNA isolation

After isolation cells were washed three times with pre-cooled PBS. Total cellular ribonucleic acid (RNA) was extracted from the different cell populations using the QuickGene RNA blood cell kit S and the QuickGene mini 80 automat (Fuji, Tokyo, Japan). Briefly, leukocyte pellets were pooled down in 1.5 mL tubes, resuspended in 520 μL of LRB (after reconstitution with adequate volume of β-mercaptoethanol) and thoroughly mixed by vortexing for 30 secondes. Finally, 250 μL of 99% ethanol

Receptor	Forward	Reverse	Sonde
CCR1	5'-TCTGACTCTTGGCACAGCAT-3'	5'-GCCACCATTACATTCCCTCTC-3'	#76
CCR2	5'-TGAGACAAGCCACAAGCTGA-3'	5'-TTCTGATAAACCGAGAACGAGAT-3'	#56
CCR3	5'-TGCAGTGCTCTTTACCCAGA-3'	5'-GGTCATTCTCAGAGTGTGGAAA-3'	#78
CCR4	5'-TTCGTGTTTTCCCTCCCTTT-3'	5'-ACCTAGCCCAAAAACCCACT-3'	#84
CCR5	5'-AACCAGGCGAGAGACTTCTCA-3'	5'-GATCCAACTCAAATTCCTTCTCA-3'	#14
CCR6	5'-AAAGGCATCTATGCCATCAACT-3'	5'-GTACCGGTCCATGCTAATGC-3'	#68
CCR7	5'-GGGGAAACCAATGAAAAGC-3'	5'-ACCTCATCTTGACACAGGCATA-3'	#77
CCR8	5'-TGCCTCCTGTTTGTATTCAGTCT-3'	5'-CAGACCACAAGGACCAGGAT-3'	#64
CCR9	5'-CACCATGACACCCACAGACT-3'	5'-TCACAGTAGAAGTCAGTGAAGTTGAA-3'	#21
CCR10	5'-AGTAGGTGGGGGAACACTGA-3'	5'-GCAAGGCACAGAGGTAGTCC-3'	#34
CCR11	5'-TGCAAATGAGAAAAACACTAAGGT-3'	5'-TGGCAAAAACAAGCTTGAAA-3'	#32

Table 2.1 – Primers used for the qPCR analysis. All primers were designed using the Roche assay design center – #number corresponds to probe from the Universal Probe Library.

was added to each tube. Tubes were thoroughly mixed by vortexing for 30 secondes and kept at 25°C for 5 minutes. After a flash spin-down, whole lysate were transferred to the cartridge and performed for one minute under pressure. Cartridges were washed three times with 750 μL WRB solution (after reconstitution with adequate volume of ethanol). Elution was performed in RNAse-free water for one minute under pressure after waiting for 30 secondes at 25°C. Total RNA concentration and purity was determined with a Nanodrop (Thermo Fisher Scientific, Waltham, USA). 260 nm absorption was compared with 280 nm absorption and the ratio optical density (OD)260 nm/OD280 nm was routinely $\geq$2. If not immediately used, total RNA was stored at -20°C.

2.2.3 cDNA transcription

complementary DNA (cDNA) from total mRNA was synthetized using the Fermentas RT-kit and oligo(dT) primers (Fermentas, Waltham, USA). Briefly, up to 1 μg of total RNA was mixed with 2 μL 10X reaction buffer, 1 μL oligo(dT) primers, 2 μL 10 nM dNTP mix and completed to 20 μL with nuclease-free water in polymerase chain reaction (PCR) tubes. Oligo(dT) primes cDNA synthesis from the poly(A) tail present at the 3'-end of eukaryotic mRNA and thus allow to obtain a copy from all mRNA previously isolated. PCR tubes were placed in a thermal cycler and warmed at 37°C for 30 minutes to allow the transcription and 10 minutes at 72°C to terminate every reaction before cooling down to 4°C. Total cDNA concentration and purity was determined with a Nanodrop (Thermo Fisher Scientific, Waltham, USA). 260 nm absorption was compared with 280 nm absorption and the ratio OD260 nm/OD280 nm was routinely $\geq$1.8. If not immediately used, total cDNA was stored at -20°C.

2.2.4 RT-qPCR analysis

The following synthetic primers (all Eurofins Scientific, Luxembourg, Luxembourg) were used for the real-time PCR (qPCR) (designed using the Roche assay design center – #number correspond to probe from the Universal ProbeLibrary) – **Table 2.1, p. 20**. The qPCR reactions were performed in optical 96-well LightCyler 480 Multiwell Plate

using the LightCylcer 480 (Roche, Basel, Switzerland). Reaction were performed in the following mix (per well): water 3 μL, sonde 0.2 μL, primers 0.4 μL + 0.4 μL (forward and reverse), master mix 5 μL (VWR International, Radnor, USA) and cDNA 2 μL. Quantity values generated for gene expression were obtained by comparison of the fluorescence generated by each sample with hypoxanthine-guanine phosphoribosyltransferase (HPRT) standard curves. As control of Treg purity, the level of FoxP3 and CD127 mRNA was quantified in isolated cell populations. As control of RNA isolation purity, qPCR were performed with 2 μL from isolated RNA solutions.

2.3 Histopathology

2.3.1 Breast cancer patient recruitment

Microarrayed tissue samples from breast cancers were used in this retrospective study. Samples were collected from patients undergoing surgery at the Gynecology unit (*Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe*, *Ludwig-Maximilians-Universität*, Munich).

All cases had been previously characterized in terms of histology (nodal status, tumor size, grade, estrogen receptor (ER)-α/progesteron receptor (PgR)-α/human epidermal growth factor receptor 2 (Her2) status at the Instituty of Pathology, *Ludwig-Maximilians-Universität*, Munich. The histological classification was determined according to a modification of the Elston and Ellis grading proposed by Bloom and Richardson (Elston and Ellis, 1991). The hormone receptor status was evaluated by immunohistochemistry. The tumor was classified as hormone receptor positive in case of positive staining in $\geq$ 10% of the tumor. Normal breast tissue was obtained from women undergoing breast reduction surgery (n= 7). Criteria for the selection of patients for the tissue microarray were adequate routinely fixed biopsy material, no history of previous malignancy but history of distant metastases during the course of the disease.

A total of 180 ductal tumors, 14 lobular tumors and 5 others were used for this study. No preselection of patients was made. All available patients suitable for the inclusion were selected. Patients were not matched for stage disease or age. Patients were diagnosed from 1986 to 2007 and almost all underwent surgery within one month (max 7 months). All patients received standards surgical treatment of either mastectomy or wide local excision with radiotherapy. Systemic adjuvant treatments were given based on clinical scores and hormone receptor status. The end of the follow-up period was October 2014. Additional patient information is listed in – **Table 3.3, 3.4, and 3.5, pp. 37, 38, 39**. Of the 199 tumors on the array, 192 presented an assessable FoxP3 staining, 193 an assessable CCL1 staining, 194 an assessable CCL22 straining, and 192 an assessable CCL27 staining.

All materials was sampled for diagnostic purposes and research was done in accordance with the legal requirements concerning confidential medical commu-

nication as well as the data protection act. Consequently, consulting the ethics committee of the medical school, *Ludwig-Maximilians-Universität*, Munich, and written informed consent from the patients prior to participation in the study was not required.

2.3.2 Ovarian cancer patient recruitment

203 tumor tissue samples were selected from the archives of the Institute of Pathology of the *Ludwig-Maximilians-Universität*, Munich for this study. All patients had undergone debulking surgery at the Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe, Munich, and had been previously characterized in terms of histology at the Institute of Pathology of the Ludwig-Maximilian-Universität, Munich.

The patients were between 27 and 87 years of age at diagnosis, with a median age of 60. tumors were graded according to the Silverberg grading system. Most tumors were graded class 3+4 (62.2%) and only very few class 1 (2.96%). Only samples classified as stage III cancers, according to the International Federation of Gynecology and Obstetrics (FIGO), were included. All histological types of epithelial ovarian carcinoma were selected, but the serous (77.1%), endometroid (8.1%) and adenocarcinoma NOS (5.3%) were by far the most commonly represented types. Selection criteria of patients for the inclusion in this study were previous inclusion and remain of adequate routinely fixed biopsy material (203 of the total 210 originally included patients) [68]. Additional patient information is listed in – **Table 3.1 and 3.2, pp. 33, 34**. Of the 203 tumors, 203 presented an assessable CCL1 staining.

All materials was sampled for diagnostic purposes and research was done in accordance with the legal requirements concerning confidential medical communication as well as the data protection act. Consequently, consulting the ethics committee of the medical school, *Ludwig-Maximilians-Universität*, Munich, and written informed consent from the patients prior to participation in the study was not required.

2.3.3 Immunohistochemistry

Paraffin wax-embedded tissue slide samples were deparaffinized in xylol for 20 minutes, washed in 100% ethanol and then incubated in methanol/H_2O_2 (3%) for 20 minutes. After rehydration in an alcohol gradient to distilled water, the slides were placed in a pressure cooker containing sodium citrate buffer (pH= 6.0) and cooked for 5 minutes. Slides were washed twice in PBS and blocked using blocking solution 1 from ZytoChem Plus horseradish peroxidase (HRP) Polymer Kit (Zytomed, Berlin, Germany) for 5 minutes. Each slide was separately incubated with a polyclonal rabbit anti-human CCL1 primary antibody (Atlas antibodies, Stockholm, Sweden) diluted 1/200 in PBS, a polyclonal rabbit anti-human CCL22 primary antibody (Peprotech, Hamburg, Germany) diluted 1/200 in PBS, a polyclonal rabbit anti-human CCL27 primary antibody (Atlas antibodies, Stockholm, Sweden) diluted

1/200 in PBS or a monoclonal mouse anti-human FoxP3 primary antibody (clone 236A/E7) (Abcam, Cambridge, USA) diluted 1/300 in PBS. Incubation of the sections with the primary antibodies lasted for 16 h at 4°C. Afterward, sections were washed twice in PBS before incubation with postblock reagent 2 (Zytomed, Berlin, Germany) for 20 minutes. The slides were then washed in PBS and then incubated with the HRP-polymer 3, containing secondary antibody coupled with detection enzyme (Zytomed, Berlin, Germany) for 30 minutes. Staining was performed using 3,3-diaminobenzidine (DAB)-substrate solution (Dako, Glostrup, Denmark) for 180 seconds. Counterstaining was carried out with Mayer's hemalaun for 2 minutes. Finally, sections were washed in tap water for 5 minutes and afterwards dehydrated in an ascending alcohol serie and washed in xylol. Slides were cover-slipped with Eukittquick-hardening mounting medium (Sigma-Aldrich, St. Louis, USA).

2.3.4 Quantification of immunohistochemistry

For each labelling, absolute numbers of positive cells were computer-aided determined. Without any knowledge of identity, pictures were systematically taken (arrays contained duplicate cores) and positive cells were automatically counted using the image J software (Version 1.49o, National Institutes of Health, Bethesda, USA). DAB labelling was extracted using the IHC Toolbox plugin and cells were then defined and counted based on their size and morphology – **Figure 2.2, p. 24**.

2.4 Statistical analysis

2.4.1 Statistical program

The statistical program R (Version 0.99.441, RStudio, Inc., Boston, USA) was used for data collection and processing as well as analysis of statistical data. Following packages were used:

- U. Ligges and M. Mächler (2003). Scatterplot3d - an R Package for Visualizing Multivariate Data. Journal of Statistical Software 8(11), 1-20. http://www.jstatsoft.org.
- R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.
- T. Therneau (2015). A Package for Survival Analysis in S. version 2.38. http://CRAN.R-project.org/package=survival.
- H. Wickham (2009). ggplot2: elegant graphics for data analysis. http://had.co.nz/ggplot2/book.

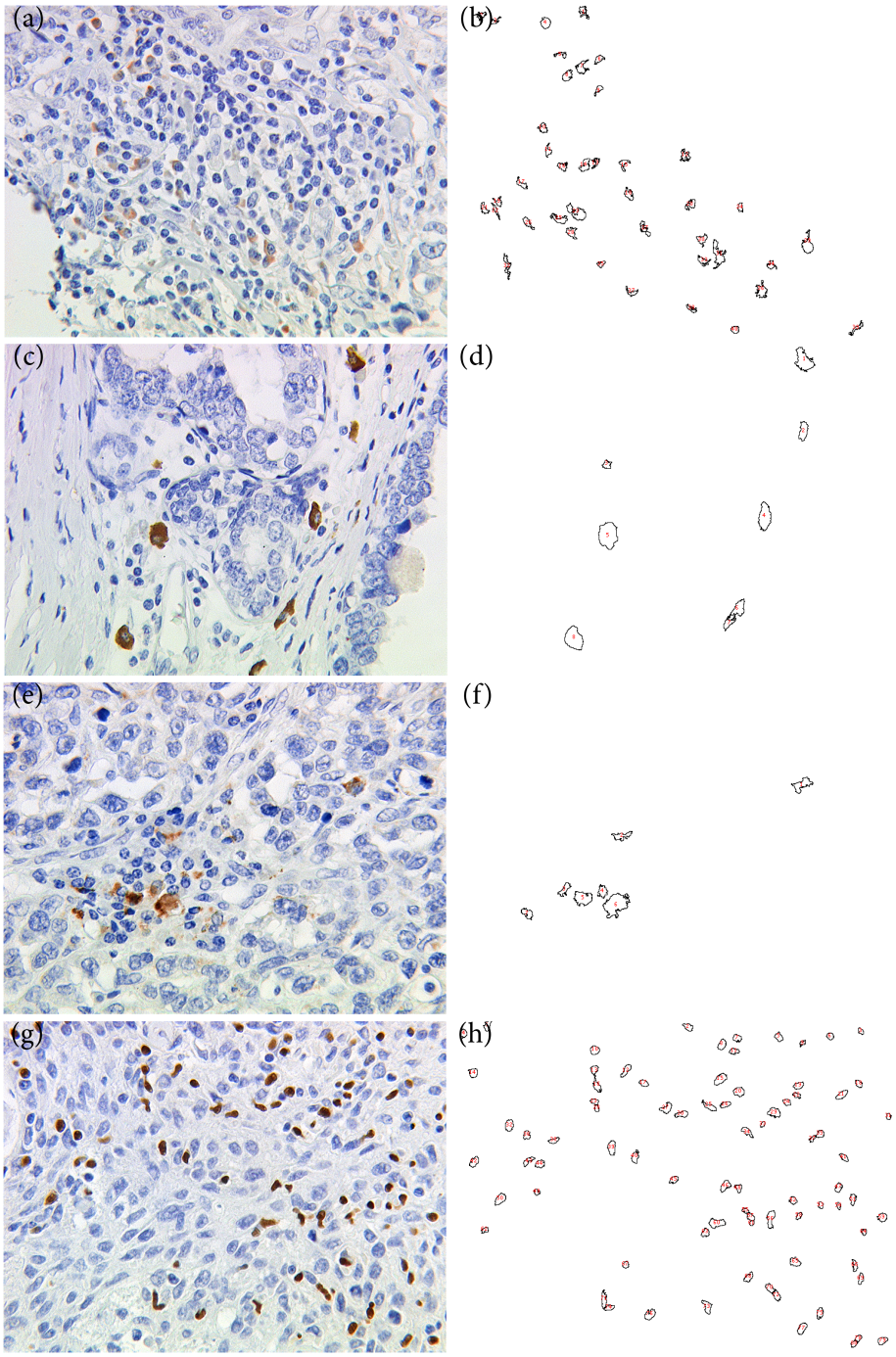

Figure 2.2 – *Representative examples of computer-aided determined labelings. (a-b) Whole breast cancer tissue section with CCL1 recognition. (c-d) Whole breast cancer tissue section with CCL22 recognition. (e-f) Whole breast cancer tissue section with CCL27 recognition. (g-h) Whole breast cancer tissue section with FoxP3 recognition. All magnifications* $\times$ *200.*

2.4.2 Statistical tests

The Kruskal-Wallis test was used to compare more than two independent groups, and the Mann-Whitney U-test was used for evaluation of two independent groups. These tests are one-way analysis of variance and analyze two or more samples which are independent from each other. Contingency tables were analyzed using the Pearson's χ^2 test. Linear regression were realize using the Pearson's product-moment correlation or Spearman's R_s based on the continuity of analyzed values. The choice between interquartile range (IQR) or standard error of the mean (SEM) is detailed in the relevant figure legends. Patients included in the statistical models are those for which all the necessary data was available. A small number of patients did not have complete data. Values with $p \leq 0.05$ were considered statistically significant.

2.4.3 Survival analysis

Given that there is no clinical defined cutoff point for the number of chemokine-expressing cells in such a context, we always selected a cutoff at the median values because this divided the patients into equal-sized groups, and does not make the assumption of an artificial cutoff for statistical analyses. We systematically analysed whether there was any correlation between cell numbers and age, nodal status, tumor size, tumor rest, grade, ER-α/PgR-α/Her2 status, adjuvant therapy, and OS. Survival was measured from the date of diagnosis to the time of death or the time the patients was last seen. The log-rank test was used to perform univariate analyses and the survival curves were estimated by the Kaplan-Meier (KM) method. Prognostic factors for survival were evaluated in multivariate analyses by Cox proportional hazards regression. The statistical tests are detailed in the relevant figure legends.

2.4.4 Kaplan-Meier curves with ggplot2

Unfortunately, the ggplot2 package does not accept a `survfit` object for representation of KM curves. In order to plot the curves with this package, following code was modified and used in this work:

```
                ####################################
                ##### Plotting KM using ggplot2 #####
                ####################################

 # Define function
ggkm <- function(sfit, returns = FALSE,
                 xlabs = "Time after Surgery (Months)", ylabs = "
                     Proportion Surviving",
                 ystratalabs = NULL, ystrataname = NULL,
                 timeby = 50, main = "Overall Survival",
                 pval = TRUE, ...) {

 # Arguments and theme
```

```
  if(is.null(ystratalabs)) {
    ystratalabs <- as.character(levels(summary(sfit)$strata))
  }
  m <- max(nchar(ystratalabs))
  if(is.null(ystrataname)) ystrataname <- ""
  times <- seq(0, max(sfit$time), by = timeby)
  .df <- data.frame(time = sfit$time, n.risk = sfit$n.risk, n.event =
      sfit$n.event, surv = sfit$surv, strata = summary(sfit, censored =
      T)$strata, upper = sfit$upper, lower = sfit$lower)
  levels(.df$strata) <- ystratalabs
  zeros <- data.frame(time = 0, surv = 1, strata = factor(ystratalabs,
      levels=levels(.df$strata)), upper = 1, lower = 1)
  .df <- rbind.fill(zeros, .df)
  d <- length(levels(.df$strata))
  p <- ggplot(.df, aes(time, surv, group = strata)) +
    geom_step(aes(colour = strata), size = .7) +
    scale_colour_manual(values = c("darkred", "black")) +
    theme_minimal() +
    theme(axis.title.x = element_text(vjust = .5)) +
    scale_x_continuous(xlabs, breaks = times, limits = c(0, max(sfit$
        time))) +
    scale_y_continuous(ylabs, limits = c(0, 1)) +
    theme(panel.grid.minor = element_blank()) +
    theme(legend.position = c( .83, .86)) +
    theme(legend.key = element_rect(colour = NA)) +
    theme(legend.title = element_text(colour = "white")) +
    labs(linetype = ystrataname) +
    theme(plot.margin = unit(c(0, 1, .5, ifelse(m < 10, 1.5, 2.5)), "
        lines")) +
    ggtitle(main)

 # Plot the curves
  print(p)
  if(returns) return(p)

}
```

Listing – Code adjustments made to plot KM curves using the ggplot2 package in R. Takes a `survfit` *object as argument.*

*
* *

Results 3

— In God we trust, all others bring data.

William E. Deming, engineer and statistician, date uncertain

3.1 Chemokine screening

3.1.1 Screening among the CC-chemokine family

Knowing that the mechanism of immunosuppression requires Treg to have close contact with target cells and that Treg use specific chemokine receptors to localize into tumors, we decided to study the clinical significance of the different Treg attracting chemokines. During the last decade, several chemokines were described to specifically attract Treg. But to our knowledge, no screening of an entire chemokine family was ever done. We therefore analyzed the migration of $CD4^+$ T cell populations in response to a panel of all CC chemokines – **Figure 3.1, p. 28**. Analysis of the CI after migration in response to a single dose of 250 ng/mL revealed that 13 out of 25 chemokines (*i.e.*, CCL1, 2, 3, 5, 7, 8, 17, 19, 20, 21, 22, 23, and CCL27) significantly attract $CD4^+$ T cells. Notably, CCL19 induced such a strong migration (median CI, 5.88 and 14.14, Treg and conventional T cells (Tcon), respectively), that it only partially appears on the top of the **Figure 3.1, p. 28** for the sake of visibility. Other chemokines seem, at least in our settings and for the 250 ng/mL tested dose, not to induce migration of the $CD4^+$ T cells.

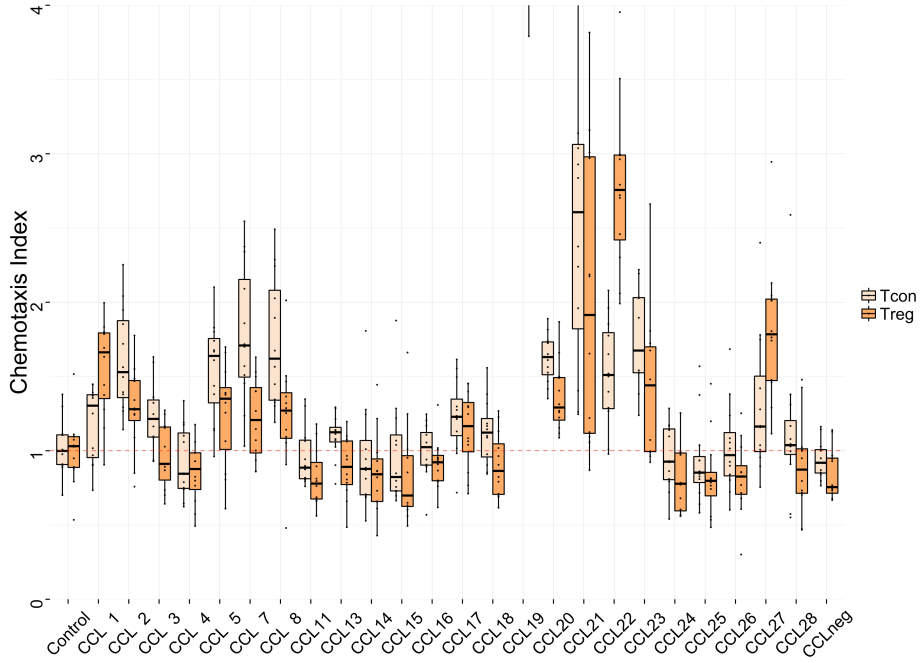

Figure 3.1 – *Chemotactic response profile of human CD4⁺ CD25^{low} Tcon and CD4⁺ CD25^{high} CD127^{low} Treg in response to all CC-chemokines (250 ng/mL). Median and IQR of four experiments performed in triplicate. Red dotted bar set at 1, no migration.*

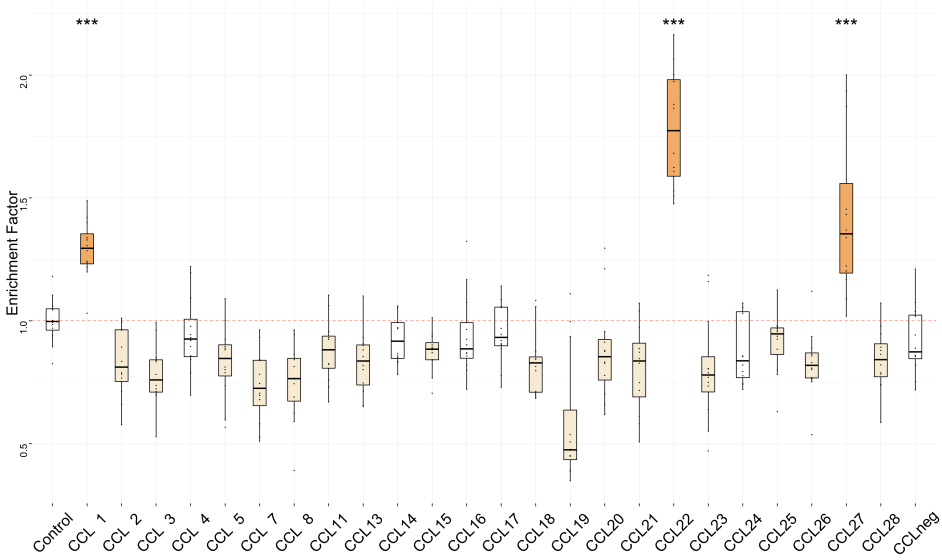

Figure 3.2 – *Enrichment after migration of human CD4⁺ CD25^{high} CD127^{low} Treg among the CD4⁺ T cell population in response to all CC chemokines (250 ng/mL). Light orange, significantly more Tcon after migration; dark orange, significantly more Treg after migration; white, no change. Median and IQR of four experiments performed in triplicate. Mann–Whitney U test. ***P< 0.001. Red dotted bar set at 1, no enrichment.*

3.1.2 CCL1, CCL22 and CCL27 specifically attract regulatory T cells

We reasoned that Treg could use particular chemokine receptors to specifically accumulate in the tumors. Work done previously on Treg migration described preferential or specific migration in response to chemokines binding on specific receptors. To determine the migration specificity, we calculate the EF among the $CD4^+$ T cell population for the same migration – **Figure 3.2, p. 28**. EF was calculated based on Treg ratio before and after migration for all chemokines and set at 1 for no migration. As it clearly appears, only CCL1, CCL22 and CCL27 were able to significantly cause a Treg enrichment after migration in response to a single dose of 250 ng/mL. Most other chemokines caused impoverishment of the Treg population (*i.e.*, CCL2, 3, 5, 7, 8, 11, 13, 15, 18, 19, 20, 21, 22, 23, 25, 26, and CCL28). A few chemokines, mostly those not inducing any significant migration, do not cause neither enrichment nor impoverishment among the $CD4^+$ T cell populations.

3.1.3 Internal control and validation of our targets

We also analyzed all chemokine receptor mRNA expression levels and compare Tcon and Treg expression levels. To determine cell population differential expressions, we normalized our data against chemokine receptor expression levels in the $CD4^-$ population – **Figure 3.4, p. 30**. Importantly, CD127 mRNA level was higher in the Tcon population, whereas FoxP3 mRNA level was higher in the Treg population. As expected, significantly higher mRNA expression levels for chemokine receptors binding to CCL1, CCL22 and CCL27 (*i.e.*, CCR4, CCR8 and CCR10) were observed among the isolated Treg population.

Finally, to support the idea that these chemokines were indeed responsible for the observed cell migrations and to control the quality of our migration assays, we took advantage of the shortened version of CCL22 (67 amino acid instead of 69 amino acid), which binds to its receptor CCR4, but does not induce migration. Consistent with this the shortened version of CCL22, noted CCLneg on the **figure 3.1, p. 28** and the **figure 3.2, p. 28** induced neither significant migration nor significant enrichment of cells among the $CD4^+$ T cell population. Migrations in response to CCL1, CCL22 and CCL27 were reproduced using the suboptimal dose of 50 ng/mL and a dose of 500 ng/mL – **Figure 3.3, p. 30**. We observed the typical dose/response effect for all three chemokines. CCL1 and CCL22 are causing much more chemotaxis than CCL27. But CCL27 leads to a very stable migration, not decreased by the chemokine receptor desensibilisation process, even for the highest dose of 500 ng/mL.

Both migration and enrichment are required for Treg accumulation, like in a tumor microenvironment. Overall, our results suggest that CCL1, CCL22 and CCL27 might be the best candidates.

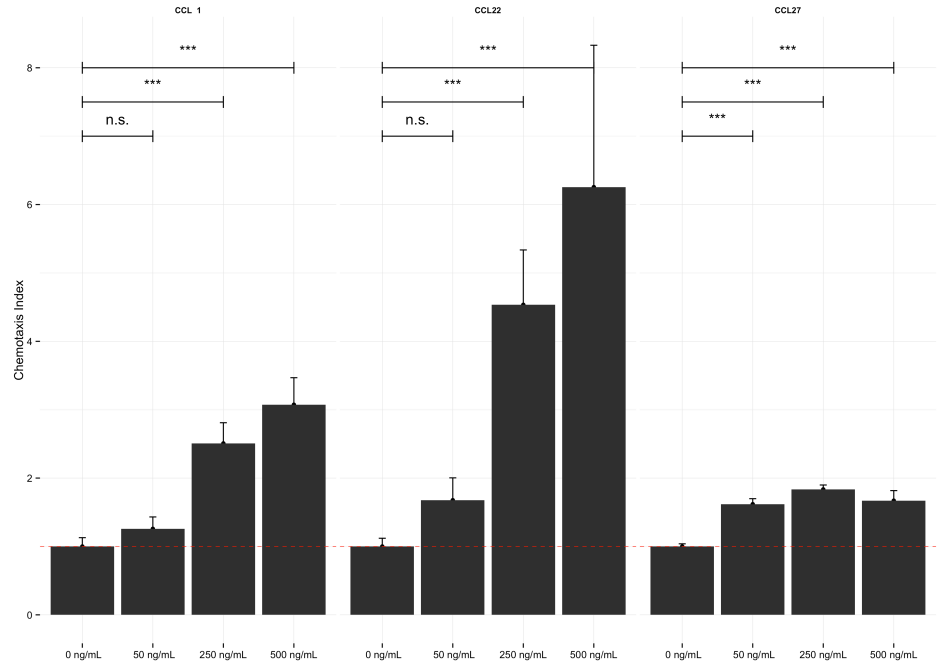

Figure 3.3 – *Chemotactic responses of Treg to CCL1, CCL22 and CCL27, respectively. Mean and SEM of three experiments performed in triplicate. Mann-Whitney U test. ***P< 0.001. Red dotted bar set at 1, no migration.*

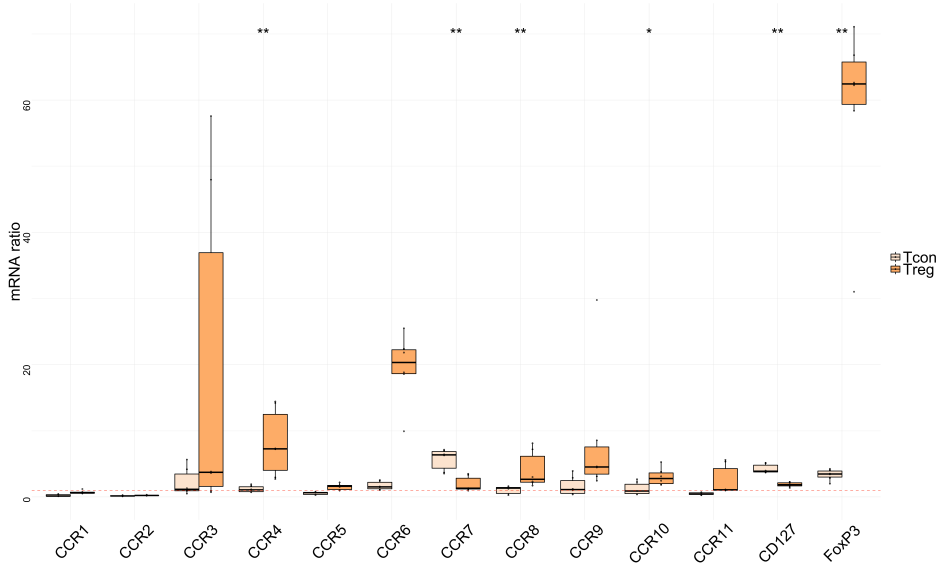

Figure 3.4 – *CC Chemokine receptor differential mRNA expression levels. All values were normalized against chemokine receptor expression levels in the CD4⁻ population. Median and IQR of three experiments performed in dupicate. Mann-Whitney U test. *P< 0.05; **P< 0.005. Red dotted bar set at 1, level in the CD4⁻ population.*

3.2 Expression of chemokines in ovarian cancer

3.2.1 First description of CCL1 expression in ovarian cancer

As can be seen in representative photomicrographs, ovarian cancer tumor sections expressed CCL1 – **Figure 3.5, p. 31**. Assessment of all tissue microarray sections showed a high degree of variability in chemokine-expressing cell total numbers. CCL1 was expressed intratumorally by tumor cells (median, 150 cells/mm^2; range, 0 to 2064) and peritumorally by immune cells (median, 169 cells/mm^2; range, 0 to 1900). In both cases expression in normal ovarian tissue was significantly lower than in tumor tissues (p= 0.028 and p= 0.024 intratumorally and peritumorally, respectively) – **Figure 3.6, p. 32**. Although there was a degree of variability in intra- and peritumoral CCL1-expressing cell total numbers, both expressions showed a correlation in ovarian cancer (p= 2.38 10^{-07}, ρ= 0.353, Pearson's product-moment correlation) – **Figure 3.7, p. 32**.

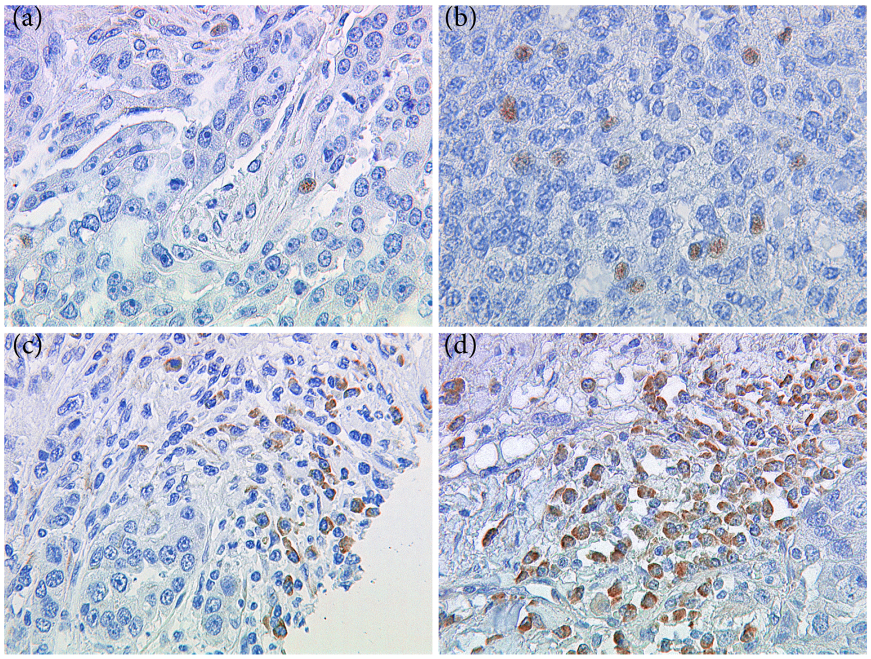

Figure 3.5 – *Representative examples of CCL1 immuno-staining in ovarian cancer. (a) Whole ovarian cancer tissue section with few CCL1-expressing tumor cells. (b) Whole ovarian cancer tissue section with numerous CCL1-expressing tumor cells. (c) Whole ovarian cancer tissue section with few CCL1-expressing immune cells. (d) Whole ovarian cancer tissue section with numerous CCL1-expressing immune cells. (a-b-c-d) magnification × 200.*

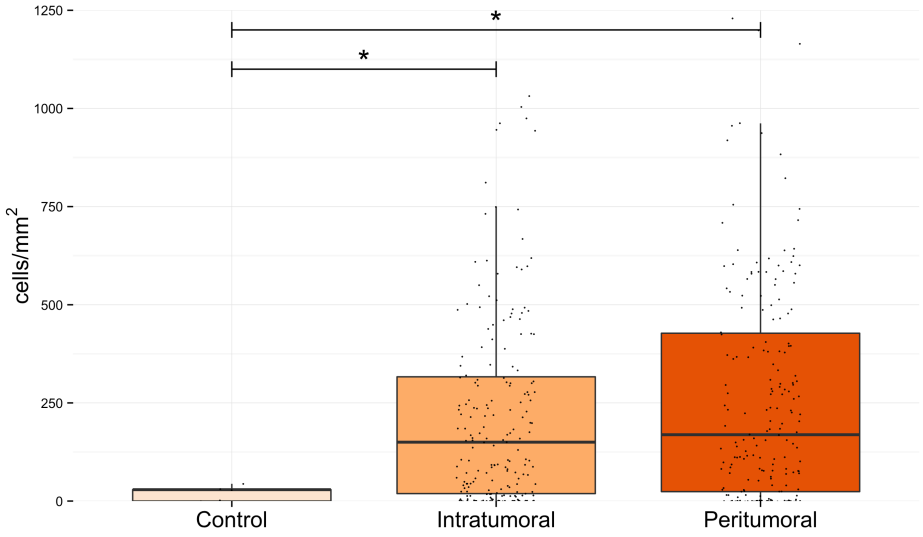

Figure 3.6 – *Total number of CCL1-expressing cells in normal ovarian tissue compared to ovarian tumor tissues. Median and IQR of CCL1 cell numbers. Mann-Whitney U test. *P< 0.05.*

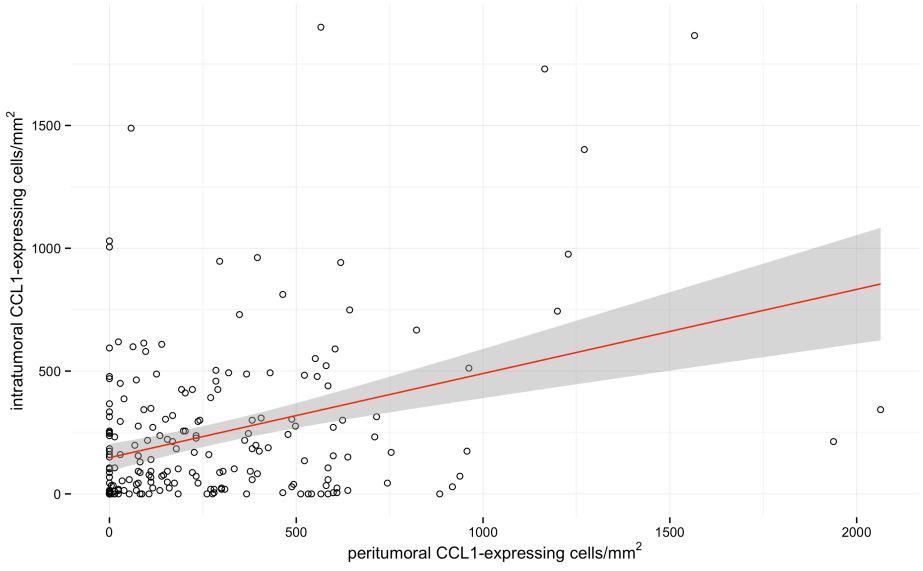

Figure 3.7 – *Linear regression representing the relation between intra- and peritumoral CCL1 expression in ovarian tumor tissues. Linear regression line in red with the 95% confidence region in light grey.*

Variable	CCL1⁺ ≤ 150† No.	%	CCL1⁺ > 150† No.	%	Significance
Total No. of patients	100	49	103	51	
Age, years					p= 0.532
≤ 60	49	24	56	28	
> 60	51	25	47	23	
Histological subtype					p= 0.359
Serous	77	39	81	40	R_s= 0.065
Mucinous	2	1	0	0	
Endometroid	10	5	6	3	
Adenocarcinoma (NOS)	5	2	5	2	
Undifferentiated	4	2	3	2	
Unknown	5	2	5	2	
Grade					p= 0.372
I	4	2	2	1	R_s= -0.064
II	28	14	39	19	
III & IV	64	31	60	30	
Unknown	4	2	2	1	
Optimal surgical debulking					p= 0.136
Radical operation	24	12	25	12	R_s= 0.105
Remaining tumor ≤ 2 cm	37	18	28	13	
Remaining tumor > 2 cm	23	11	19	10	
Sample laparotomy	1	1	1	1	
Unknown	18	9	27	13	
OS, 15 years			Worse		**p= 0.011**
OS, 5 years			Worse		**p= 0.011**

NOTE. † refers to total number of intratumoral CCL1⁺ cells/mm^2
The χ^2 test was used for categorical variables. R_s, Spearman rank-correlation.
Boldfacing indicates significant p-values.
A small number of patients did not have complete data.

Table 3.1 – Correlation between total number of intratumoral CCL1⁺ cells and clinicopathologic features in invasive ovarian cancer

3.2.2 CCL1 significantly more expressed in cancer tissues

Furthermore, we noted that CCL1-expressing cell distribution was positively skewed not only in terms of total number but also in terms of compartiments. CCL1-expressing cells were frequently present peritumorally, in lymphoid-enriched areas but not detectable in adjacent stromal compartments – **Figure 3.5, p. 31**. In all cases expressing cells were immune cells of lymphoid and myeloid lineages.

Moreover, CCL1 was also expressed intratumorally by tumor cells distributed among the tumor core and present in almost all cases (found in 84% of the tumors) – **Figure 3.5, p. 31**. For all further calculations, positive cell total numbers were determined in representative positive areas of the sections. Expressions by tumor cell themselves was commonly observed and comparable in intensity with the immune cell labeling. Thus, we added them from recognition when the computer-aided determination was run.

Variable	CCL1⁺ ≤ 150† No.	%	CCL1⁺ > 150† No.	%	Significance
Total No. of patients	100	49	103	51	
Age, years					p= 0.424
≤ 60	53	26	52	26	
> 60	43	21	55	27	
Histological subtype					p= 0.988
Serous	75	37	83	41	R_s= 0.001
Mucinous	1	1	1	1	
Endometroid	6	3	10	5	
Adenocarcinoma (NOS)	7	3	3	2	
Undifferentiated	2	1	5	2	
Unknown	5	2	5	2	
Grade					p= 0.265
I	4	2	2	1	R_s= 0.080
II	34	17	33	17	
III & IV	56	28	68	33	
Unknown	3	1	3	1	
Optimal surgical debulking					p= 0.372
Radical operation	26	13	23	11	R_s= 0.063
Remaining tumor ≤ 2 cm	32	16	33	16	
Remaining tumor > 2 cm	18	9	24	12	
Sample laparotomy	0	0	2	1	
Unknown	20	10	25	12	
OS, 15 years			Worse		**p= 0.001**
OS, 5 years			Worse		**p= 0.001**

NOTE. † refers to total number of peritumoral CCL1⁺ cells/mm^2
The χ^2 test was used for categorical variables. R_s, Spearman rank-correlation.
Boldfacing indicates significant p-values.
A small number of patients did not have complete data.

Table 3.2 – Correlation between total number of peritumoral CCL1⁺ cells and clinicopathologic features in invasive ovarian cancer

3.2.3 Association of CCL1 expression with clinicopathologic parameters

Correlations between total numbers of intra- and peritumoral CCL1-expressing cells and clinicopathologic parameters are presented in **Table 3.1 and 3.2, pp. 33, 34**, respectively. The total number of intratumoral CCL1-expressing cells was not significantly correlated with the patient's age, histological subtype, grade of the tumor and surgical debulking – **Table 3.1, p. 33**. Furthermore, we didn't observe any significant correlation between total number of peritumoral CCL1-expressing cells and the patient's age, histological subtype, grade of the tumor and surgical debulking – **Table 3.2, p. 34**. Thus, CCL1 expression seems to occur independently from these commonly used clinical markers.

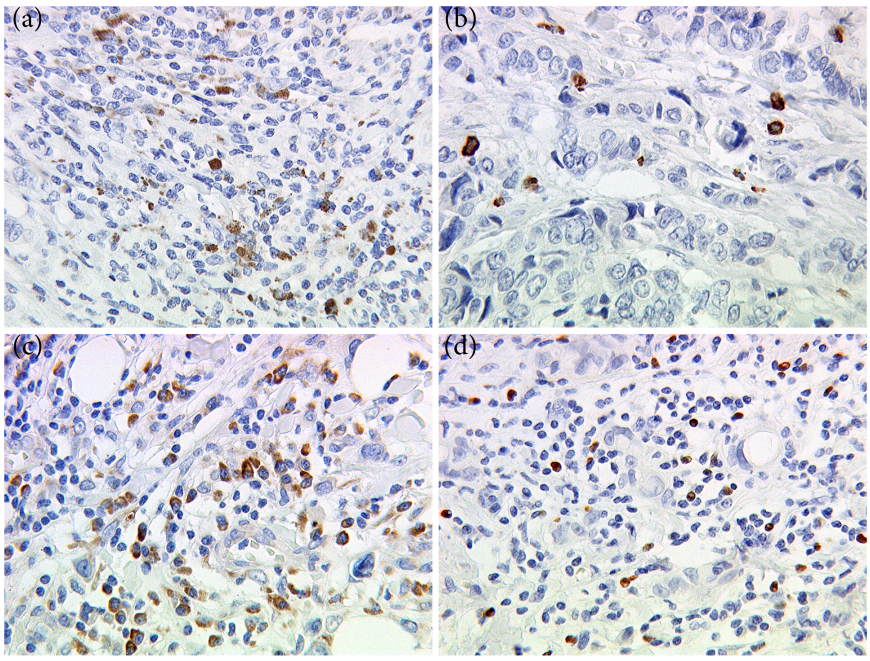

FIGURE 3.8 – *Representative examples of CCL1, CCL22, CCL27, and FoxP3 immuno-staining in breast cancer. (a) Whole breast cancer tissue section with CCL1-expressing cells. (b) Whole breast cancer tissue section with CCL22-expressing cells. (c) Whole breast cancer tissue section with CCL27-expressing cells. (d) Whole breast cancer tissue section with FoxP3-expressing cells. (a-b-c-d) magnification* $\times$ *200.*

3.3 Expression of chemokines in breast cancer

3.3.1 CCL1, CCL22 and CCL27 all expressed in breast cancer

As can be seen in representative photomicrographs, breast cancer tumor sections expressed CCL1, CCL22 and CCL27 – **Figure 3.8, p. 35**. Assessment of all tissue microarray sections showed a high degree of variability in chemokine-expressing cell total numbers. This was particularly true for CCL1 (median, 116 cells/mm^2; range, 0 to 1784) and CCL27 (median, 102 cells/mm^2; range, 0 to 1378). In both cases expression in normal breast tissue was significantly lower than in tumor tissue (p= 0.031 and p= 0.002 for CCL1 and CCL27, respectively) – **Figure 3.9, p. 36**. In contrast, although there was a degree of variability in CCL22-expressing cell total numbers in tumors (median, 29 cells/mm^2; range, 0 to 189), fewer expressing cells were observed than for CCL1 and CCL27. Moreover, the total number of cells expressing CCL22 in tumor tissue was not significantly different from normal breast tissue (p= 0.359) – **Figure 3.9, p. 36**.

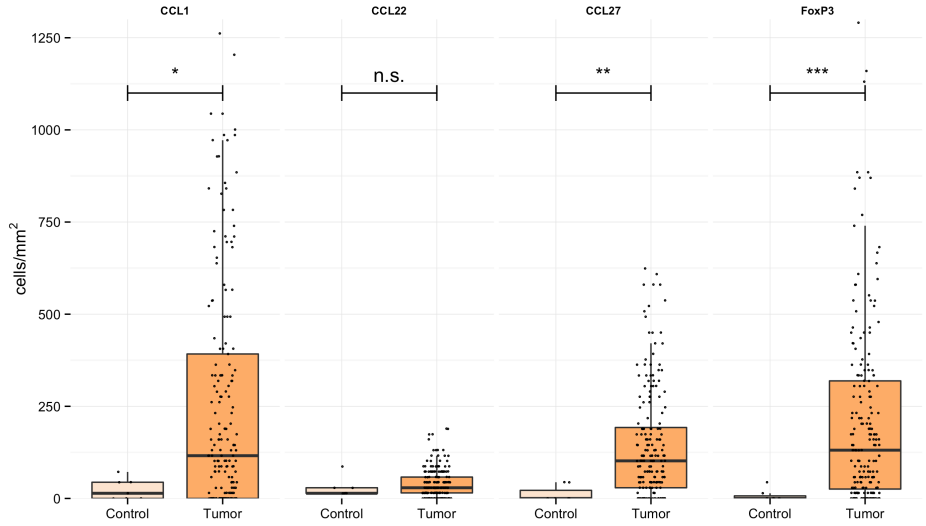

Figure 3.9 – *Total number of CCL1-, CCL22-, CCL27-, and FoxP3-expressing cells in normal breast tissue compared to breast tumor tissue. Median and IQR of all chemokines and FoxP3 cell numbers. Mann-Whitney U test. *P< 0.05; **P< 0.005; ***P< 0.001.*

3.3.2 CCL1 and CCL27 significantly more expressed in cancer tissues

Furthermore, we noted that CCL1-, CCL22- and CCL27-expressing cell distributions were positively skewed not only in terms of total number but also in terms of compartiments. CCL1- and CCL27-expressing cells were frequently present in lymphoid-enriched areas and not detectable in adjacent stromal compartments – **Figure 3.8, p. 35**. On the contrary, CCL22-expressing cells were more equally distributed in the tumor and likely to be detected in adjacent stromal compartments – **Figure 3.9, p. 36**. For all further calculations, positive cell total numbers were determined in representative positive areas of the sections. In all cases expressing cells were immune cells of lymphoid and myeloid lineages. Expressions by tumor cell themselves was only anecdotally observed and not comparable in intensity with the immune cell labeling. Thus, we excluded them from recognition when the computer-aided determination was run.

3.3.3 FoxP3$^+$ cells significantly more infiltrated in cancer tissues

To further characterize possible associations between Treg infiltration and the expression of our candidate chemokines, we labeled FoxP3 on the same tissue microarray sections – **Figure 3.8, p. 35**. Like for the chemokine, the total number of infiltrating Treg presented a high degree of variability (median, 131 cells/mm^2; range, 0 to 1624). Compared with normal breast tissue infiltration, Treg infiltration in tumor tissue was significantly higher (p< 0.001) – **Figure 3.8, p. 35**. Treg

Variable	$CCL1^+ \leq 100^\dagger$ No.	%	$CCL1^+ > 100^\dagger$ No.	%	Significance
Total No. of patients	87	45	106	55	
Age, years					p= 0.209
≤ 50	26	13	42	22	
> 50	61	32	64	33	
Nodal status					p= 0.085
Negative	18	10	11	6	
Positive	63	36	85	48	
Tumor size, cm					p> 0.999
≤ 2	17	9	21	11	
> 2	69	37	82	43	
Grade					p= 0.094
II	41	21	36	19	
III	45	24	68	36	
ER-α status					**p< 0.001**
Negative	30	16	64	34	
Positive	55	30	38	20	
PgR-α status					p= 0.795
Negative	50	27	63	33	
Positive	35	19	39	21	
Her2 status					p= 0.088
Negative	62	33	64	34	
Positive	22	11	41	22	
OS, 15 years			Worse		**p= 0.033**
OS, 5 years			Worse		**p= 0.031**

NOTE. † refers to total number of $CCL1^+$ cells/mm^2
Abbreviations: ER-α, estrogen receptor-alpha; PgR-α, progesterone receptor-alpha; Her2, human epidermal growth factor receptor 2.
The χ^2 test was used for categorical variables. Boldfacing indicates significant p-values.
A small number of patients did not have complete data.

Table 3.3 – Correlation between total number of $CCL1^+$ cells and clinicopathologic features in invasive breast cancer

were commonly localized in the vicinity of tumor cells, but were likely to locally accumulate in lymphoid-enriched areas.

3.3.4 Association of CCL1, CCL22 and CCL27 expressions with clinicopathologic parameters

Correlations between total numbers of CCL1-, CCL22- and CCL27-expressing cells and clinicopathologic parameters are presented in **Table 3.3, 3.4, and 3.5, pp. 37, 38, 39**, respectively. The total number of CCL1-expressing cells was not significantly correlated with the nodal status, Her2 status and tumor grade. Furthermore, total number of CCL1-expressing cells strongly correlated with ER-α status (p< 0.001), showing a clear inversion of the ratio ER-α^+/ER-α^- between the groups – **Table 3.3, p. 37**. The total number of CCL27-expressing cells only weakly correlated with the tumor grade – **Table 3.5, p. 39**. In contrast, no correlation was found with the total number of CCL22-expressing cells – **Table 3.4, p. 38**.

Interestingly, there was a significant relationship between the Treg infiltration in tumors and chemokine-expressing cell total numbers – **Table 3.6, p. 40**. In

Variable	CCL22⁺ ≤ 25† No.	%	CCL22⁺ > 25† No.	%	Significance
Total No. of patients	74	39	116	61	
Age, years					p= 0.949
≤ 50	25	13	41	22	
> 50	49	26	75	39	
Nodal status					p= 0.312
Negative	14	8	14	8	
Positive	55	32	91	52	
Tumor size, cm					p= 0.548
≤ 2	13	7	25	13	
> 2	61	33	87	47	
Grade					p= 0.811
II	28	15	46	25	
III	46	25	67	35	
ER-α status					p= 0.932
Negative	36	20	58	31	
Positive	36	20	54	29	
PgR-α status					p= 0.256
Negative	48	26	64	35	
Positive	24	13	48	26	
Her2 status					p= 0.320
Negative	51	27	72	39	
Positive	21	11	43	23	
OS, 15 years			Same		p= 0.460
OS, 5 years			Same		p= 0.570

NOTE. † refers to total number of CCL22⁺ cells/mm^2
Abbreviations: ER-α, estrogen receptor-alpha; PgR-α, progesterone receptor-alpha; Her2, human epidermal growth factor receptor 2.
The χ^2 test was used for categorical variables. Boldfacing indicates significant p-values.
A small number of patients did not have complete data.

Table 3.4 – Correlation between total number of CCL1⁺ cells and clinicopathologic features in invasive breast cancer

Pearson's product-moment correlations, we found significant associations between CCL1- and CCL27-expressing cell total numbers and total number of infiltrating Treg (p< 0.001, both), but not between CCL22-expressing cell total number and total number of infiltrating Treg (p= 0.748). The association with CCL1- and CCL27-expressing cell total numbers was further significant in a multivariate linear model (p< 0.001 and p= 0.023, for CCL1 and CCL27, respectively) – **Table 3.6, p. 40**, as plotted in 3d – **Figure 3.10, p. 40**.

Both CCL1 and CCL27 could therefore actively recruit Treg into the tumors and our results strongly suggest that we might gain considering them together instead of separately.

Variable	CCL27⁺ ≤ 80† No.	%	CCL27⁺ > 80† No.	%	Significance
Total No. of patients	83	43	109	57	
Age, years					p> 0.999
≤ 50	29	15	39	20	
> 50	54	28	70	37	
Nodal status					p= 0.177
Negative	16	9	12	7	
Positive	61	35	87	49	
Tumor size, cm					p= 0.963
≤ 2	17	9	21	11	
> 2	64	34	86	46	
Grade					p= 0.074
II	39	21	36	19	
III	43	23	71	37	
ER-α status					p= 0.318
Negative	38	20	57	31	
Positive	44	24	47	25	
PgR-α status					p= 0.690
Negative	48	26	65	35	
Positive	34	18	39	21	
Her2 status					p= 0.153
Negative	58	31	67	35	
Positive	22	12	42	22	
OS, 15 years			Trend		p= 0.150
OS, 5 years			Worse		**p= 0.031**

NOTE. † refers to total number of CCL27⁺ cells/mm^2
Abbreviations: ER-α, estrogen receptor-alpha; PgR-α, progesterone receptor-alpha; Her2, human epidermal growth factor receptor 2.
The χ^2 test was used for categorical variables. Boldfacing indicates significant p-values.
A small number of patients did not have complete data.

Table 3.5 – Correlation between total number of CCL1⁺ cells and clinicopathologic features in invasive breast cancer

3.4 Chemokines correlate with worse overall survival

3.4.1 CCL1 expression predict poor patient outcome in ovarian cancer

The median follow-up period was 32 months (range, 1 to 233), during which 145 patients died from ovarian cancer. Patient groups were defined in respect to the total number of chemokine-expressing cells (*i.e.*, higher or lower than respective medians). Patients with high intra- or peritumoral CCL1-expressing cell total numbers presented strongly reduced median survivals (53 months versus 29 and 60 months versus 30, for intra- and peritumoral, respectively) – **Figure 3.11 and 3.12, pp. 41, 41**. Moreover, the OS difference was significant over a long period of time. Survival of patients with high intratumoral CCL1-expressing cell total number was worse after 5 years as well as after 15 years follow-up (p= 0.011 both, for survival after 5 and 15 years, respectively) – **Table 3.1, p. 33**. This was also the case for patients with high total number of peritumoral CCL1-expressing cell (p= 0.001 both, for survival after 5 and 15 years, respectively) – **Table 3.2, p. 34**.

	Linear regression (Pearson)			
FoxP3	Number of pairs	ρ	p-value	
CCL1	189	0.4981	$2.28\ 10^{-13}$	***
CCL22	184	0.0237	0.748	
CCL27	186	0.3580	$4.57\ 10^{-07}$	***
	Multiple linear regression			
FoxP3	Estimate	Std. error	p-value	
Intercept	91.518	24.544	$2.56\ 10^{-4}$	***
CCL1	0.2949	0.0505	$2.29\ 10^{-8}$	***
CCL27	0.2942	0.1286	0.023	*

ρ, Pearson's correlation coefficient.

*Table 3.6 – Pearson's product-moment correlation and multiple linear regression fit to total number of FoxP3-expressing cells using total number of CCL1-, CCL22- and CCL27-expressing cells as predictors. *P< 0.05; ***P< 0.001.*

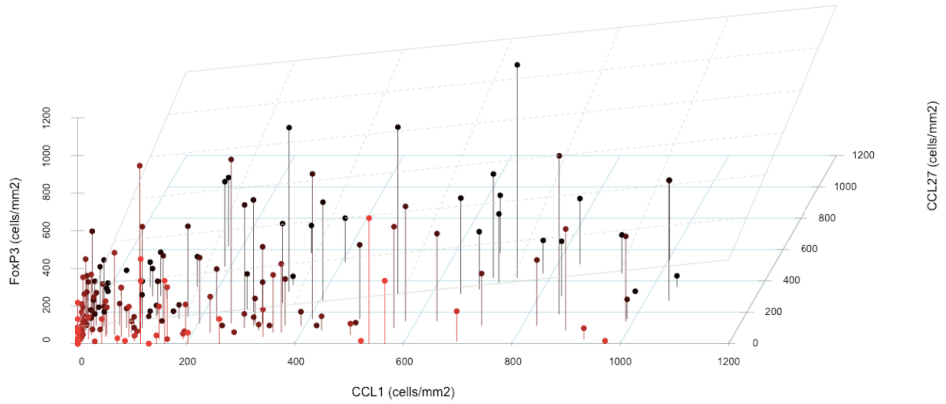

Figure 3.10 – *Multiple linear regression fit to total number of FoxP3-expressing cells using total number of CCL1- and CCL27-expressing cells as predictors. The third axis represents the total number of CCL27-expressing cells; red spots, low CCL27-expression, black spots, high CCL27-expression.*

Consistent with the correlation between intra- and peritumoral expressions, the analysis for patient groups based on both intra- and peritumorally expressing cell total numbers showed no difference in the results. Patients with high intra- and peritumoral expression of CCL1 were regrouped and compared with the others (*i.e.*, patients with high expression in none or only one of the two areas).

Median survival of these high-risk patients was comparatively reduced (26 months versus 53) – **Figure 3.13, p. 42**. The OS difference was highly significant over the 15 years follow-up (p< 0.001 both, for survival after 5 and 15 years, respectively).

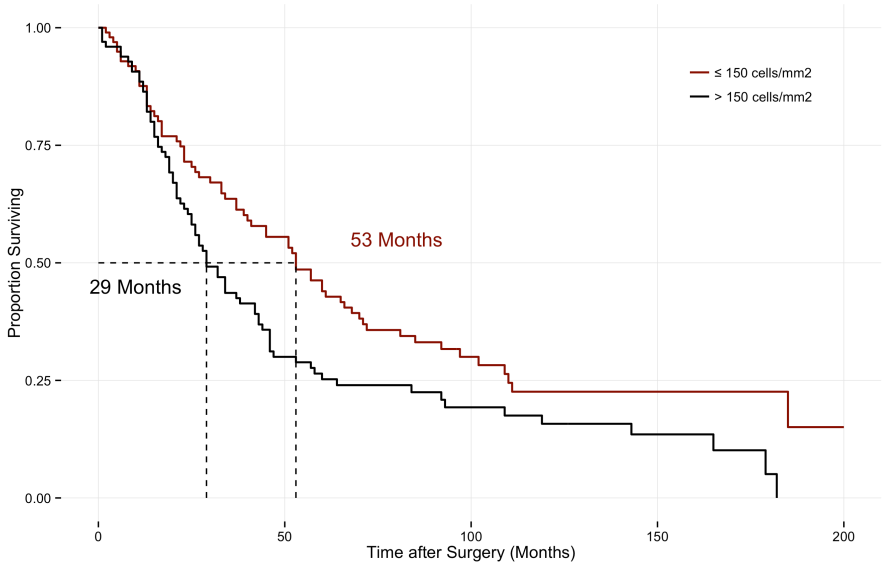

FIGURE 3.11 – *KM plots of ovarian cancer OS, stratified by a cutoff at the median values for CCL1 expressed intratumorally.*

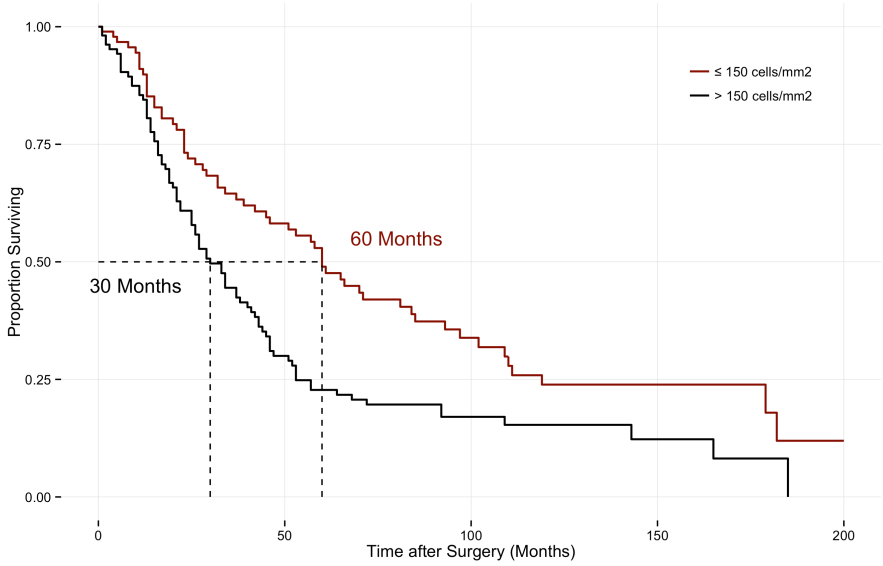

FIGURE 3.12 – *KM plots of ovarian cancer OS, stratified by a cutoff at the median values for CCL1 expressed intratumorally.*

3.4.2 Prognostic significance of CCL1-expression in ovarian cancer

Based on these results, we investigated the prognostic significance of high CCL1-expression in ovarian cancer. Multivariate Cox proportional hazards model showed that high intratumoral expression of CCL1 was independently associated with worse

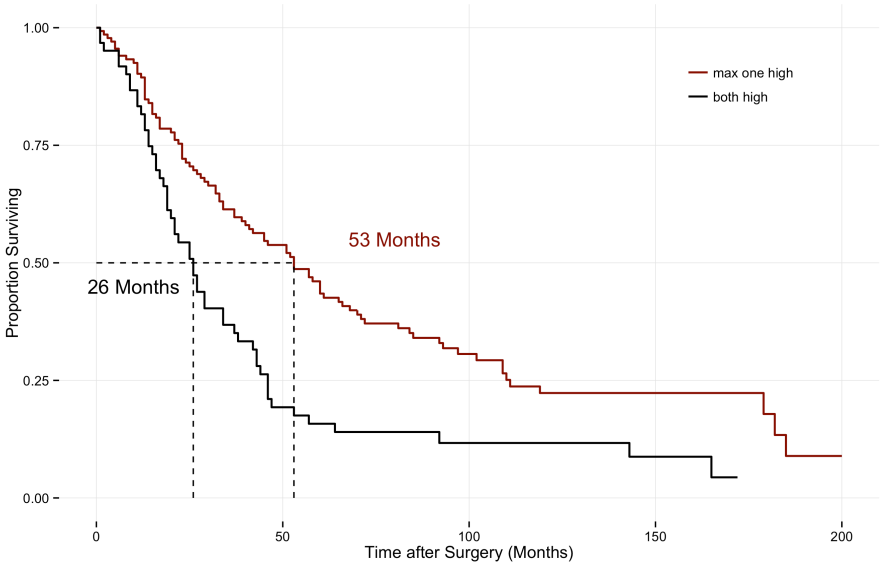

Figure 3.13 – *KM plots of ovarian cancer OS, stratified by a global cutoff for CCL1-expressing cells (*i.e., *patients highly expressing CCL1 both intra- and peritumorally against the others).*

prognosis (hazard ratio, 1.60; 95% confidence interval (Conf. Int.), 1.13 to 2.62; p= 0.008) – **Table 3.7, p. 42**. Peritumoral expression of CCL1 was also independently associated with worse prognosis (hazard ratio, 1.59; 95% Conf. Int., 1.13 to 2.25; p= 0.008) – **Table 3.8, p. 43**. Interestingly, with exception of the adjuvant therapy (hazard ratio, 0.68; 95% Conf. Int., 0.42 to 1.10; p= 0.113), all other common markers included were independently associated with worse prognosis, according to the current knowledge. Similar results were obtained when considering only the first 5 years after surgery.

		Conf. Int. hazard ratio†			
Variable	Hazard ratio†	Lower .95	Upper .95	p-value	
Tumor grade	1.62	1.13	2.33	0.009	**
Patients' age	1.74	1.21	2.50	0.003	**
Tumor rest $\leq$ 2 cm	1.98	1.25	3.12	0.003	**
Tumor rest > 2 cm	2.62	1.54	4.46	< 0.001	***
Adjuvant therapy	0.68	0.42	1.10	0.113	
CCL1 intratumoral	1.60	1.13	2.62	0.008	**

† Obtained using the Cox proportional hazards model.

*Table 3.7 – Multivariate Cox proportional hazards model showing hazard ratios for patient conferred by grade, patients' age, tumor rest size, adjuvant therapies and higher total numbers of intratumoral CCL1-expressing cells. **P< 0.005; ***P< 0.001.*

Thus our results show that high total numbers of CCL1-expressing cells in ovarian cancer correlated with a significantly worse survival over 15 years follow-up and

Variable	Hazard ratio†	Conf. Int. hazard ratio†		p-value	
		Lower .95	Upper .95		
Tumor grade	1.54	1.07	2.21	0.020	*
Patients' age	1.69	1.18	2.42	0.004	**
Tumor rest $\leq$ 2 cm	1.69	1.07	2.66	0.023	*
Tumor rest > 2 cm	2.25	1.34	3.80	0.002	**
Adjuvant therapy	0.61	0.38	.99	0.046	*
CCL1 peritumoral	1.59	1.13	2.25	0.008	**

† Obtained using the Cox proportional hazards model.

*Table 3.8 – Multivariate Cox proportional hazards model showing hazard ratios for patient conferred by grade, patients' age, tumor rest size, adjuvant therapies and higher total numbers of peritumoral CCL1-expressing cells. *P< 0.05; **P< 0.005.*

was independently associated with worse prognosis.

3.4.3 CCL1 and CCL27 expression predict poor patient outcome in breast cancer

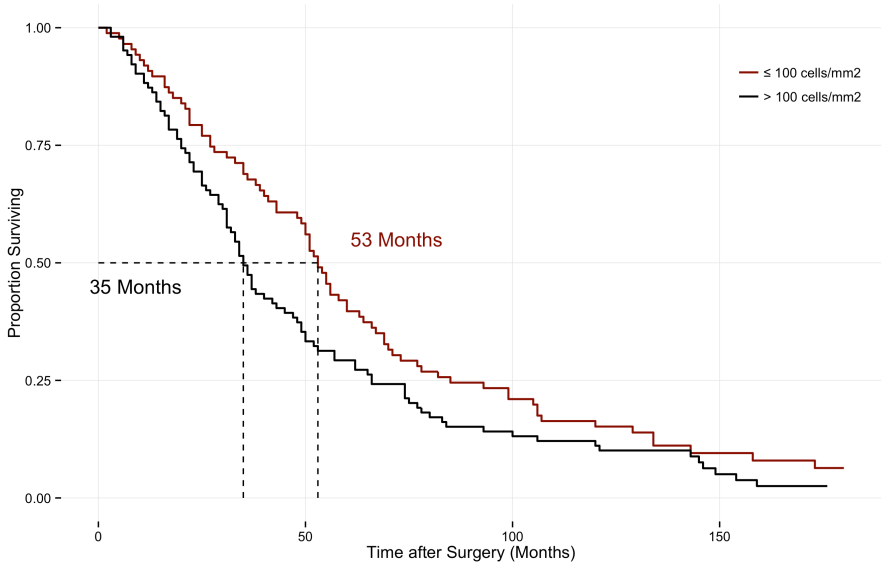

FIGURE 3.14 – *KM plots of breast cancer OS, stratified by a cut-off at the median values for CCL1.*

The median follow-up period was 41 months (range, 1 to 201), during which 185 patients died from invasive breast cancer. Patient groups were defined in respect to the total number of chemokine-expressing cells (*i.e.*, higher or lower than respective medians). Patients with high CCL1- or CCL27-expression cell total numbers in their tumor presented strongly reduced median survivals (53 months versus 35

and 36, for high CCL1- and CCL27-expression, respectively) – **Figure 3.14 and 3.16, pp. 43, 45**. However, high total number of CCL22-expressing cells did not significantly predict a worse outcome (40 months versus 48) – **Figure 3.15, p. 44**. Moreover, in the case of CCL1 and CCL27 expression, the OS difference was significant over a long period of time. Survival of patients with high CCL1-expressing cell total number was worse after 5 years as well as after 15 years follow-up (p= 0.031 and p= 0.033, for survival after 5 and 15 years, respectively) – **Table 3.3, p. 37**. This was, however, not the case for patients with high total number of CCL27-expressing cell. Survival difference was similarly signifiant after 5 years, but not any more after 15 years, (p= 0.031 and p= 0.150, for survival after 5 and 15 years, respectively) – **Table 3.5, p. 39**.

Applying the same analysis to patient groups based on both CCL1- and CCL27-expressing cell total numbers showed the most significant result. Patients with high expression of both CCL1 and CCL27 were regrouped and compared with the others (*i.e.*, patients with high expression of none or only one of the two chemokines). Median survival of these high-risk patients was dramatically reduced (33 months versus 55) – **Figure 3.17, p. 45**. Furthermore, the OS difference was highly significant over the 15 years follow-up (p< 0.001 and p= 0.002, for survival after 5 and 15 years, respectively).

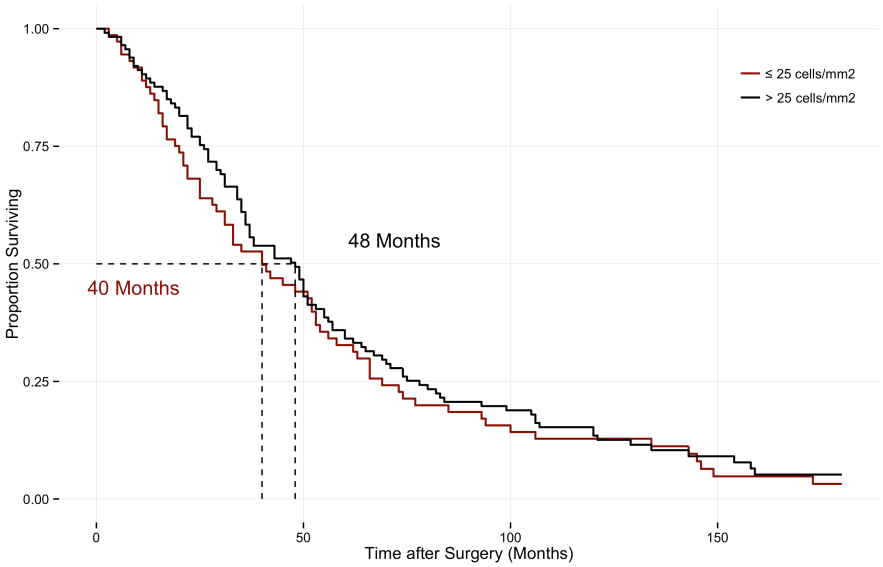

Figure 3.15 – *KM plots of breast cancer OS, stratified by a cut-off at the median values for CCL22.*

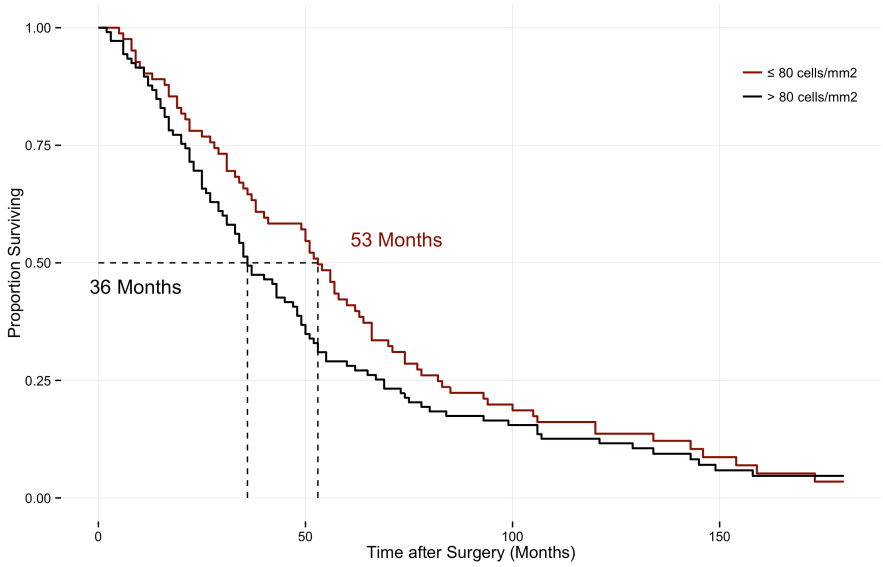

FIGURE 3.16 – *KM plots of breast cancer OS, stratified by a cut-off at the median values for CCL27.*

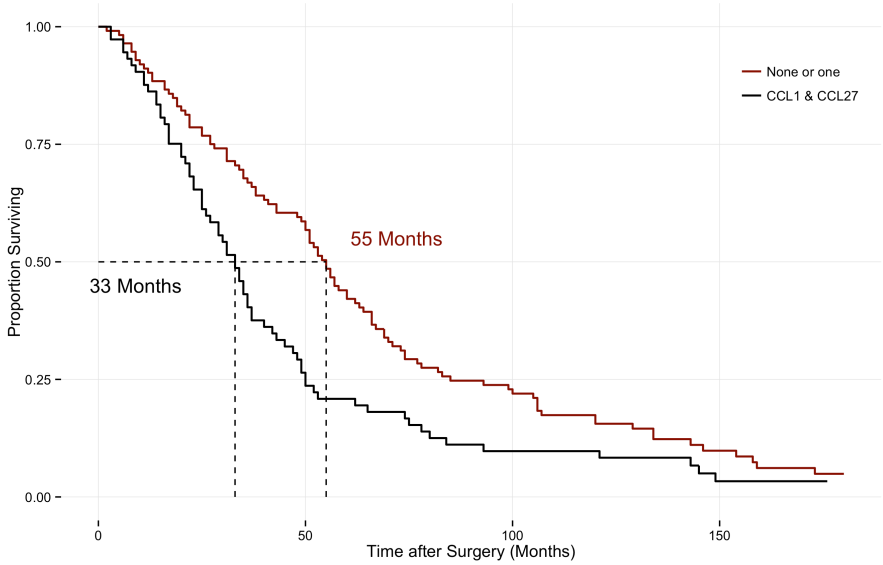

FIGURE 3.17 – *KM plots of breast cancer OS, stratified by addition of both CCL1- and CCL27-risk factors (*i.e., *patients highly expressing both CCL1 and CCL27 against the others).*

3.4.4 Prognostic significance of CCL1- and CCL27-expression in breast cancer

Based on these results, we investigated the prognostic significance of both high CCL1- and CCL27-expression in breast cancer. Multivariate Cox proportional hazards model showed that high expression of both CCL1 and CCL27 was in-

dependently associated with worse prognosis (hazard ratio, 1.53; 95% Conf. Int., 1.02 to 2.03; p= 0.016) – **Table 3.9, p. 46**. Interestingly, with exception of the tumor grade (hazard ratio, 1.44; 95% Conf. Int., 1.02 to 2.03; p= 0.037), no other common markers included were independently associated with worse prognosis (p= 0.415, p= 0.669, p= 0.787, p= 0.999, and p= 0.240, for tumor size and ER-α, PgR-α, and Her2 status, respectively). Similar results were obtained when considering only the first 5 years after surgery.

		Conf. Int. hazard ratio†			
Variable	Hazard ratio†	Lower .95	Upper .95	p-value	
Tumor grade > 1	1.44	1.02	2.03	0.037	*
Patients' age > 50	1.15	1.83	1.59	0.415	
Tumor size > 2 cm	0.91	0.61	1.37	0.669	
ER-α status	1.06	0.69	1.64	0.787	
PgR-α status	0.99	0.65	1.53	0.999	
Her2 status	1.23	0.87	1.73	0.240	
CCL1 & CCL27	1.53	1.02	2.03	0.016	*

† Obtained using the Cox proportional hazards model.

*Table 3.9 – Multivariate Cox proportional hazards model showing hazard ratios for patient conferred by grade, patients' age, tumor size, ER-α status, PgR-α status, Her2 status and higher total numbers of CCL1- and CCL27-expressing cells. The parameter nodal status is not included in multivariate analysis because tumors capacity to form metastases is not an independent clinical parameter in our cohort. *P< 0.05.*

Thus our results show that high total numbers of both CCL1- and CCL27-expressing cells in invasive breast cancer correlated with a significantly worse survival over 15 years follow-up and were independently associated with worse prognosis.

*
* *

Discussion 4

— There cannot however be the least doubt, that the higher organisms, as they are now constructed, contain within themselves the germs of death.

August Weissmann, philosopher of biology, 1889

4.1 Chemokines specifically attracting regulatory T cells

4.1.1 A brief history of chemokines in cancer

The effect of chemokines on Treg recruitment and tumor escape for patients with breast cancer is poorly described. Although previous studies associated increased intratumoral number of $CD4^+$ $CD25^+$ Treg with CCL22 and CCL28 and reported adversely affected prognosis in ovarian cancer, before our current work the clinical importance of other chemokines was unknown [15, 16].

Pioneer work was done fifteen years ago and documents the migration of Treg in response to several CC- and CXC-chemokines [67]. Iellem *et. al.* described the strong and preferential migration of Treg in response to CCL1, CCL17 and CCL22 binding CCR8 and CCR4 (both CCL17 and CCL22), respectively. Simultaneously, CCL22 was shown to be expressed by tumor cells and macrophages and associated with specific recruitment of Treg in ovarian cancer [15].

Here, our results demonstrated that two other chemokines, CCL1 and CCL27 could potentially also attract Treg into tumors. To the best of our knowledge, we are the first investigating the prognostic significance of CCL1 and CCL27 in human ovarian and breast cancer. Our results strongly suggest that the total number

of CCL1- and CCL27-expressing cells has an important role in Treg recruitment in ovarian and breast cancer, as described for CCL22 and CCL28 in ovarian cancer [15, 16].

4.1.2 Our candidates: CCL1, CCL22 and CCL27

Soon after CCL22 became the first candidate by which tumors may foster immune privilege, CCR10 was added to the list of receptors allowing Treg to migrate in response to chemokine [69]. It was then rapidly confirmed that CCL28 acts under tumor hypoxia conditions to recruit Treg in ovarian cancer [16].

Our investigations focused on a broader spectrum of Treg responsiveness to chemokines that could potentially allow them to access tumor tissues. This approach allows, besides comparison of determined CI and EF between chemokines, the validation of CCL1 in a Treg specific recruitment role [67].

Moreover, we provide evidence that CCL27 should be as well considered as good candidate. CCL27 should therefore be added to the list of chemokine possibly secreted in tumor to recruit Treg. CCL27 description fits yet well with the current knowledge as it binds CCR10 known to be preferentially expressed on Treg, in a same way as CCL28 [69, 70]. Furthermore, CCL27 mediates, via binding to CCR10, chemotactic responses of skin-associated lymphocytes and is induced by TNF-α and IL-1β [71].

4.1.3 About the selectivity of our screening

Although particularly striking at first, the determination of only three chemokines (*i.e.*, CCL1, CCL22 and CCL27) specifically attracting Treg should be considered in the context of our analysis. We intended to select the most relevant chemokines under discriminant migration conditions.

The recruitment of Treg by several other chemokines into tumors or inflammatory sites has been suggested, like for CCL17 and CCL18 [72, 73]. However, we used whole PBMC in our migration assays and occasionally observed a slightly negative T cell migration. This points out that our conditions were particularly discriminant and other cell populations could have slowed T cells in their migration.

Unexpectedly, we observed no migration for CCL28, which is, however, besides CCR10, also binding to CCR3 [74]. Consistant with this, cells recruited through binding to CCR3 could have handicapped T cell migrations. Moreover, it seems to have been the case for most of the chemokines not inducing migration in our conditions (*i.e.*, CCL4, 11, 13, 14, 15, 16, 24, 26, and CCL28). All of them bind to the very redundant chemokine receptors CCR1, 2, 3, or CCR5. Other receptors for CCL17 and CCL18 are not known, but our results suggest it as a possibility. Indeed, in human, CCL17 and other chemokines (*i.e.*, CCL22 and CX3CL1) belongs to a cluster on chromosome 16 [75]. CCL18 belongs to the big MIP-cluster, like CCL4, CCL13, CCL14, CCL15, CCL16, and consists of a fusion of two CCL3-like genes [76, 77]. Thus, they both could potentially bing to more than one receptor

– not like our three candidates – and this may explain our results, even if they are likely to attract Treg otherwise.

4.2 The case of ovarian cancer

4.2.1 CCL1 produced by tumor and immune cells

Based on work done previously, we decided to explore the chemokine expressions of our candidates in ovarian cancer [15, 16]. Unfortunately, due to the few samples available, we were only able to realize one labeling for this cohort. Labelings for FoxP3 and CD8 have been previously published [68].

In the same manner as for the already documented expressions of CCL22 and CCL28, we found an expression of CCL1 in tumor tissue [15, 16]. Moreover, this chemokine is expressed by tumor cells as well as by infiltrating immune cells. Production of CCL22 by tumor cells following detection by immune cells was described *in vitro*, and associated with the recruitment of Treg [78]. Importantly, we also observed and quantified CCL1 expression by infiltrated immune cells. Both expressions were significantly higher in tumor tissue compared to the basal expression in normal ovarian tissue.

4.2.2 Both expressions correlated with reduced overall survival

High total number of CCL1-expressing cells correlated with reduced OS. The nature of the producing cells, as well as their localizations - intra- or peritumoral - does not seem to influence the results of the KM analysis. Moreover, both correlated with the Treg infiltration previously described (Pearson's product-moment correlation; p= 0.050 and p= 0.013, intra- and peritumoral expression, respectively), consistent with a possible recruitment into the tumor of Treg by CCL1 [68].

Recently, higher plasma CCL22 levels of women at stage IV FIGO, associated with higher peritoneal fluid Treg percentage was also described [79]. We, however, observed no correlation between total number of CCL1-expressing cells and commonly used clinical markers. This could be due to the fact, that we only included women classified at stage FIGO III and should be explored in the future, since the CCL1-production kinetic by tumor cells or immune cells could plays a role in the disease.

4.2.3 CCL1 could serve as a new biomarker in ovarian cancer

Here we provide the first demonstration that CCL1-expression recruits $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells, which can dampen effector T cell function and promote tumour progression. This finding reinforces the link between chemokines, Treg and peripheral tolerance.

This clearly appears in our Cox proportional hazards model analysis, which showed that a higher total number of both CCL1-expressing cells was independently associated with a higher risk of death (hazard ratio, 1.60; 95% Conf. Int., 1.13 to 2.62; p= 0.008) in a model that included the usual parameters evaluated in clinical studies. All other common parameters (*i.e.*, tumor grade, patient age, tumor rest) were significantly associated with patient worse outcome. Adjuvant therapy to the surgery presented a reduced hazard ratio (0.68; 95% Conf. Int., 0.42 to 1.10; p= 0.113), although not significant. Thus leading us to conclude, that these patients might had benefit from an associated immunotherapy targeting CCL1.

This hypothesis is strongly supported by the observation of Treg migration in response to CCL1 in the past, and the association of Treg infiltration and reduce OS by other groups [15, 16, 67]. It was suggested, that anti-CCR4 treatment augments anti-tumor immunity in cancer patients by selectively depleting Treg cells [80, 81]. Our data suggest, that the same effect could possibly be reached with an anti-CCR8 treatment. Moreover, CCR8 was implicated in donor Treg survival and is critical for the prevention of murine graft-versus-host disease [82]. This implies, that blockade of CCR8, the receptor for CCL1, could represent a two edged sword therapeutic option, affecting Treg migration as well as their survival.

4.3 The case of breast cancer

4.3.1 CCL22 similarly produced in tumor tissue

Surprisingly, the total number of CCL22-expressing cells in tumors was not significantly higher than in normal breast tissue. However, our computer-aided cell recognition focuses on activated T cells, DC, monocytes, or macrophages [67, 83].

Although CCL22 production by tumor cells was documented, we excluded them from recognition. They represented in breast cancer a minor light expressing tumor cell population and we decided to focus on expressing immune cell populations in our study [78, 84]. Thus, since our methods differ, it may not be surprising that a high total number of CCL22-expressing cells did not correlate with total number of infiltrating Treg in tumors and reduced OS.

4.3.2 CCL1 and CL27 produced in tumor tissue

On the contrary, CCL1 and CCL27 both correlate with total number of infiltrating Treg in tumor and reduced OS. In a recent study, Hoelzinger *et. al.* described that the blockade of CCL1 inhibits Treg suppressive function without affecting T effector responses in a murine model [85]. More recently, Das *et. al.* suggested that tumor cell entry into the lymph node is controlled by CCL1 [86]. Without presuming a role in T cell migration, these data support at least the specificity of the CCL1 action on Treg among immune cells and suggest possible other roles for this chemokine on tumor cells.

High total number of CCL1-expressing cells also strongly correlate with ER-α in a same way as described for Treg, which supports our hypothesis, that it could attract these cells [53]. During the last ten years, CCL27 was implicated several times in tumor progression and immune escape in cutaneous malignant melanoma [87, 88, 89, 90].

4.3.3 CCL1 and CCL27 could be targeted in immunotherapy

Furthermore, CCL1 and CCL27 fit the total number of infiltrating Treg as predictors in a multivariable analysis. These investigations have led us to the hypothesis that the high expression of two chemokines, instead of only one, may enhance the recruitment of Treg and facilitates the creation of an immunosuppressive milieu.

This hypothesis is strongly supported by the observation of a synergistic rather than an additive effect in response to a combination of suboptimal doses of CCL1 and CCL22 [67]. Prerequisite was the high total number of CCL1- and CCL27-expressing cells found in breast cancers, which could act synergistically to recruit Treg, binding to both CCR8 and CCR10. This idea was supported by the reduced OS observed for patients with high total numbers of both CCL1- and CCL27-expressing cells. Both chemokines seem to be required because recruitment could not occur via a distinct chemokine axis, as very recently suggested in a murine model [91].

This was confirmed in our Cox proportional hazards model analysis, which showed that a higher total number of both CCL1- and CCL27-expressing cells was independently associated with a higher risk of death (hazard ratio, 1.50; 95% Conf. Int., 1.07 to 2.10; p= 0.019) in a model that included the usual parameters evaluated in clinical studies. Neither tumor size nor receptor status were significantly associated with patient outcome. The patients in our cohort had all documented metastases during the course of their disease. It may be worth to mention, since at least CCL1 was implicated in tumor cell entry into the lymph node [86]. A possible explanation for the worse prognosis may be due, in part, to this association with the metastatic process.

4.4 Conclusion

4.4.1 Comparison between ovarian and breast cancer chemokine expressions

Our work presents advantages compared to former studies. We realize a large chemokine screening among the CC-chemokine family. This allows us, besides a better comparison among these chemokines and their effect on Treg, to exclude the possibility for all others to recruit Treg specifically. Fact being corroborated by previous studies, which described only CCR4, CCR8 and CCR10 (receptors for our three candidates, CCL22, CCL1, and CCL27, respectively) specifically expressed

on Treg [15, 16, 67]. In fact, our own work on chemokine receptors fails to find other receptors overexpressed on Treg, thus leading us to conclude to the probable absence of other candidate.

Additionally, we provided here the possibility to compare the effect of chemokines on Treg recruitment in two cancer types. In both cases, we found a comparable effect of CCL1 on the patients' OS, as well as almost identical cell distributions per mm^2 (150 versus 116 cells/mm^2, in ovarian and breast cancer, respectively). Moreover, our Cox proportional hazards model analysis revealed a similar effect of chemokine productions in the two cancer types (hazard ratio, 1.60 versus 1.50, in ovarian and breast cancer, respectively). Importantly, the hazard ratio was comparable to the other common clinical parameters in the case of ovarian cancer, thus leading us to conclude that CCL1 might be a good candidate for adjuvant immunotherapy.

Finally, the case of breast cancer enabled us to compare the effect of more than one chemokine and led us to propose, that they might act together to recruit Treg. Patients with a high expression of both CCL1 and CCL27 presenting a significantly higher risk of death (hazard ratio of 1.50). In this cohort, all patients had an history of metastasis and commonly used clinical markers were incapable of risk predictions. Thus, it might be worse to block more than one chemokine receptor. It was also the opportunity to explore the CCL22 production of the immune cell alone, since we observed almost no expression by the tumor cells. This was the most striking difference compared to ovarian cancer, where tumor cells were independently massively producing chemokine (*i.e.*, CCL1 in our work, or CCL22 and CCL28, described by others) leading to worse survival of patients [15, 16].

4.4.2 Limitation of our investigations

There are limitations of our investigation. It is a retrospective evaluation; however, we investigated a prespecified hypothesis largely documented in prospective murine studies, and we supported our data by complete migration analysis. Since in our breast cancer cohort all patients had a documented history of metastases, we had to exclude the nodal status in the multivariate Cox model. Tumors invasion into lymph node have not been an independent clinical parameter. In our analysis we presuppose that FoxP3 is a valid Treg marker. There have been, however, publications questioning this assumption in the past [92, 93]. Some evidence also suggests that $FoxP3^+$ Treg subpopulations may be involved in tumor suppressive functions [94, 95]. For now FoxP3 remains the most established and understood Treg cell marker we know [35, 96, 97, 98].

4.4.3 Concluding remark

Validation of our findings in additional investigations might open the way for new therapeutic approaches of the combination of conventional chemotherapy with immunotherapy. Blockade of only one chemokine or chemokine receptor may not be sufficient to overcome a Treg associated immunosuppressive milieu. Our results in-

dicate that depleting both CCL1 and CCL27 or blocking both CCR8 and CCR10 might improve the efficacy of adoptive T cell transfer. This study strongly supports further investigation of this line of potential therapeutic intervention.

*
* *

Appendix 5

— Nous trouvons de tout dans notre mémoire. Elle est une espèce de pharmacie, de laboratoire de chimie, où on met au hasard la main tantôt sur une drogue calmante, tantôt sur un poison dangereux.

Marcel Proust, novelist, 1923

5.1 *Curiculum vitæ*

Dr. Christoph Paul Freier

315 S Peoria St, Chicago, IL 60607, USA
+1 (312) 889-4772 • christoph@freier.fr

Pharmacist

Personal Details

Date of birth: December 6, 1985
Place of birth: la Garenne-Colombes, France
Citizenship: German and French

Highlights

Interdisciplinary expertise in preclinical oncology and immunology with emphasis for the role of regulatory T cells in gynecological human tumors

Fully fluent in Unix, Python and R for data analysis and acted as a consultant for programming and statistical analysis of research projects

Pharmacist expertise with leadership capacity: worked in community pharmacies, supervision of students, initiation of projects and collaborations

Skills

Oncology	**Biomarker characterisations** using uni- and multivariable predictive models in ovarian and breast cancers
Immunology	**Comprehensive expertise** in lymphocyte manipulation, culture, migration assays, and advanced FACS skills
Pathology	**Mastered immunohistochemistry**, from single IHC to multiple fluorescence through computer-aided quantification
Genomics	**Working knowledge** with Bowtie, Tophat, Cufflinks, CummRbund, and Samtools for differential gene expression analysis
Statistics	**Coded myself** PCA, ROC curves, KM and COX proportional hazards modelling, and mentored students in statistics
Bioprogramming	**Programmed and used algorithms** for statistical analysis, high throughput sequencing and sincle-cell RNA-seq

Current Occupation

Since 2015	**Postdoctoral Research Associate** at the University of Illinois at Chicago, IL, USA Involved in a combination of bench work, model system and uses of computational languages for innovative single-cell RNA-seq experiments

Working Experience

2011 – 2015	**Ph.D.** at the *Ludwig-Maximilians-Universität* of Munich, Germany **Thesis:** role of regulatory T cells and associated chemokines in human diseases **Supervisor:** Prof. Dr. *rer. nat.* Udo Jeschke
05/15 – 10/15	**Pharmacist** in a **german pharmacy** **Routine shifts** in the evenings and week-ends
2010 – 2011	**Master 2** at **INSERM**[1] **unit 747** "*pharmacologie, toxicologie et signalisation cellulaire*" of Prof. Dr. Dr. Robert Barouki
2007 – 2011	**Assistant Pharmacist** in **french pharmacies** **Part time** during semester, **full time** during summer breaks **Perfectly familiarized** with analyzing, delivering and advising drug prescriptions and non-prescription drugs
06/10 – 09/10	**Master 1** at **INSERM**[1] **unit 996** "*cytokines, chimiokines et immunopathologie*" of Prof. Dr. Dominique Emilie
06/08 – 09/08	**Internship** at **INSERM**[1] **unit 749** "*signalisation dans la prolifération cellulaire et l'apoptose*" of Prof. Dr. Jacques Bertoglio

Education

Passed 2012	***Diplôme d'état de docteur en pharmacie*** **(Pharm.D.)** at the *université Paris-Sud*, France **Supervisor:** Prof. Dr. Dr. Marc Pallardy
Passed 2011	**Master of Science (M.Sc.):** human toxicology, risk evaluation and vigilance at the *université Paris-Sud*, France
2005 – 2011	**Pharmaceutical studies** at the *université Paris-Sud*, France

[1] French National Institute of Health and Medical Research

Language Knowledge

French	**native**
German	**near native**
English	**fluent, business fluent**

Others

Laboratory	Large panel of technical skills: WB, RT-qPCR, ELISA, vector construction, protein expression and purification, *etc*
Informatics	Office suite, Adobe suite, LaTeX, IDLE, and RStudio Accustomed to OSX and various GNU/Linux flavors
Coding	Unix, Python, and R for data mining, data wrangling, data analysis, and statistical programming
Memberships	BLAK (Bavarian Chamber of Pharmacists) DGfI (German Society for Immunology)

Grants and Awards

2011 – 2015	DFG[2] Ph.D. stipend
2014	HSTM - 16th Hamburger Symposium on Tumor Markers Oral presentation and **poster price, 1st place**

References

These persons are familiar with my professional qualifications and my character:

Pr. Dr. Dr. Marc Pallardy
Pharm.D. mentor
Université Paris XI
Chatenay-Malabry, France email: marc.pallardy@u-psud.fr

Prof. Dr. *rer. nat.* Udo Jeschke
Ph.D. mentor
Ludwig-Maximilians-Universität
Munich, Germany email: udo.jeschke@med.uni-muenchen.de

Chicago, March 1, 2016

[2] German National Research Society

5.2 *Eidesstattliche Versicherung*

Ich erkläre hiermit an Eides statt,

dass ich die vorliegende Dissertation mit dem Thema

Role of regulatory T cells and associated chemokines in human gynecological tumors

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, den 28 September 2015 Christoph Paul Freier

*
* *

List of Figures

List of Tables

List of Acronyms

APC antigen presenting cells 4

BSA bovine serum albumin 19

CD cluster of differentiation 4

cDNA complementary DNA 20

CI chemotaxis index 18

Conf. Int. confidence interval 42

CTLA4 cytotoxic T lymphocyte-associated antigen 4 5

Da Dalton 10

DAB 3,3-diaminobenzidine 23

DC dendritic cells 3

EF enrichment factor 18

EDTA ethylenediaminetetraacetic acid 19

ER estrogen receptor 21

FIGO International Federation of Gynecology and Obstetrics 22

FoxP3 forkhead box P3 6

GM-CSF granulocyte-macrophage colony-stimulating factor 4

GPCR G protein-coupled receptors 8

Her2 human epidermal growth factor receptor 2 21

HPRT hypoxanthine-guanine phosphoribosyltransferase 21

HRP horseradish peroxidase 22

ICAM intercellular adhesion molecule 15

IFN interferon 12

IL interleukin 4

IQR interquartile range 25

IU international unit 17

KM Kaplan-Meier 25

KO knockout 9

MDNCF monocyte-derived neutrophil chemotactic factor 8

M molar 17

mRNA messenger RNA 11

NAP neutrophil-activating peptide 8

OD optical density 20

OS overall survival iii

PAF platelet activating factor 8

PBMC peripheral blood mononuclear cells 17

PBS phosphate-buffered saline 17

PCR polymerase chain reaction 20

PD1 programmed cell death protein 1 5

PDL1 programmed cell death ligand 1 5

PgR progesteron receptor 21

qPCR real-time PCR 20

RNA ribonucleic acid 19

RPMI Roswell Park Memorial Institute medium 17

SEM standard error of the mean 25

Tcon conventional T cells 27

TCR T cell receptor 4

Bibliography

[1] R. S. Goldszmid and G. Trinchieri, "The price of immunity," *Nat Immunol*, vol. 13, pp. 932–8, Oct 2012.

[2] O. Leavy, "New mechanism of tolerance induction in cancer," *Nat Rev Immunol*, vol. 7, pp. 580–580, Aug 2007.

[3] S. J. Allen, S. E. Crown, and T. M. Handel, "Chemokine: receptor structure, interactions, and antagonism," *Annu Rev Immunol*, vol. 25, pp. 787–820, Apr 2007.

[4] G. E. White, A. J. Iqbal, and D. R. Greaves, "CC chemokine receptors and chronic inflammation–therapeutic opportunities and pharmacological challenges," *Pharmacol Rev*, vol. 65, pp. 47–89, Jan 2013.

[5] D. Rossi and A. Zlotnik, "The biology of chemokines and their receptors," *Annu Rev Immunol*, vol. 18, pp. 217–42, Apr 2000.

[6] B. Bajoghli, "Evolution and function of chemokine receptors in the immune system of lower vertebrates," *Eur J Immunol*, vol. 43, pp. 1686–92, Jul 2013.

[7] D. G. DeNardo and L. M. Coussens, "Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression," *Breast Cancer Res*, vol. 9, p. 212, Aug 2007.

[8] C. G. Drake, E. Jaffee, and D. M. Pardoll, "Mechanisms of immune evasion by tumors," *Adv Immunol*, vol. 90, pp. 51–81, May 2006.

[9] W. H. Fridman, R. Remark, J. Goc, N. A. Giraldo, E. Becht, S. A. Hammond, D. Damotte, M.-C. Dieu-Nosjean, and C. Sautès-Fridman, "The immune microenvironment: a major player in human cancers," *Int Arch Allergy Immunol*, vol. 164, pp. 13–26, May 2014.

[10] D. Hanahan and R. A. Weinberg, "Hallmarks of cancer: the next generation," *Cell*, vol. 144, pp. 646–74, Mar 2011.

[11] W. H. Fridman, F. Pagès, C. Sautès-Fridman, and J. Galon, "The immune contexture in human tumours: impact on clinical outcome," *Nat Rev Cancer*, vol. 12, pp. 298–306, Apr 2012.

[12] W.-H. Fridman, M.-C. Dieu-Nosjean, F. Pagès, I. Cremer, D. Damotte, C. Sautès-Fridman, and J. Galon, "The immune microenvironment of human tumors: general significance and clinical impact," *Cancer Microenviron*, vol. 6, pp. 117–22, Aug 2013.

[13] N. A. Giraldo, E. Becht, R. Remark, D. Damotte, C. Sautès-Fridman, and W. H. Fridman, "The immune contexture of primary and metastatic human tumours," *Curr Opin Immunol*, vol. 27, pp. 8–15, Apr 2014.

[14] Q. Gao, S.-J. Qiu, J. Fan, J. Zhou, X.-Y. Wang, Y.-S. Xiao, Y. Xu, Y.-W. Li, and Z.-Y. Tang, "Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection," *J Clin Oncol*, vol. 25, pp. 2586–93, Jun 2007.

[15] T. J. Curiel, G. Coukos, L. Zou, X. Alvarez, P. Cheng, P. Mottram, M. Evdemon-Hogan, J. R. Conejo-Garcia, L. Zhang, M. Burow, Y. Zhu, S. Wei, I. Kryczek, B. Daniel, A. Gordon, L. Myers, A. Lackner, M. L. Disis, K. L. Knutson, L. Chen, and W. Zou, "Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival," *Nat Med*, vol. 10, pp. 942–9, Sep 2004.

[16] A. Facciabene, X. Peng, I. S. Hagemann, K. Balint, A. Barchetti, L.-P. Wang, P. A. Gimotty, C. B. Gilks, P. Lal, L. Zhang, and G. Coukos, "Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and T(reg) cells," *Nature*, vol. 475, pp. 226–30, Jul 2011.

[17] H. R. Ali, E. Provenzano, S.-J. Dawson, F. M. Blows, B. Liu, M. Shah, H. M. Earl, C. J. Poole, L. Hiller, J. A. Dunn, S. J. Bowden, C. Twelves, J. M. S. Bartlett, S. M. A. Mahmoud, E. Rakha, I. O. Ellis, S. Liu, D. Gao, T. O. Nielsen, P. D. P. Pharoah, and C. Caldas, "Association between CD8+ T-cell infiltration and breast cancer survival in 12,439 patients," *Ann Oncol*, vol. 25, pp. 1536–43, Aug 2014.

[18] C. Denkert, S. Loibl, A. Noske, M. Roller, B. M. Müller, M. Komor, J. Budczies, S. Darb-Esfahani, R. Kronenwett, C. Hanusch, C. von Törne, W. Weichert, K. Engels, C. Solbach, I. Schrader, M. Dietel, and G. von Minckwitz, "Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer," *J Clin Oncol*, vol. 28, pp. 105–13, Jan 2010.

[19] M. Tosolini, A. Kirilovsky, B. Mlecnik, T. Fredriksen, S. Mauger, G. Bindea, A. Berger, P. Bruneval, W.-H. Fridman, F. Pagès, and J. Galon, "Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, Th2, Treg, Th17) in patients with colorectal cancer," *Cancer Res*, vol. 71, pp. 1263–71, Feb 2011.

[20] R. J. Greenwald, G. J. Freeman, and A. H. Sharpe, "The B7 family revisited," *Annu Rev Immunol*, vol. 23, pp. 515–48, Apr 2005.

[21] I. Melero, W. W. Shuford, S. A. Newby, A. Aruffo, J. A. Ledbetter, K. E. Hellström, R. S. Mittler, and L. Chen, "Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors," *Nat Med*, vol. 3, pp. 682–5, Jun 1997.

[22] J. Mitsui, H. Nishikawa, D. Muraoka, L. Wang, T. Noguchi, E. Sato, S. Kondo, J. P. Allison, S. Sakaguchi, L. J. Old, T. Kato, and H. Shiku, "Two distinct mechanisms of augmented antitumor activity by modulation of immunostimulatory/inhibitory signals," *Clin Cancer Res*, vol. 16, pp. 2781–91, May 2010.

[23] A. H. Sharpe, E. J. Wherry, R. Ahmed, and G. J. Freeman, "The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection," *Nat Immunol*, vol. 8, pp. 239–45, Mar 2007.

[24] D. M. Pardoll, "The blockade of immune checkpoints in cancer immunotherapy," *Nat Rev Cancer*, vol. 12, pp. 252–64, Apr 2012.

[25] B. Li, M. VanRoey, C. Wang, T.-h. T. Chen, A. Korman, and K. Jooss, "Anti-programmed death-1 synergizes with granulocyte macrophage colony-stimulating factor–secreting tumor cell immunotherapy providing therapeutic benefit to mice with established tumors," *Clin Cancer Res*, vol. 15, pp. 1623–34, Mar 2009.

[26] F. S. Hodi, M. Butler, D. A. Oble, M. V. Seiden, F. G. Haluska, A. Kruse, S. Macrae, M. Nelson, C. Canning, I. Lowy, A. Korman, D. Lautz, S. Russell, M. T. Jaklitsch, N. Ramaiya, T. C. Chen, D. Neuberg, J. P. Allison, M. C. Mihm, and G. Dranoff, "Immunologic and clinical effects of antibody blockade of cytotoxic T lymphocyte-associated antigen 4 in previously vaccinated cancer patients," *Proc Natl Acad Sci U S A*, vol. 105, pp. 3005–10, Feb 2008.

[27] G. A. Rabinovich, D. Gabrilovich, and E. M. Sotomayor, "Immunosuppressive strategies that are mediated by tumor cells," *Annu Rev Immunol*, vol. 25, pp. 267–96, Jul 2007.

[28] M. Vanneman and G. Dranoff, "Combining immunotherapy and targeted therapies in cancer treatment," *Nat Rev Cancer*, vol. 12, pp. 237–51, Apr 2012.

[29] M. L. Disis, "Immune regulation of cancer," *J Clin Oncol*, vol. 28, pp. 4531–8, Oct 2010.

[30] L. M. Coussens and Z. Werb, "Inflammation and cancer," *Nature*, vol. 420, pp. 860–7, Dec 2002.

[31] V. Shankaran, H. Ikeda, A. T. Bruce, J. M. White, P. E. Swanson, L. J. Old, and R. D. Schreiber, "IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity," *Nature*, vol. 410, pp. 1107–11, Apr 2001.

[32] M. J. Smyth, G. P. Dunn, and R. D. Schreiber, "Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity," *Adv Immunol*, vol. 90, pp. 1–50, Mar 2006.

[33] S. M. A. Mahmoud, E. C. Paish, D. G. Powe, R. D. Macmillan, M. J. Grainge, A. H. S. Lee, I. O. Ellis, and A. R. Green, "Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer," *J Clin Oncol*, vol. 29, pp. 1949–55, May 2011.

[34] E. M. Shevach, "Certified professionals: CD4(+)CD25(+) suppressor T cells," *J Exp Med*, vol. 193, pp. F41–6, Jun 2001.

[35] W. Liu, A. L. Putnam, Z. Xu-Yu, G. L. Szot, M. R. Lee, S. Zhu, P. A. Gottlieb, P. Kapranov, T. R. Gingeras, B. Fazekas de St Groth, C. Clayberger, D. M. Soper, S. F. Ziegler, and J. A. Bluestone, "CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells," *J Exp Med*, vol. 203, pp. 1701–11, Jul 2006.

[36] T. Azuma, T. Takahashi, A. Kunisato, T. Kitamura, and H. Hirai, "Human CD4+ CD25+ regulatory T cells suppress NKT cell functions," *Cancer Res*, vol. 63, pp. 4516–20, Aug 2003.

[37] P. Trzonkowski, E. Szmit, J. Myśliwska, A. Dobyszuk, and A. Myśliwski, "CD4+CD25+ T regulatory cells inhibit cytotoxic activity of T CD8+ and NK lymphocytes in the direct cell-to-cell interaction," *Clin Immunol*, vol. 112, pp. 258–67, Sep 2004.

[38] H. W. Lim, P. Hillsamer, A. H. Banham, and C. H. Kim, "Cutting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells," *J Immunol*, vol. 175, pp. 4180–3, Oct 2005.

[39] J. Schulze Zur Wiesch, A. Thomssen, P. Hartjen, I. Tóth, C. Lehmann, D. Meyer-Olson, K. Colberg, S. Frerk, D. Babikir, S. Schmiedel, O. Degen, S. Mauss, J. Rockstroh, S. Staszewski, P. Khaykin, A. Strasak, A. W. Lohse, G. Fätkenheuer, J. Hauber, and J. van Lunzen, "Comprehensive analysis of frequency and phenotype of T regulatory cells in HIV infection: CD39 expression of FoxP3+ T regulatory cells correlates with progressive disease," *J Virol*, vol. 85, pp. 1287–97, Feb 2011.

[40] S. Sakaguchi and N. Sakaguchi, "Thymus and autoimmunity. Transplantation of the thymus from cyclosporin A-treated mice causes organ-specific autoimmune disease in athymic nude mice," *J Exp Med*, vol. 167, pp. 1479–85, Apr 1988.

[41] M. Papiernik, M. L. de Moraes, C. Pontoux, F. Vasseur, and C. Pénit, "Regulatory CD4 T cells: expression of IL-2R alpha chain, resistance to clonal deletion and IL-2 dependency," *Int Immunol*, vol. 10, pp. 371–8, Apr 1998.

[42] M. S. Jordan, A. Boesteanu, A. J. Reed, A. L. Petrone, A. E. Holenbeck, M. A. Lerman, A. Naji, and A. J. Caton, "Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide," *Nat Immunol*, vol. 2, pp. 301–6, Apr 2001.

[43] K. A. Weissler and A. J. Caton, "The role of T-cell receptor recognition of peptide: MHC complexes in the formation and activity of FoxP3+ regulatory T cells," *Immunol Rev*, vol. 259, pp. 11–22, May 2014.

[44] L. Zou, B. Barnett, H. Safah, V. F. Larussa, M. Evdemon-Hogan, P. Mottram, S. Wei, O. David, T. J. Curiel, and W. Zou, "Bone marrow is a reservoir for CD4+CD25+ regulatory T cells that traffic through CXCL12/CXCR4 signals," *Cancer Res*, vol. 64, pp. 8451–5, Nov 2004.

[45] M. A. Schneider, J. G. Meingassner, M. Lipp, H. D. Moore, and A. Rot, "CCR7 is required for the in vivo function of CD4+ CD25+ regulatory T cells," *J Exp Med*, vol. 204, pp. 735–45, Apr 2007.

[46] G. Borland and W. Cushley, "Positioning the immune system: unexpected roles for alpha6-integrins," *Immunology*, vol. 111, pp. 381–3, Apr 2004.

[47] B. Engelhardt, "Molecular mechanisms involved in T cell migration across the blood-brain barrier," *J Neural Transm*, vol. 113, pp. 477–85, Apr 2006.

[48] J. Wang, A. Ioan-Facsinay, E. I. H. van der Voort, T. W. J. Huizinga, and R. E. M. Toes, "Transient expression of FoxP3 in human activated nonregulatory CD4+ T cells," *Eur J Immunol*, vol. 37, pp. 129–38, Jan 2007.

[49] S. Sakaguchi, N. Sakaguchi, M. Asano, M. Itoh, and M. Toda, "Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases," *J Immunol*, vol. 155, pp. 1151–64, Aug 1995.

[50] F. Ramsdell and S. F. Ziegler, "FoxP3 and scurfy: how it all began," *Nat Rev Immunol*, vol. 14, pp. 343–9, May 2014.

[51] M. Miyara, Y. Yoshioka, A. Kitoh, T. Shima, K. Wing, A. Niwa, C. Parizot, C. Taflin, T. Heike, D. Valeyre, A. Mathian, T. Nakahata, T. Yamaguchi, T. Nomura, M. Ono, Z. Amoura, G. Gorochov, and S. Sakaguchi, "Functional

delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor," *Immunity*, vol. 30, pp. 899–911, Jun 2009.

[52] D. A. A. Vignali, L. W. Collison, and C. J. Workman, "How regulatory T cells work," *Nat Rev Immunol*, vol. 8, pp. 523–32, Jul 2008.

[53] G. J. Bates, S. B. Fox, C. Han, R. D. Leek, J. F. Garcia, A. L. Harris, and A. H. Banham, "Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse," *J Clin Oncol*, vol. 24, pp. 5373–80, Dec 2006.

[54] D. Mougiakakos, C. C. Johansson, E. Trocme, C. All-Ericsson, M. A. Economou, O. Larsson, S. Seregard, and R. Kiessling, "Intratumoral forkhead box P3-positive regulatory T cells predict poor survival in cyclooxygenase-2-positive uveal melanoma," *Cancer*, vol. 116, pp. 2224–33, May 2010.

[55] H. Tao, Y. Mimura, K. Aoe, S. Kobayashi, H. Yamamoto, E. Matsuda, K. Okabe, T. Matsumoto, K. Sugi, and H. Ueoka, "Prognostic potential of FoxP3 expression in non-small cell lung cancer cells combined with tumor-infiltrating regulatory T cells," *Lung Cancer*, vol. 75, pp. 95–101, Jan 2012.

[56] T. Yoshimura, "Discovery of IL-8/CXCL8 (the story from Frederick)," *Front Immunol*, vol. 6, p. 278, Jun 2015.

[57] B. Wang, C. Esche, A. Mamelak, I. Freed, H. Watanabe, and D. N. Sauder, "Cytokine knockouts in contact hypersensitivity research," *Cytokine Growth Factor Rev*, vol. 14, pp. 381–9, Oct 2003.

[58] G. E. Rovati, V. Capra, and R. R. Neubig, "The highly conserved DRY motif of class A G protein-coupled receptors: beyond the ground state," *Mol Pharmacol*, vol. 71, pp. 959–64, Apr 2007.

[59] L. Chen, H. M. Linden, B. O. Anderson, and C. I. Li, "Trends in 5-year survival rates among breast cancer patients by hormone receptor status and stage," *Breast Cancer Res Treat*, vol. 147, pp. 609–16, Oct 2014.

[60] J. Ferlay, H.-R. Shin, F. Bray, D. Forman, C. Mathers, and D. M. Parkin, "Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008," *Int J Cancer*, vol. 127, pp. 2893–917, Dec 2010.

[61] C. E. DeSantis, C. C. Lin, A. B. Mariotto, R. L. Siegel, K. D. Stein, J. L. Kramer, R. Alteri, A. S. Robbins, and A. Jemal, "Cancer treatment and survivorship statistics, 2014," *CA Cancer J Clin*, vol. 64, pp. 252–71, Jun 2014.

[62] G. C. Jayson, E. C. Kohn, H. C. Kitchener, and J. A. Ledermann, "Ovarian cancer," *Lancet*, vol. 384, pp. 1376–88, Oct 2014.

[63] E. R. Woodward, H. V. Sleightholme, A. M. Considine, S. Williamson, J. M. McHugo, and D. G. Cruger, "Annual surveillance by CA125 and transvaginal ultrasound for ovarian cancer in both high-risk and population risk women is ineffective," *BJOG: An International Journal of Obstetrics and Gynaecology*, vol. 114, pp. 1500–9, Dec 2007.

[64] L. Zhang, J. R. Conejo-Garcia, D. Katsaros, P. A. Gimotty, M. Massobrio, G. Regnani, A. Makrigiannakis, H. Gray, K. Schlienger, M. N. Liebman, S. C. Rubin, and G. Coukos, "Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer," *N Engl J Med*, vol. 348, pp. 203–13, Jan 2003.

[65] G. P. Dunn, A. T. Bruce, H. Ikeda, L. J. Old, and R. D. Schreiber, "Cancer immunoediting: from immunosurveillance to tumor escape," *Nat Immunol*, vol. 3, pp. 991–8, Nov 2002.

[66] G. P. Dunn, L. J. Old, and R. D. Schreiber, "The immunobiology of cancer immunosurveillance and immunoediting," *Immunity*, vol. 21, pp. 137–48, Aug 2004.

[67] A. Iellem, M. Mariani, R. Lang, H. Recalde, P. Panina-Bordignon, F. Sinigaglia, and D. D'Ambrosio, "Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells," *J Exp Med*, vol. 194, pp. 847–53, Sep 2001.

[68] C. Hermans, D. Anz, J. Engel, T. Kirchner, S. Endres, and D. Mayr, "Analysis of FoxP3+ T-regulatory cells and CD8+ T-cells in ovarian carcinoma: location and tumor infiltration patterns are key prognostic markers," *PLoS One*, vol. 9, p. e111757, Nov 2014.

[69] B. Eksteen, A. Miles, S. M. Curbishley, C. Tselepis, A. J. Grant, L. S. K. Walker, and D. H. Adams, "Epithelial inflammation is associated with CCL28 production and the recruitment of regulatory T cells expressing CCR10," *J Immunol*, vol. 177, pp. 593–603, Jul 2006.

[70] B. Homey, W. Wang, H. Soto, M. E. Buchanan, A. Wiesenborn, D. Catron, A. Müller, T. K. McClanahan, M. C. Dieu-Nosjean, R. Orozco, T. Ruzicka, P. Lehmann, E. Oldham, and A. Zlotnik, "Cutting edge: the orphan chemokine receptor G protein-coupled receptor-2 (GPR-2, CCR10) binds the skin-associated chemokine CCL27 (CTACK/ALP/ILC)," *J Immunol*, vol. 164, pp. 3465–70, Apr 2000.

[71] B. Homey, H. Alenius, A. Müller, H. Soto, E. P. Bowman, W. Yuan, L. McEvoy, A. I. Lauerma, T. Assmann, E. Bünemann, M. Lehto, H. Wolff, D. Yen, H. Marxhausen, W. To, J. Sedgwick, T. Ruzicka, P. Lehmann, and A. Zlotnik, "CCL27-CCR10 interactions regulate T cell-mediated skin inflammation," *Nat Med*, vol. 8, pp. 157–65, Feb 2002.

[72] Y. Mizukami, K. Kono, Y. Kawaguchi, H. Akaike, K. Kamimura, H. Sugai, and H. Fujii, "CCL17 and CCL22 chemokines within tumor microenvironment are related to accumulation of FoxP3+ regulatory T cells in gastric cancer," *Int J Cancer*, vol. 122, pp. 2286–93, May 2008.

[73] C. Chenivesse, Y. Chang, I. Azzaoui, S. Ait Yahia, O. Morales, C. Plé, A. Foussat, A.-B. Tonnel, N. Delhem, H. Yssel, H. Vorng, B. Wallaert, and A. Tsicopoulos, "Pulmonary CCL18 recruits human regulatory T cells," *J Immunol*, vol. 189, pp. 128–37, Jul 2012.

[74] J. Pan, E. J. Kunkel, U. Gosslar, N. Lazarus, P. Langdon, K. Broadwell, M. A. Vierra, M. C. Genovese, E. C. Butcher, and D. Soler, "A novel chemokine ligand for CCR10 and CCR3 expressed by epithelial cells in mucosal tissues," *J Immunol*, vol. 165, pp. 2943–9, Sep 2000.

[75] H. Nomiyama, T. Imai, J. Kusuda, R. Miura, D. F. Callen, and O. Yoshie, "Human chemokines fractalkine (SCYD1), MDC (SCYA22) and TARC (SCYA17) are clustered on chromosome 16q13," *Cytogenet Cell Genet*, vol. 81, pp. 10–1, Feb 1998.

[76] Y. Tasaki, S. Fukuda, M. Iio, R. Miura, T. Imai, S. Sugano, O. Yoshie, A. L. Hughes, and H. Nomiyama, "Chemokine PARC gene (SCYA18) generated by fusion of two MIP-1alpha/LD78alpha-like genes," *Genomics*, vol. 55, pp. 353–7, Feb 1999.

[77] S. A. Islam, M. F. Ling, J. Leung, W. G. Shreffler, and A. D. Luster, "Identification of human CCR8 as a CCL18 receptor," *J Exp Med*, vol. 210, pp. 1889–98, Sep 2013.

[78] J. Faget, C. Biota, T. Bachelot, M. Gobert, I. Treilleux, N. Goutagny, I. Durand, S. Léon-Goddard, J. Y. Blay, C. Caux, and C. Ménétrier-Caux, "Early detection of tumor cells by innate immune cells leads to T(reg) recruitment through CCL22 production by tumor cells," *Cancer Res*, vol. 71, pp. 6143–52, Oct 2011.

[79] I. Wertel, J. Surówka, G. Polak, B. Barczyński, W. Bednarek, J. Jakubowicz-Gil, A. Bojarska-Junak, and J. Kotarski, "Macrophage-derived chemokine CCL22 and regulatory T cells in ovarian cancer patients," *Tumour Biol*, Feb 2015.

[80] M. Roselli, V. Cereda, M. G. di Bari, V. Formica, A. Spila, C. Jochems, B. Farsaci, R. Donahue, J. L. Gulley, J. Schlom, and F. Guadagni, "Effects of conventional therapeutic interventions on the number and function of regulatory T cells," *Oncoimmunology*, vol. 2, p. e27025, Oct 2013.

[81] D. Sugiyama, H. Nishikawa, Y. Maeda, M. Nishioka, A. Tanemura, I. Katayama, S. Ezoe, Y. Kanakura, E. Sato, Y. Fukumori, J. Karbach,

E. Jäger, and S. Sakaguchi, "Anti-CCR4 mAb selectively depletes effector-type FoxP3+CD4+ regulatory T cells, evoking antitumor immune responses in humans," *Proc Natl Acad Sci U S A*, vol. 110, pp. 17945–50, Oct 2013.

[82] J. M. Coghill, K. A. Fowler, M. L. West, L. M. Fulton, H. van Deventer, K. P. McKinnon, B. G. Vincent, K. Lin, A. Panoskaltsis-Mortari, D. N. Cook, B. R. Blazar, and J. S. Serody, "CC chemokine receptor 8 potentiates donor Treg survival and is critical for the prevention of murine graft-versus-host disease," *Blood*, vol. 122, pp. 825–36, Aug 2013.

[83] H. L. Tang and J. G. Cyster, "Chemokine up-regulation and activated T cell attraction by maturing dendritic cells," *Science*, vol. 284, pp. 819–22, Apr 1999.

[84] Y.-Q. Li, F.-F. Liu, X.-M. Zhang, X.-J. Guo, M.-J. Ren, and L. Fu, "Tumor secretion of CCL22 activates intratumoral Treg infiltration and is independent prognostic predictor of breast cancer," *PLoS One*, vol. 8, p. e76379, Oct 2013.

[85] D. B. Hoelzinger, S. E. Smith, N. Mirza, A. L. Dominguez, S. Z. Manrique, and J. Lustgarten, "Blockade of CCL1 inhibits T regulatory cell suppressive function enhancing tumor immunity without affecting T effector responses," *J Immunol*, vol. 184, pp. 6833–42, Jun 2010.

[86] S. Das, E. Sarrou, S. Podgrabinska, M. Cassella, S. K. Mungamuri, N. Feirt, R. Gordon, C. S. Nagi, Y. Wang, D. Entenberg, J. Condeelis, and M. Skobe, "Tumor cell entry into the lymph node is controlled by CCL1 chemokine expressed by lymph node lymphatic sinuses," *J Exp Med*, vol. 210, pp. 1509–28, Jul 2013.

[87] O. Simonetti, G. Goteri, G. Lucarini, A. Filosa, T. Pieramici, C. Rubini, G. Biagini, and A. Offidani, "Potential role of CCL27 and CCR10 expression in melanoma progression and immune escape," *Eur J Cancer*, vol. 42, pp. 1181–7, May 2006.

[88] A. Pivarcsi, A. Müller, A. Hippe, J. Rieker, A. van Lierop, M. Steinhoff, S. Seeliger, R. Kubitza, U. Pippirs, S. Meller, P. A. Gerber, R. Liersch, E. Buenemann, E. Sonkoly, U. Wiesner, T. K. Hoffmann, L. Schneider, R. Piekorz, E. Enderlein, J. Reifenberger, U.-P. Rohr, R. Haas, P. Boukamp, I. Haase, B. Nürnberg, T. Ruzicka, A. Zlotnik, and B. Homey, "Tumor immune escape by the loss of homeostatic chemokine expression," *Proc Natl Acad Sci U S A*, vol. 104, pp. 19055–60, Nov 2007.

[89] H. Kai, T. Kadono, T. Kakinuma, M. Tomita, H. Ohmatsu, Y. Asano, Y. Tada, M. Sugaya, and S. Sato, "CCR10 and CCL27 are overexpressed in cutaneous squamous cell carcinoma," *Pathol Res Pract*, vol. 207, pp. 43–8, Jan 2011.

[90] C. Monteagudo, D. Ramos, A. Pellín-Carcelén, R. Gil, R. C. Callaghan, J. M. Martín, V. Alonso, A. Murgui, L. Navarro, S. Calabuig, J. A. López-Guerrero, E. Jordá, and A. Pellín, "CCL27-CCR10 and CXCL12-CXCR4 chemokine ligand-receptor mRNA expression ratio: new predictive factors of tumor progression in cutaneous malignant melanoma," *Clin Exp Metastasis*, vol. 29, pp. 625–37, Aug 2012.

[91] B. Ondondo, E. Colbeck, E. Jones, K. Smart, S. N. Lauder, J. Hindley, A. Godkin, B. Moser, A. Ager, and A. Gallimore, "A distinct chemokine axis does not account for enrichment of FoxP3(+) CD4(+) T cells in carcinogen-induced fibrosarcomas," *Immunology*, vol. 145, pp. 94–104, May 2015.

[92] M. Beyer and J. L. Schultze, "Regulatory T cells in cancer," *Blood*, vol. 108, pp. 804–11, Aug 2006.

[93] K. Wing and S. Sakaguchi, "Regulatory T cells exert checks and balances on self tolerance and autoimmunity," *Nat Immunol*, vol. 11, pp. 7–13, Jan 2010.

[94] H.-Y. Zhang and H. Sun, "Up-regulation of FoxP3 inhibits cell proliferation, migration and invasion in epithelial ovarian cancer," *Cancer Lett*, vol. 287, pp. 91–7, Jan 2010.

[95] T. Zuo, L. Wang, C. Morrison, X. Chang, H. Zhang, W. Li, Y. Liu, Y. Wang, X. Liu, M. W. Y. Chan, J.-Q. Liu, R. Love, C.-G. Liu, V. Godfrey, R. Shen, T. H.-M. Huang, T. Yang, B. K. Park, C.-Y. Wang, P. Zheng, and Y. Liu, "FoxP3 is an X-linked breast cancer suppressor gene and an important repressor of the Her-2/ErbB2 oncogene," *Cell*, vol. 129, pp. 1275–86, Jun 2007.

[96] J. D. Fontenot, M. A. Gavin, and A. Y. Rudensky, "FoxP3 programs the development and function of CD4+CD25+ regulatory T cells," *Nat Immunol*, vol. 4, pp. 330–6, Apr 2003.

[97] M. Ahmadzadeh, A. Felipe-Silva, B. Heemskerk, D. J. Powell, Jr, J. R. Wunderlich, M. J. Merino, and S. A. Rosenberg, "FoxP3 expression accurately defines the population of intratumoral regulatory T cells that selectively accumulate in metastatic melanoma lesions," *Blood*, vol. 112, pp. 4953–60, Dec 2008.

[98] M. Kmieciak, M. Gowda, L. Graham, K. Godder, H. D. Bear, F. M. Marincola, and M. H. Manjili, "Human T cells express CD25 and FoxP3 upon activation and exhibit effector/memory phenotypes without any regulatory/-suppressor function," *J Transl Med*, vol. 7, p. 89, Oct 2009.

Index

*

* *