

# Tumor Immunotherapy by Utilizing a Double-Edged Sword, Chemokines

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Tumor immunotherapy by utilizing a double-edged sword, chemokines

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**Abstract**

Both innate and adaptive immune responses have an essential role in protection against tumor cells. Various types of immune cells such as dendritic cells and lymphocytes contribute to the establishment of immune responses to tumor cells. Chemokines, a family consisting of more than 40 related chemoattractant proteins, have a crucial role in the control of the recruitment of immune cells needed for the induction and activation of tumor immunity. Based on these properties, several chemokines have been utilized in pre-clinical models to augment tumor immunity by enhancing the migration and activation of immune cells. Paradoxically, tumor tissues use chemokines to evade immunosurveillance by attracting immune suppressive cells. Moreover, chemokines can mediate survival and migration of tumor cells, and promote new blood vessel formation, thereby leading to tumor progression and metastasis. Thus, a number of therapeutic strategies have been proposed to target chemokines, in order to reduce tumor progression and metastasis, although these strategies have not yet been translated to clinical situations. Here, we will briefly summarize the pre-clinical results obtained by using and/or targeting chemokines to combat tumors and discuss the potential efficacy of these methods.

## Introduction

Chemokines are heparin-binding proteins characterized by the presence of 4 cysteine residues in the conserved positions [1](Moser 2004). Two intermolecular disulfide bonds are formed between the first and third cysteines, and between the second and fourth cysteines, and these bonds result in the formation of triple-stranded  $\beta$ -sheet structures, while the carboxyl-terminal region forms an  $\alpha$ -helix form [2](Fernandez 2002). Thus, although overall sequence similarities are not high among chemokines, they exhibit a similar three-dimensional structure. Chemokines exert their biological activities by binding their cognate receptors, which belong to G-protein coupled receptor (GPCR) with 7-span transmembrane portions [1]. Thus, the target cell specificity of each chemokine is determined by the expression pattern of its corresponding receptor. At their high concentrations, chemokines tend to dimerize by forming hydrogen bonds between their  $\beta$ -sheet structures (Jansma 2009)[3]. The current consensus is that monomeric forms of chemokines are sufficient for receptor binding to induce cell migration. It still remains elusive on the functions of dimerized chemokines, although the dimer is assumed to be associated with other complex functional roles (Jansma 2009)[3]. Moreover, through the carboxyl-terminal region with the capacity to bind heparin, chemokines can bind to proteoglycans and glycosaminoglycans with a high avidity. Consequently, most chemokines are produced as a secretory proteins, but upon their secretion, they can be immobilized on endothelium cells and in extracellular matrix by interacting with proteoglycans and glycosaminoglycans (Fernandez 2002)[2]. The immobilization facilitates the generation of a concentration gradient, which is crucial for inducing the target cells to migrate in a directed way.

Based on their structure, chemokines are classified into 4 subgroups, namely, CXC, CC, CX<sub>3</sub>C and C (Moser 2004) [1] (Table 1). The first 2 cysteines are separated by 1 and 3 amino acids in CXC and CX<sub>3</sub>C chemokines, respectively, while the first 2 cysteines are adjacent in CC chemokine. The C chemokine lacks the second and the fourth cysteines. Systematic chemokine nomenclature is based on their cysteine subclass roots, followed by “L” for “ligand” (Zlotnik 2000)[4]. The numbers correspond generally to the same number

used in the corresponding gene nomenclature. Because most chemokine receptors can bind to a single chemokine subclass, the nomenclature system of chemokine receptors is rooted by the chemokine subclass specificity, followed by “R” for “receptor” and the number (Zlotnik 2000)[4] (Table 1). The CXC chemokines are further grouped based on the presence or the absence of a 3-amino acid sequence, glutamic acid-leucine-arginine (the “ELR” motif), immediately preceding the CXC sequence (Vandercappellen 2008)[5]. In general, CXC chemokines with the ELR motif can bind CXCR1 and/or CXCR2, and exhibit an angiogenic and a neutrophil chemotactic activity (Vandercappellen 2008)[5].

Chemokines can be classified as inflammatory, homeostatic, or both, based on their expression pattern (Mantovani 2006)[6]. Various types of inflammatory stimuli induce the expression of inflammatory chemokines, which have a crucial role in the infiltration of inflammatory cells including granulocytes and monocytes/macrophages. Representative inflammatory chemokines are CXC chemokines with ELR motif and CCL2. On the contrary, homeostatic chemokines are expressed constitutively in specific tissues or cells. They are involved in organogenesis of various organs including lymph nodes, as they have key roles in stem cell migration. Moreover, most homeostatic chemokines can regulate the trafficking of immune cells such as lymphocytes and dendritic cells, and eventually adaptive immunity.

The human and mouse genomes contain over 44 and 38 different chemokine genes, respectively (Nomiyama 2010)[7]. There is a difference in gene numbers with some ambiguities of orthologous relationship between the human and mouse chemokine family. These observations would indicate that the chemokine gene family has been rapidly evolving, resulting in species-specific expansions and contractions. A notable difference has been found in one of the major chemokine, CXCL8, and its receptors, CXCR1 and CXCR2. Mice and rats do not possess a homolog of the *CXCL8/IL-8* gene, which is present in other species including humans, rabbits, cats, and dogs (Nomiyama 2010)[7]. Moreover, the *CXCR1* and *CXCR2* genes encode functional receptor proteins in humans, whereas there still remains a question on the presence of functional CXCR1 in mice or rats (Moepps 2006)[8]. Different expression patterns between humans and mice were observed also on

other chemokine receptors such as CCR1 (Su 1996)[9]. These observations should be taken into consideration when the findings obtained with mouse models are extrapolated to human conditions.

### **Chemokine receptor signaling (Figure 1)**

Approximately 20 signaling chemokine receptors have been identified as well as several non-signaling receptors (Table 1) (Allen 2007)[10]. The presence of a DRY motif in the second transmembrane region is responsible for the ability of chemokine receptors to signal upon ligand binding, and non-signaling signaling receptors lack this motif. Chemokine receptors are coupled with heterotrimeric  $G\alpha\beta\gamma$  proteins bound to intracellular loops. The  $G\alpha$  subunit contains a GTPase domain involved in binding and hydrolysis of GTP. In the inactive state, the  $G\alpha$  subunit binds GDP, and interacts directly with the intracellular loop of chemokine receptors and with  $G\beta$  subunit, which in turn forms a tight complex with  $G\gamma$  subunit. A two-step model has been proposed for activation of the receptor (Fernandez 2002)[2]. In the first step, a chemokine specifically recognizes and binds the receptor. Consequently, the amino-terminus of the chemokine interacts with the receptor, leading to the activation of the receptor. Simultaneously, ligand binding induces internalization of the chemokine receptor by using the clathrin-mediated pathway or the lipid rafts/caveole-dependent internalization routes (Neel 2005)[11]. Internalized receptors are recycled and reappear on the cell surface quickly. However, it still remains controversial on the necessity of internalization and recycling for chemokine-mediated signaling and chemotaxis.

The activation induces dissociation of GDP from  $G\alpha$  and replacement of GTP.  $G\alpha$ -GTP eventually dissociates from the receptor and the  $G\beta\gamma$  heterodimer, and both complexes activate a series of downstream effectors (Figure 1). Generated  $G\beta\gamma$  heterodimer recruits and activates phosphatidylinositol 3-kinase- $\gamma$  (PI3K- $\gamma$ ), which in turn generates phosphatidylinositol 3,4,5-trisphosphate (PIP<sub>3</sub>) (Servant 2000)[12]. PIP<sub>3</sub> activates protein kinase B (Akt) as well as small GTPase such as Rac and Rho (Figure 1). In addition, active  $G\alpha$  and  $G\beta\gamma$  facilitate the polarization of the cells with the leading edge (pseudopodium) in

the front and the formation of a trailing tail (uropod) at the back. PI3K and Rac accumulate at the leading edge to induce actin polymerization and F-actin formation (Ridley 2003)[13]. Simultaneously, Rho and its effector molecules accumulate at the trailing edge to facilitate actomyosin contraction and tail retraction, thereby leading to the migration of the cells.

GPCR-mediated signals can be down-regulated by regulators of G protein signaling (RGS) proteins. RGS proteins are a family consisting of 20 members and can activate GTPase activities. RGS proteins directly interact with GTP-bound  $G\alpha$  subunit to catalyze GTP hydrolysis and G protein downregulation and eventually decrease the half-life of the active GTP-bound state of  $G\alpha$ . RGS1, RGS3, and RGS4 attenuated CXCL8-mediated signals in neutrophils (Druey 1996)[14] while RGS1 and RGS13 reduces CXCL12- and CXCL13-mediated signals in B cells (Shi 2002, Le 2005)[15, 16].

The binding of a chemokine to its corresponding receptor exposes the tyrosine residue in DRY motif in the second transmembrane region (Mellado 1998, Rodríguez-Frade 2001)[17, 18]. This exposure allows access of Janus kinase, which activates the receptor by tyrosine phosphorylation. Simultaneous activation of Janus kinase leads to the recruitment of STAT (signal transducers and activators of transcription) and eventually STAT-mediated expression of the target genes [17, 18](Mellado 1998, Rodríguez-Frade 2001) (Figure 1). Moreover, this pathway requires ligand-induced homodimerization of chemokine receptors, as observed on other GPCRs that can frequently exist as dimers and/or high-order oligomers (Breitwieser 2004)[19]. In the case of CCR5, Ile52 in transmembrane region-1 (TM1) and Val150 in TM4 are key residues in the interaction surface between CCR5 molecules (Hernanz-Falcon 2004)[20]. Moreover, mutation in these residues generates nonfunctional receptors that cannot dimerize or trigger signaling. Similar regions in CCR2 receptor are required for CCL2-induced homodimerization and subsequent activation (Rodríguez-Frade 1999)[18].

It is widely accepted that even distantly related GPCRs can form heterodimers (Breitwieser 2004)[19]. Indeed, heterodimerization is also observed among several chemokine receptors including CCR2, CCR5, CXCR2, and CXCR4 (Rodríguez-Frade 2001)[21]. For example, the heterodimerization of CCR2 with CCR5 cooperates to trigger

calcium influx at concentrations 10- to 100-fold lower than the threshold for either CCL2, a ligand for CCR2, and or CCL5, a ligand for CCR5 (Mellado 2001)[22]. However, it recruits a dissimilar signaling pathway such as  $G_{\alpha/11}$  association and delays activation of PI3-K. The consequences are triggering of cell adhesion rather than chemotaxis. In the case of CCR2/CXCR4 heterodimers, specific antagonists of one receptor inhibit the binding of chemokines to other receptor both in recombinant cell lines and primary leukocytes (Sohy 2007)[23]. This results in a significant functional cross-inhibition in terms of calcium mobilization and chemotaxis. Thus, chemokine receptor antagonists can regulate allosterically the functions of receptors, which they do not directly bind. These observations may have important implications for the effects of these antagonists.

Ras and its downstream signaling pathway, mitogen activated protein kinase (MAPK)/Erk kinase pathway, can be activated by several chemokine receptors including CXCR1, CXCR2 (Knall 1996)[24], and CXCR4 (Barbero 2003)[25] (Figure 1). The activation is frequently observed in tumor cells and leads to gene expression and cell proliferation. Moreover, activation of CXCR4 stimulates ovarian cancer cell growth through transactivation of the epidermal growth factor receptor (Porcile 2005)[26]. The activation of these signaling pathways may favor tumor cell proliferation.

### **Effector cells in tumor immunity and chemokines**

Accumulating evidence indicates the presence of cytotoxic T lymphocytes (CTLs) that can specifically recognize tumor-associated antigens (TAA) and attack tumor cells in humans as well as in mice (Knutson 2005)[27] (Figure 2). In this immunological approach to cancer, antigen-presenting cells can deliver TAAs and prime TAA-specific T cells. Dendritic cells (DCs) are professional antigen-presenting cells and can express on their cell surface major histocompatibility complex (MHC) class I and II molecules, and as well as co-stimulatory molecules, all of which assist in T cells activation (Palucka 2012)[28]. DCs are widely distributed over peripheral tissues, and DCs in peripheral tissues are in an immature state and have a high capacity to endocytose various materials (Sozaani 2005)[29]. In periphery, DCs capture exogenous and endogenous antigens including tumor



cell-derived antigens (Figure 2). When DCs capture antigens in the absence of inflammatory cues such as Toll-like receptor-mediated signals, they failed to increase the expression of co-stimulatory molecules and to present antigens efficiently. On the contrary, when DCs capture antigens in the presence of inflammatory stimuli, they change to a mature state with a loss of endocytosis ability and start to migrate into the T cell areas of regional lymph nodes via afferent lymphatic venules under the guidance of chemokines (Figures 2 and 3). Mature DCs process the antigens into the peptides presented on MHC molecules, exhibit enhanced expression of co-stimulatory molecules, and induce primary immune responses through antigen presentation to T cells in the regional lymph node (Sozaani 2005)[29] (Figure 3). Immature DCs in peripheral tissues express various chemokine receptors including CCR1, CCR2, CCR4, CCR5, CCR6, CCR8, and CXCR4, whereas mature DCs express a limited set of chemokine receptors, CCR7 and CXCR4 (Sozaani 2005) [29] (Figure 3).

CCR7 and its ligands, CCL19 and CCL21, have a pivotal role in DC migration to lymph nodes in both steady state and inflammatory conditions (Förster, 1999)[30] although the contribution of another chemokine receptor, CCR8, cannot be excluded (Qu 2004)[31]. Antigen-pulsed CCR7<sup>+/+</sup> but not CCR7<sup>-/-</sup> DCs migrate efficiently to the draining lymph nodes when an antigen is injected intravenously (Martin-Fontecha 2003)[32]. Moreover, DC migration is markedly enhanced when intranodal CCL21 expression is augmented by pretreatment with interleukin (IL)-1 or tumor necrosis factor (TNF). Furthermore, the magnitude and quality of T cell response is proportional to the number of antigen-carrying DCs in the lymph nodes (Martin-Fontecha 2003)[32]. Furthermore, DCs can produce the chemokines which affect the trafficking and functions of natural killer (NK) cells, a main executor of innate immunity-mediated tumor cell killing (Sozaani 2005) [29].

Once generated in the regional lymph nodes, TAA-specific CTLs should migrate to tumor sites to kill tumor cells (Figure 2). Numerous clinical studies have indicated that the presence of CD3<sup>+</sup> or CD8<sup>+</sup> tumor-infiltrating lymphocytes (TILs) has a positive prognostic influence on survival (Gooden 2011)[33]. Most TILs are deemed to possess cytotoxic activities against tumor cells. Evidence is accumulating to indicate that several

chemokines regulate the migration of CTLs into tumor sites. CXCR3 is deemed to be a major chemokine receptor expressed by TILs. In a mouse model, increased expression of ligands for CXCR3, CXCL9 and CXCL10, can elicit antitumor response accompanied with an enhanced infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes (Pan 2006)[34]. In line with this observation, in human gastric and colorectal cancer, TILs express CXCR3 (Musha 2005, Ohtani 2009, Muthuswamy 2012).[35, 36, 37]. Moreover, high levels of CXCL9 and CXCL10, ligands for CXCR3, are produced by stromal cells, mainly macrophages (Ohtani 2009).[36]. CD8<sup>+</sup> TILs also express CCR5 (Musha 2005, Muthuswamy 2012)[35, 37]. Concomitantly, CD8<sup>+</sup> TIL numbers correlate well with the expression of CCL5, a ligand for CCR5, by tumor tissues (Muthuswamy 2012)[37]. TILs express other chemokine receptors, CX3CR1 and the expression of its ligand, CX3CL3, is elevated in tumor cells in colorectal cancer tissues (Ohta 2005)[38]. Furthermore, the expression level of CXCL16 also correlates with CD4<sup>+</sup> and CD8<sup>+</sup> TIL numbers with a better prognosis although cells expressing CXCR6, a receptor for CXCL16, are not identified (Hojo 2007)[39]. Thus, CXCL9, CXCL10, CXCL16, CCL5, and CX3CL1 can be used to efficiently mobilize CTLs from regional lymph nodes to tumor tissues with an objective to enhance CTL-mediated tumor destruction.

NK cells are unconventional lymphocytes and were initially identified as a leukocyte to kill tumor cells without any antigen stimulation (Vivier 2008)[40]. Mouse and human NK cells can in vitro kill a broad range of tumor cells of both hematopoietic and non-hematopoietic origin by utilizing perforin and secreting interferon (IFN)- $\gamma$  (Vivier 2008)[40]. Moreover, in vivo, mouse NK cells can eliminate many transplantable and spontaneous tumors. Distinct sets of chemokine receptors are utilized for NK cell trafficking (Table 1). NK cells migrate to lymph nodes mainly by utilizing CXCR3 and CCR7, while their migration to the inflamed tissues including tumor sites involves CCR1, CCR2, CCR5, CXCR3, and CX3CR1 (Walzer 2011)[41]. Thus, the ligands for these receptors can regulate NK cell trafficking and augment their functions. However, in colorectal tumor tissues, NK cells are scarce despite a significant lymphocyte infiltration, even in the presence of high levels of CXCL9, CXCL10, CCL3, CCL4, CCL5, and CX3CL1

(Halama 2011)[42]. These observations suggest that NK cell migration into tumor tissues is impaired early during tumor development by the mechanism that do not affect TIL trafficking.

### **Chemokine-mediated enhancement in tumor immunity**

As discussed above, the establishment of tumor immunity is a process consisting of multiple steps; migration of DCs to tumor sites, capture of tumor antigens by DCs, migration of DCs to regional lymph nodes, antigen presentation to effector cells by DCs in regional lymph nodes, and migration of effector cells to tumor sites (Figure 2). Chemokines have profound effects on tumor immunity, particularly migration steps.

The appearance of apoptotic cells induces the migration of immature dendritic cells to the tumor tissues. Accumulated immature dendritic cells capture TAAs and migrate to draining lymph nodes, where DCs present antigens to induce specific CTLs (Figure 2). Tumor-infiltrating DCs expressed CCR1 and CCR5, and a ligand for these receptors, CCL3, was abundantly detected in mouse bearing hepatocellular carcinoma (HCC) (Iida 2008)[43]. Moreover, DCs in tumor sites and lymph nodes, and subsequent cytotoxicity generation were reduced in CCR1-, CCR5-, or CCL3-deficient mice (Iida 2008)[43]. These observations may mirror the capacity of CCL3 to mobilize immature DCs to peripheral blood from bone marrow by interacting with CCR1 or CCR5 (Zhang 2004)[44]. Actually, systemic administration of CCL3 increased the numbers of DCs in peripheral blood and tumor tissues, and concomitantly augmented antitumor effects after radiofrequency ablation of murine HCCs (Iida 2010)[45]. These observations suggest that CCL3 may be effective to enhance tumor immunity by inducing the migration of immature DCs through peripheral blood to dying tumor cells.

The interaction between CCR7 and its ligands, CCL19 and CCL21, regulates DC migration to lymph nodes for antigen presentation to naïve T cells, which also utilize CCR7-mediated mechanisms to enter T cell zone (Förster, 1999)[30]. Moreover, CCL19 and CCL21 can attract NK cells to the lymph node. These observations suggest the potency of these chemokines to enhance acquired and innate immunity against various antigens

including TAAs. Indeed, when CCL21 was injected into a regional lymph node of SV40-transgenic mice that developed bilateral multifocal lung adenocarcinomas, it increased CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes as well as DCs at lymph nodes and tumor sites, and eventually led to a marked reduction in tumor burdens with enhanced survival (Sharma 2001)[46]. Similar results were also obtained when CCL19 was injected intranodally into SV40-transgenic mice (Hillinger 2006)[47].

*Ex vivo* generated DC have a very limited capacity to move from the injected sites to locally draining lymph nodes (Chang 2002)[48]. This limitation may account for a clinical weakness in DC-based vaccines. The capacity of CCL19 and CCL21 to effectively induce DC migration prompted the use of these chemokines to modify *ex vivo* generated DCs. Intratumoral injection of *CCL21* gene-modified DCs resulted in tumor growth inhibition that was significantly better than unmodified control DCs (Kirk 2001a)[49], together with intratumoral accumulation of DCs and T cells (Kirk 2001b)[50]. Moreover, even when *CCL21* gene-modified DCs were pulsed with tumor lysates and subsequently injected subcutaneously to tumor-free sites in tumor-bearing mice, it elicited an antitumor response (Kirk 2001a)[49]. These promising preclinical results have led to ongoing phase I clinical trials (Baratelli 2008)[51].

Intratumoral administration of *CCL21* gene-modified DCs reduced tumor burden in spontaneous murine lung carcinoma, accompanied with extensive T cell infiltration, and the enhanced elaboration of IFN- $\gamma$ , IL-12, CXCL9, and CXCL10 (Yang 2004)[52]. Moreover, *in vivo* depletion of either CXCL9 or CXCL10 significantly reduced the antitumor efficacy of *CCL21* gene-modified DCs. This may mirror the fact that CXCR3 is highly expressed by activated effector CD8<sup>+</sup> T cells and Th1-type CD4<sup>+</sup> T cells (Groom 2011)[53]. CXCL10 gene transduction into tumor cells had few effects on *in vitro* tumor cell proliferation but *in vivo* elicited a potent T cell-dependent antitumor response (Luster 1993)[54]. Likewise, tumor cells expressing CXCL10, induced the infiltration of tumor-specific cytotoxic T cells into the tumor site (Yang 2006)[55]. Moreover, tumor cells induced these cytotoxic T cells to proliferate and to produce high level of IFN- $\gamma$ , while CXCL10 expanded these tumor-specific T cells. Gene transduction of another ligand for

CXCR3, CXCL11, into tumor cells, also retarded *in vivo* tumor growth accompanied by intratumoral infiltration of CD8<sup>+</sup> cells (Hensbergen 2005)[56]. As T cells rapidly acquire CXCR3 expression upon activation with IL-2 (Groom 2011)[53], combined strategy of systemic IL-2 with intratumor CXCL9 administration was proven to be more efficacious than either cytokine alone, for augmenting tumor-associated immunity (Pan 2006)[34]. Thus, CXCR3-binding chemokines can be utilized to redirect the migration of effector T cells to tumor sites.

Muthuswamy observed that colorectal tumors with reduced accumulation of CD8<sup>+</sup> effector cells express low levels of CXCL10 and CCL5, the chemokine with potent chemoattractant activities for CD8<sup>+</sup> effector cells (2012)[37]. They demonstrated that a combination of IFN- $\alpha$  and a TLR3 ligand, poly-I:C, can uniformly enhance the production of CXCL10 and CCL5. Moreover, these effects can be optimized by the further addition of cyclooxygenase (COX)-2 inhibitors. Of interest is that this triple combination also consistently suppresses the production of a ligand for CCR4, CCL22, a chemokine associated with Treg infiltration. Thus, this strategy can enhance the intratumoral trafficking of CD8<sup>+</sup> effector T cells and can simultaneously reduce that of Treg cells, thereby augmenting local tumor immunity.

CCL2 protein was initially isolated as a factor which can augment monocyte-mediated tumor cytostatic activity and can exhibit monocyte chemotactic activity (Matsushima 1989)[57]. Indeed, tumor formation was suppressed *in vivo* but not *in vitro* when the tumor was genetically engineered to express *CCL2* gene (Rollins 1991)[58]. *CCL2*-expressing cells elicited a predominantly monocytic infiltrate at the site of injection, suggesting the roles of infiltrating monocytes in tumor rejection process (Rollins 1991)[58]. In addition to monocytes/macrophages, a receptor for *CCL2*, *CCR2*, is expressed by additional types of leukocytes such as NK cells (Table 1). *CCL2* gene transduction into tumor cells retarded tumor growth *in vivo* by inducing NK infiltration into tumor sites (Nokihara 2000)[59]. Moreover, NK cell infiltration was associated with elevated Th1 response in tumor sites (Tsuchiyama 2007)[60], suggesting that *CCL2* can regulate the

infiltration and activation of Th1 cells in tumor sites through NK cell recruitment and activation.

Tumor formation was also suppressed *in vivo* when mouse lymphoma cell lines were transduced with the gene of another chemokine, CX3CL1 (Lavergne 2003)[61]. This antitumor response was abolished in NK cell-deficient beige mice but not in T- and B-cell-deficient Rag1<sup>-/-</sup> mice, indicating the indispensable roles of NK but not T cells. Gene therapy using *CX3CL1* gene could activate T cells as well as NK cells to exert its antitumor responses (Tang 2007, Zeng 2007)[62, 63]. Moreover, intratumoral injection of a DNA plasmid coding for a chimeric immunoglobulin presenting CX3CL1 chemokine domain provided strong antitumor activity (Lavergne 2003)[64]. The administration of this fusion protein with tumor antigens, induced a strong *in vivo* antigen-specific T cell proliferation and effector function, accompanied with myeloid DC accumulation (Iga 2007)[64]. Thus, CX3CL1 can redirect T cells and DCs as well as NK cells, thereby augmenting adaptive immunity to tumor antigens.

In order to enhance the capacity to move to tumor sites by utilizing the chemokine(s) produced by tumor cells, several groups genetically engineered T cells to express the corresponding chemokine receptor. The Reed-Sternberg cells of Hodgkin lymphoma predominantly produce CCL17 and CCL22, which preferentially attract CCR4-expressing Th2 and Treg cells (van den Berg 1999)[65]. On the contrary, effector CD8<sup>+</sup> T cells lack CCR4. When CD8<sup>+</sup> cells were forced to express CCR4, these cells migrated more efficiently to Hodgkin lymphoma site. Moreover, tumor formation was more effectively inhibited by the administration of T lymphocytes expressing CCR4 and a chimeric antigen receptor directed to the Hodgkin lymphoma-associated antigen CD30 (di Stasi 2009)[66]. Similarly, CCL2 was highly secreted by malignant pleural mesothelioma cells, but CCR2 was minimally expressed on activated human T cells transduced with a chimeric antibody receptor (CAR) directed to mesothelioma tumor antigen, mesothelin (mesoCAR T cells) (Moon 2011)[67]. *CCR2* gene-transduced mesoCAR T cells exhibited enhanced antitumor responses accompanied with augmented T cell infiltration into tumor sites, when they were given intravenously [67]. This novel gene therapy technology using a

chemokine receptor can effectively enhance the migration of adoptively transferred T cells into tumor sites, where a corresponding chemokine is expressed abundantly.

### **Reversal of suppressor cell-mediated immune suppression by targeting chemokines**

Tumor immunity can frequently induce immune suppressive mechanisms to dampen the “immunity to self”. Thus, tumor immunity can be reduced by the action of several negative immunoregulatory receptors such as cytotoxic T lymphocyte antigen-4 (CTLA-4) and the programmed death receptor-1 (PD-1)-PD ligand-1 (PD-L1) axis. Indeed, evidence is accumulating to indicate that the antagonizing monoclonal antibodies to CTLA-4, PD-1, or PD-L1, are effective against various types of cancer even at advanced stages (Sarnaik 2009, Ribas 2012)[68, 69]. These observations indicate that targeting tumor-induced immune suppression can be effective to enhance tumor immunity.

Tumor tissues contain the leukocytes that can diminish tumor immunity. The most predominant subset is tumor-associated macrophages (TAMs) (Sica 2008)[70]. Circulating monocytes are mostly the precursor of these TAMs and are attracted into tumor sites, by chemotactic factors including CCL2, CCL5, CCL7, CCL8, CXCL12, and macrophage colony stimulating factor (M-CSF), which are produced in tumor tissues (Sica 2008)[70] (Figure 4). In human colorectal cancer tissues, macrophage accumulation increases with tumor stages and correlates with CCL2 expression in tumor sites (Bailey 2007)[71]. Thus, CCL2-induced TAM infiltration can have a pro-tumorigenic activity.

Hypoxia in tumor microenvironment induces TAMs to produce abundantly vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), two potent angiogenic factors (Sica 2008)[70]. Moreover, a fraction of TAMs can be incorporated into tumor vasculature (Kim 2009)[72]. TAMs are frequently polarized into M2 phenotypes under the influence of various factors present in tumor microenvironment, such as IL-4, IL-10, and prostaglandins (PGs) (Ruffell 2012) [73]. M2 phenotype is characterized by the expression of arginase (Arg)-1 and inducible NO synthase (iNOS), the enzymes responsible for the generation of reactive oxygen species (ROS), which can inhibit CTL proliferation [73](Ruffell 2012). TAMs can additionally produce IL-10 and

TGF- $\beta$  to promote the generation of another immunosuppressive cells, regulatory T cells (Treg) (Sica 2008)[70], while they can also produce CCL22 to induce intratumoral Treg migration (Curiel 2004)[74]. Moreover, a fraction of TAMs express B7-H4 on their surface to inhibit CTL proliferation (Kryczek 2006)[75]. These properties endow TAMs with an immunosuppressive capacity. Thus, TAMs can promote tumor progression by inducing angiogenesis and suppression of adaptive and innate anti-tumor immunity (Figure 4).

Systemic delivery of neutralizing anti-CCL2 antibody attenuated tumor burdens in human prostate cancer-bearing mice although its effects of TAMs have not been examined (Loberg 2007)[76]. Combined treatment of azoxymethane and repeated dextran sodium sulfate solution ingestion caused multiple tumors in murine colons, together with a massive infiltration of monocytes/macrophages expressing COX-2, an enzyme crucially involved in colon carcinogenesis (Popivanova 2009)[77]. CCL2 was abundantly detected in colon tissues and induced CCR2-positive COX-2 expressing monocytes/macrophages to infiltrate colon tissues and blocking CCL2 retarded tumor progression with reduced macrophage infiltration (Popivanova 2009)[77]. CCL2 also recruited monocytes to pulmonary metastatic sites of murine breast cancer [78]. As a consequence, infiltrated monocytes promoted the extravasation of tumor cells, a prerequisite step for metastasis, in a process that required monocyte-derived VEGF and CCL2 blockade markedly reduced lung metastasis (Qian 2011).

Myeloid-derived suppressor cells (MDSCs) is are an additional type of cells characterized by a strong ability to suppress various T cell functions (Condamine 2011)[79]. MDSCs represent a heterogenic population of immature myeloid cells that consists of precursors of macrophages, granulocytes, and dendritic cells. In mice, MDSCs are characterized by the co-expression of two distinct myeloid-cell lineage differentiation antigens, Gr-1 and CD11b in mouse (Condamine 2011)[79]. In humans, MDSCs are defined as CD14<sup>-</sup>CD11b<sup>+</sup> cells or as cells that express the common myeloid marker CD33 but lack the expression markers of mature myeloid and lymphoid markers. Similarly as TAMs do, MDSCs express Arg-1 and iNOS, and produce immunosuppressive cytokines such as TGF- $\beta$ 1 and IL-10, thereby inhibiting T cell response (Condamine 2011)[79]



(Figure 4). CCL2 recruits MDSCs in several types of mouse cancer including Lewis lung carcinoma, MethA sarcoma, melanoma and lymphoma (Huang 2007)[80]. Moreover, CCL2-mediated MDSC accumulation can negatively regulate the entry of adoptively transferred activated CD8<sup>+</sup> cells into tumor sites (Lesokhin 2012)[81]. However, CCR2 deficiency caused conversion of the MDSC phenotype to neutrophil lineage without affecting tumor growth (Sawanobori 2008)[82], probably because MDSC contains a subset of immature neutrophils (Brandau 2011)[83]. CXCL5 and CXCL12 also induced MDSC infiltration in mouse mammary adenocarcinoma (Yang 2008)[84]. In ascites isolated from human ovarian cancer patients, PGE<sub>2</sub> induced CXCL12 production and the expression of its receptor, CXCR4, and the CXCL12-CXCR4 axis subsequently induced the accumulation of MDSCs (Obermajer 2011)[85]. Due to the heterogeneity of MDSCs (Condamine 2011)[79], it remains elusive on the relevance of this observation.

Treg cells are characterized by the expression of CD4 and CD25 on their cell surface with the expression of a transcription factor, Foxp3 (Nishikawa 2010)[86]. Treg cells are polarized from CD4<sup>+</sup> naïve T cells in thymus or periphery, and are physiologically engaged in the maintenance of immunological self-tolerance. A large number of Treg cells often infiltrate into tumors and systemic removal of Treg cells enhances natural as well as vaccine-induced anti-tumor T cell immunity [86](Nishikawa 2010). Intratumoral CD8<sup>+</sup>/Foxp3<sup>+</sup> ratio but not absolute Foxp3<sup>+</sup> cell numbers correlated inversely with survival (Gooden 2011)[33]. Thus, the relative ratio of Treg to CD8<sup>+</sup> CTL but not absolute Treg number can have impacts on immune tolerance to tumor cells.

Treg cells express CCR4 and its ligand, CCL22, mainly regulates intratumoral Treg infiltration in various tumors [86] (Nishikawa 2010) (Figure 4). Indeed, intratumoral CCL22 expression correlated well with Foxp3 expression in colorectal carcinoma tissues (Muthuswamy 2012)[37]. Hypoxia induced the expression of another chemokine, CCL28, in colorectal tumor cells (Facciabene, 2011)[87]. CCL28 seemed to utilize mainly CCR10 to induce Treg migration into tumor sites (Figure 4) although CCL28 was reported to utilize both CCR3 and CCR10 as its receptors (Table 1). Moreover, infiltrating Treg cells can produce VEGF to promote tumor neovascularization (Facciabene, 2011)[87].

Furthermore, anti-CCL2 antibody augmented cancer immunotherapy against non-small cell lung cancer in mice when it was administered in combination with a tumor vaccine (Fridelender 2010)[88]. This enhanced tumor immunity was associated with reduced intratumoral Tregs and increased numbers of intratumoral CD8<sup>+</sup> cells that are more activated and more antitumor antigen-specific. These observations illustrate that targeting these chemokines can reduce intratumoral Treg cells, resulting in the enhancement of tumor immunity.

Adult T cell leukemia (ATL) cells are also characterized by robust expression of CCR4 and can migrate *in vitro* to CCL17 and CCL22, ligands for CCR4 [89] (Yoshie 2002). By using genetic engineering methods, humanized monoclonal antibody to CCR4 has been defucosylated to exert more potent antibody-dependent cytotoxicity (ADCC) (Ishida 2011)[90]. The resultant antibody is capable of removing CCR4-expressing ATL cells in peripheral blood and bone marrow mainly by ADCC. Thus, this antibody may also be effective to reduce intratumoral Treg cell numbers in solid tumors, thereby augmenting T cell-mediated cytotoxicity against tumor cells.

Recently, CCR1-expressing CD34<sup>+</sup> immature myeloid cells have been detected in murine intestinal tumors with SMAD4 deficiency (Kitamura 2007)[91]. These cells expressed abundantly MMP9MMP-9 and MMP2MMP-2, and were involved in invasion. Moreover, a CCR1 antagonist suppressed colon cancer liver metastasis by blocking accumulation of this CD34<sup>+</sup> immature myeloid cells (Kitamura 2010)[92].

### **Other strategies of antitumor therapy targeting chemokines**

Chemokines were originally identified as factors affecting leukocyte migration and activation (Oppenheim 1990)[93]. Subsequent studies revealed that chemokines have effects on non-leukocytic cells including tumor cells and endothelial cells (Figure 5). Indeed, several chemokines can directly induce cancer cells to express pro-tumorigenic genes and to proliferate. CXCL8 can induce the proliferation of human gastric cancer cells (Kitadai 2000)[94], esophageal cancer cells (Wang 2006)[95], and melanoma cells (Singh 2009)[96]. CXCR4 activation also caused the proliferation of various cancer cells including

ovarian, glioma, melanoma, lung, renal, and thyroid cancer cells (Teicher 2010)[97]. Likewise, CCR6 and CXCR6 can promote the proliferation of colorectal cancer cells (Ghadjar 2009)[98] and prostate cancer cells (Darsh-Yahana 2009)[99], respectively. Furthermore, the activation of CXCR4, CCR10, or CCR7 axis delivered surviving signals to various types of malignant cells (Murakami 2003, Wang 2008, Bertran 2009, Righi 2011, Messmer 2011)[100, 101, 102, 103, 104]. Thus, the inhibition of these chemokines may directly reduce *in vivo* tumor cell proliferation.

Metastasis is a complicated process wherein cancer cells extravasate from the original tissues, move inside bloodstream and/or lymphatics, invade to and grow in distant organs. The first step of metastasis, extravasation from the original tissues, requires epithelial-mesenchymal transition (EMT) (Bertran 2009)[102]. Accumulating evidence indicates the crucial roles of CXCL12 [102](Bertran 2009) and CXCL8 in EMT (Fernando 2011)[105]. Moreover, when tumor cells enter circulation, tumor cells are prone to anoikis, which is a form of cell death arising from the lack of the support from extracellular matrix and is a major block in the metastatic spread of various types of cancer cells. CXCL12 and a CCR7 ligand, CCL21, can reduce the sensitivity of cancer cells to anoikis by regulating pro-apoptotic Bmf and anti-apoptotic Bcl-xL proteins (Kochetkova 2009)[106].

CXCR4, CCR7, CCR9, CXCR1, and CXCR2 were detected in tumor cells and their ligands induced the chemotaxis of the corresponding receptor-expressing cells (Müller 2001, Buonamici 2009, Amersi 2008, Waugh 2008, Messner 2011, Zhang 2012)[107, 108, 109, 110, 111]. Specific chemokine receptor-expressing tumor cells may migrate to organs with high expression levels of respective chemokines along a concentration gradient (Müller 2001)[107]. However, there remains a question on the presence of a concentration gradient between primary and metastatic sites. Alternatively, cancer cells themselves are actively promoting their own metastasis and tropism by producing chemokines (Shields 2007)[112]. Moreover, the arrival of tumor cells in a specific organ is passive and chemokine receptor expression provides tumor cells with an advantage to survive and grow in another ligand-rich metastatic microenvironment (Zhang 2009)[113]. Nevertheless,

several chemokines can serve as inducers of metastasis to distant organs and therefore, may be a good target for controlling metastasis.

Neovascularization is crucial for tumor growth, progression, and metastasis (Fidler 1994)[114]. The ELR motif-positive CXC chemokines, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8 can directly promote the migration and proliferation of endothelial cells and eventually neovascularization, mainly interacting with CXCR2, but not CXCR1 (Keeley 2011)[115] (Figure 5). Indeed, the administration of anti-CXCL8 reduced the tumor sizes of human non-small cell lung cancer cells which are injected into severe combined immune deficient (SCID) mice in advance (Arenberg 1996)[116]. The reduction in tumor size was associated with a decline in tumor-associated vascular density and was accompanied by a decrease in spontaneous lung metastasis.

CXCL12 is not an ELR-positive CXC chemokine but exhibits potent angiogenic effects (Kryczek 2007)[117]. In addition, three CC chemokines, CCL2, CCL11, and CCL16 have also been implicated in tumor neovascularization (Gálvez 2005, Salcedo 2001, Strasly 2004)[118, 119, 120]. Indeed, CCR2, a specific receptor for CCL2, was expressed by endothelial cells and CCL2 exerted its angiogenic activity in a membrane type 1 (MT1)-MMP-dependent manner (Gálvez 2005) [118] (Figure 5). TAMs and MDSCs are recruited at tumor sites mainly by CCL2 and promote angiogenesis by producing a wide variety of angiogenic factors such as VEGF, TGF- $\beta$ , CXCL8, platelet-derived growth factor (PDGF), and MMP such as MMP-2MMP-2 and MMP-9MMP-9. Moreover, recruited TAMs and MDSCs may acquire endothelial cell phenotypes and can be incorporated into the newly formed vascular structure (Rehman 2003)[121]. Thus, targeting these chemokines may be effective to control tumor neovascularization.

CXCL4 and interferon-inducible ELR motif-negative CXC chemokines such as CXCL9, CXCL10, and CXCL11 inhibit the angiogenesis induced by ELR motif-positive CXC chemokines, VEGF, and bFGF (Maione 1990, Romagnani 2001)[122, 123]. The anti-angiogenic effects of these chemokines are mediated by a common receptor, CXCR3 (Figure 5) and targeted expression of CXCL9 or intratumoral CXCL9 administration retarded *in vivo* tumor growth by inhibiting tumor-derived angiogenesis (Addison 2000,

Pan 2006)[34, 124]. Thus, these chemokines can be effective for tumor therapy by inhibiting neovascularization as well as inducing CXCR3-expressing cytotoxic T cell infiltration.

### Perspective

Chemokines regulate the trafficking of leukocytes including immune cells in the presence of a concentration gradient and Chemokines have a crucial role in the control of the recruitment of immune cells needed for the induction and activation of tumor immunity. As we described above, based on these properties, several chemokines have been utilized in pre-clinical models to augment tumor immunity by enhancing the migration and activation of immune cells. Most of these trials, however, have not yet been translated into clinical trials. However, trafficking of a particular type of immune cells is regulated simultaneously by several distinct chemokines in a redundant manner (Table 1). Thus, it still remains to be investigated which chemokine(s) is the most suitable for inducing the trafficking of the targeted immune cells, to exert efficient immune response to tumors. ~~Chemokines regulate the trafficking of leukocytes including immune cells in the presence of a concentration gradient.~~ In order to obtain a local high concentration, gene therapy techniques were used in most trials and this may preclude the translation into clinical trials. Thus, it is mandatory to develop a drug delivery system to supply efficiently a chemokine protein into the targeting site.

Moreover, it is embarrassing that the same chemokine can induce tumor progression as well as protection against a tumor. One representative chemokine is CCL2, which can destroy tumor tissues when administered to tumor tissues by using gene therapy technology. It, however, exhibits a wide variety of actions involved in promotion of tumor progression and metastasis, and targeting CCL2 was proven to be effective for reducing tumor burdens and metastasis in several murine models. This paradox may be explained by the assumption that endogenously produced CCL2 can act on the cells present in tumor tissues but cannot cause a concentration gradient sufficient to attract immune cells from

outside of the tumor tissues. Otherwise, Alternatively, the responsiveness of immune effector cells to CCL2 may be much lower than that of immune suppressive cells, endothelial cells, and tumor cells. This may favor endogenous chemokine-mediated generation of pro-tumorigenic microenvironments rather than antitumor immune response. Thus, we should also clarify the local concentration of the chemokine, which is required for the responsiveness of immune effector cells but not that of immune suppressive cells, endothelial cells, and tumor cells. Based on the information, we should devise a method to sustain a local chemokine concentration sufficient to attract immune effector cells to elicit immune response to tumor. Alternatively, genetic modification of immune effector cells with a chemokine receptor gene can confer a capacity to respond more efficiently to a chemokine on immune effector cells. Thus, this may be an attractive maneuver to change chemokine-mediated pro-tumorigenic environments, where a particular chemokine is present abundantly, into an effective immune surveillance situationsystem, where the abundantly expressed chemokine can attract immune effector cells to exert immune responses..



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Chemokine Receptor	Chemokines	Receptor Expression in		
		Leukocytes	Epithelium	Endothelium
CXCR1	CXCL6, 8	PMN	+	-
CXCR2	CXCL1, 2, 3, 5, 6, 7, 8	PMN	+	+
CXCR3	CXCL4, 9, 10, 11	Th1, NK	-	+
CXCR4	CXCL12	Widespread	+	+
CXCR5	CXCL13	B	-	-
CXCR6	CXCL16	activated T	+	-
CXCR7	CXCL12, CXCL11	Widespread	+	+
Unknown	CXCL14 (acts on monocytes)			
CCR1	CCL3, 4, 5, 7, 14, 15, 16, 23	Mo, M $\phi$ , iDC, NK	+	+
CCR2	CCL2, 7, 8, 12, 13	Mo, M $\phi$ , iDC, NK activated T, B	+	+
CCR3	CCL5, 7, 11, 13, 15, 24, 26, 28	Eo, Ba, Th2	-	+
CCR4	CCL2, 3, 5, 7, 22	iDC, Th2, NK, T, M $\phi$	-	-
CCR5	CCL3, 4, 5, 8	Mo, M $\phi$ , NK, Th1 activated T	+	-
CCR6	CCL20	iDC, activated T, B	+	-
CCR7	CCL19, 21	mDC, M $\phi$ , naïve T activated T	+	-
CCR8	CCL1, 4, 17	Mo, iDC, Th2, Treg	-	-
CCR9	CCL25	T	+	-
CCR10	CCL27, 28	activated T, Treg	+	-
Unknown	CCL18 (acts on mDC and naïve T)			
CX3CR1	CX3CL1	Mo, iDC, NK, Th1	+	-
XCR1	XCL1, 2	T, NK	-	-
Miscellaneous-----scavenger receptors for chemokines				
Duffy antigen	CCL2, 5, 11, 13, 14 CXCL1, 2, 3, 7, 8			
D6	CCL2, 3, 4, 5, 7, 8, 12 CCL13, 14, 17, 22			

Table 1. The human chemokine system. Leukocyteonyms are as follows: Ba, basophil; Eo, eosinophil; iDC, immature dendritic cell; mDC, mature dendritic cell; Mo, monocyte; M $\phi$ , macrophage; NK, natural killer cell; Th1, type I helper T cell; Th2, type II helper T cell; Treg, regulatory T cell.

Legends to Figures

Figure 1. Intracellular signaling pathway of chemokines.

Figure 2. Tumor immunity generation.

Figure 3. Maturation stages of dendritic cells.

Figure 4. Biological effects of chemokines on suppressive leukocytes in tumors

Figure 5. Biological effects of chemokines on tumor and endothelial cells.

Figure 1 Mukaida

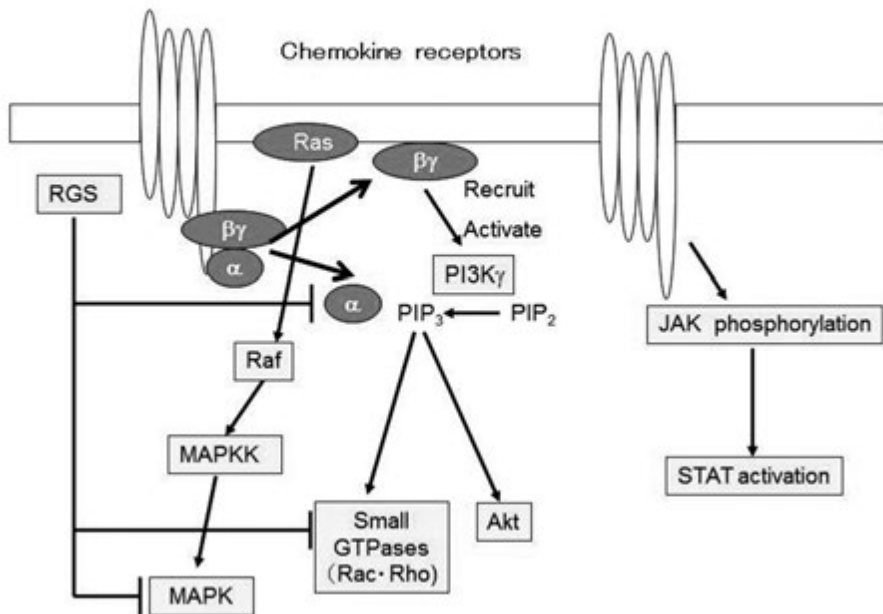


Figure 2 Mukaida

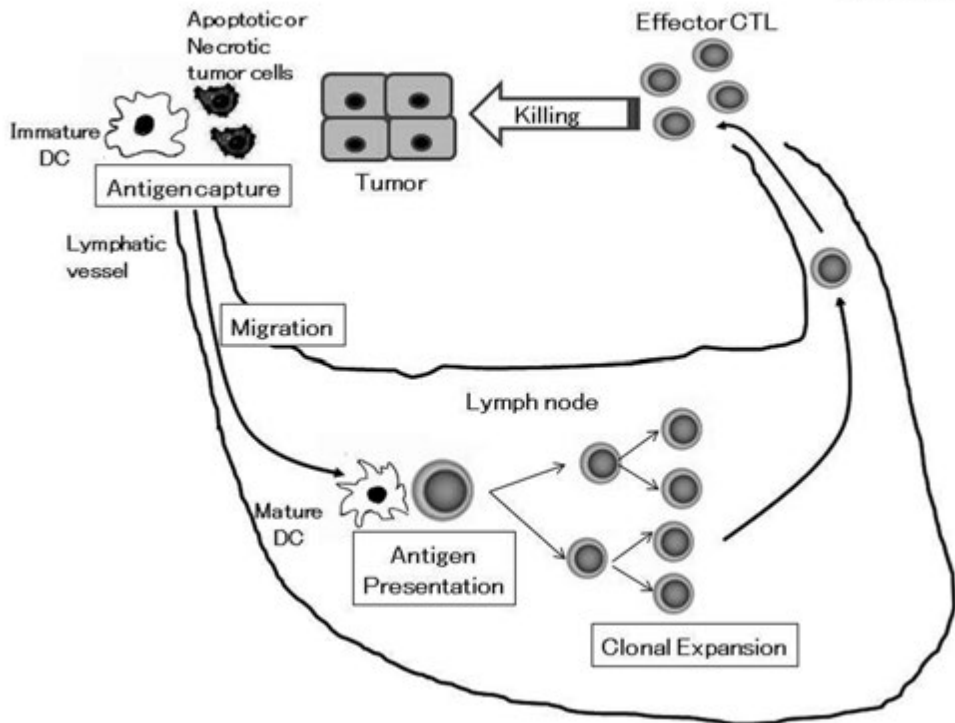
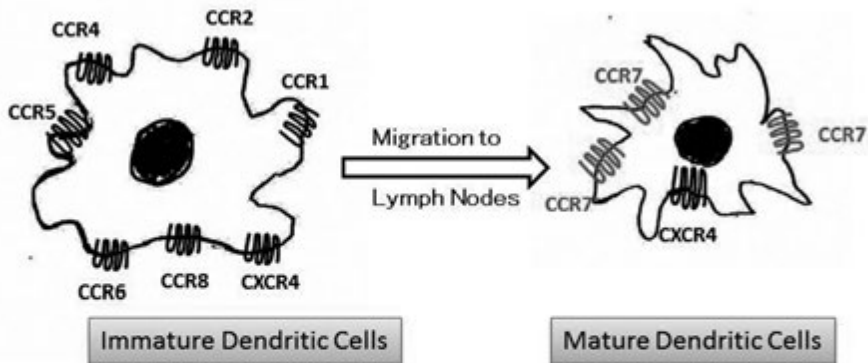


Figure 3 Mukaida



Antigen Capture Capacity	High	Low
Antigen Presentation Capacity	Low	High
MHC class II Expression	Low	High
Co-stimulatory Molecule Expression	Low	High

Figure 4 Mukaida

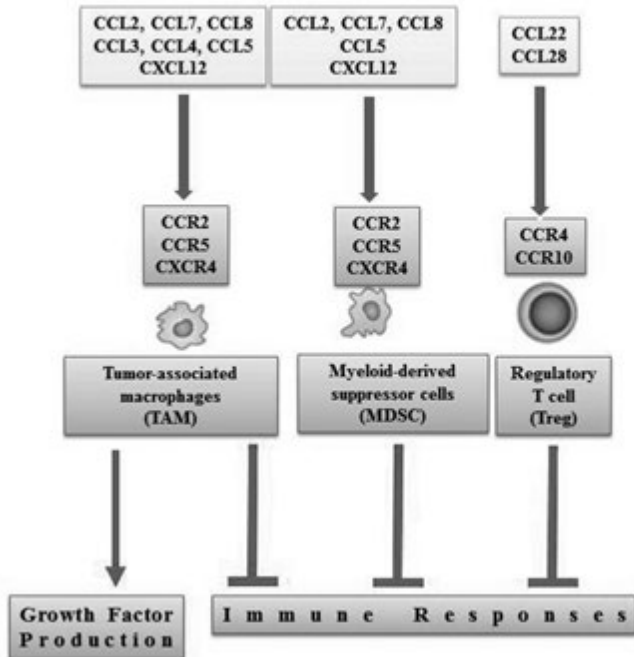


Figure 5 Mukaida

