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Chemokines as adjuvants for immunotherapy: Implications for immune activation with CCL3

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Abstract

Introduction—Immunotherapy embodies any approach that manipulates the immune system for therapeutic benefit. In this regard, various clinical trials have employed direct vaccination with patient-specific dendritic cells or adoptive T cell therapy to target highly aggressive tumors. Both modalities have demonstrated great specificity, an advantage that is unmatched by other treatment strategies. However, their full potential has yet to be realized.

Areas covered—In this review, we provide an overview of chemokines in pathogen and anti-tumor immune responses and discuss further improving immunotherapies by arming particular chemokine axes.

Expert Commentary—The chemokine macrophage inflammatory protein-1 alpha (MIP-1 α , CCL3) has emerged as a potent activator of both innate and adaptive responses. Specifically, CCL3 plays a critical role in recruiting distinct immune phenotypes to intratumoral sites, is a pivotal player in regulating lymph node homing of dendritic cell subsets, and induces antigen-specific T cell responses. The recent breadth of literature outlines the various interactions of CCL3 with these cellular subsets, which have now served as a basis for immunotherapeutic translation.

Keywords

Chemokines; macrophage inflammatory protein-1 alpha; MIP-1 α ; CCL3; immunotherapy; antitumor; antigen-specific T cell; dendritic cell

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1. Chemokines in pathogen and antitumor immune responses

The field of chemokine biology has provided evidence that these expressed proteins occur in distinct patterns as part of distinct profiles of immune responses, whereby immune cells and their subsets express certain chemokines in a location-dependent manner [1,2]. Chemokines comprise a group of small proteins (7–15 kDa) that bind to G-protein coupled receptors (GPCRs) on various immune cell types. To date, there are about fifty known chemokine members which are divided into four sub-families with conserved N-terminal cysteine motifs (disulfide bonds) and the presence or absence of intervening amino acids [3]. There are two major sub-classes, which consist of the CXC (or α) and CC (or β) chemokines and two minor sub-classes, the C (or γ) and CX₃C chemokines (or δ). They share significant structural homology (C, CC, CXC and CX₃C) and are typically classified by their expression pattern: homeostatic, constitutive, or inducible [4]. Furthermore, chemokine ligands often share receptors, leading to redundant, additive, and competitive effects that can lead to ineffective interventions that modulate such redundant molecules. Nonetheless, through understanding these tumor-chemokine interactions, researchers and clinicians also have been able to utilize chemokines in immunotherapy to boost immune-cell trafficking to the tumor microenvironment and lymphoid tissues. Some of the most promising chemokine axes for cancer immunotherapy are CCL19/CCL21-CCR7; CCL2, CCL3, CCL5 and their cognate receptors (CCR1, CCR2, CCR4, and CCR5); CCL16 (liver-expressed chemokine [LEC]); and CXCL12-CXCR4 and CXCR7 [4–9]. These chemokine axes and sites of predominant function are summarized in Table 1 and are herein discussed in further detail.

1.1 Trafficking to draining lymph nodes

To counter the low levels of migration seen both endogenously and upon adoptive transfer of cellular subsets, investigators have focused on increasing the homing of antigen-presenting cells (APCs) to draining lymph nodes (DLNs), where they engage with and effectively prime naïve T and B cells. Certain chemokines have been shown to be vital for this process to occur. The predominant axis for skin-derived DC entry via the lymphatics is CCR7 binding to CCL19 and CCL21 on afferent lymphatic vessels [6,10]. Another key chemokine for trafficking is CCL2 (MCP-1), which, during inflammation in the skin, drains to local lymph nodes (LNs) and recruits large numbers of blood-borne monocytes to LNs via high endothelial venules (HEVs) [11]. Both CCL3 and CCL4 were required for the education of naïve CD8⁺ T cells in the draining lymph nodes, using the OVA protein antigen as a marker of antigen-specific immune responses. The activation of these naïve T-cells selectively depended on sites of OVA-specific CD4⁺ T cell and incoming DC engagement, where local production of both CCL3 and CCL4 occurred. Blockade of both CCL3 and CCL4 markedly reduced the ability of CD4⁺ T cells to promote memory CD8⁺ T-cell generation, underscoring the presence of this chemokine pair in the differentiation on antigen-specific CD8⁺ T cell immune responses [12].

1.2 Recruitment of immune cells into the tumor

Preclinical studies by Crittenden *et al.* have selectively used chemokines to recruit immature DCs to the tumor site with the hypothesis of enhanced antigen-specific tumor responses that would be generated. Because CCR1, CCR5, and CCR6 are routinely expressed on murine

bone marrow-derived DCs, investigators chose CCL3 and CCL20 (predominant ligands for these receptors) to recruit immune cells into tumors. Using coexpression with HSVtk, a viral enzyme that converts ganciclovir from a prodrug to a toxic agent that kills the cells, thus releasing tumor antigen, investigators were able to demonstrate that localized chemokine induction with concomitant bystander antigen release acted in concert to provide superior antitumor responses in animal models, where chemokine or HSVtk expression alone was not sufficient to engender the same responses [13].

Within the previously-considered immunoprivileged CNS, immune effector cells or activated endothelium have demonstrated the capacity to produce certain chemokines that mediate chemotaxis and trafficking across the blood brain barrier. With respect to CNS trafficking, studies have shown that the β -chemokines CCL2 and CCL3 play an important role in CNS inflammatory states. Both these chemokines are upregulated in multiple sclerosis (MS) lesions and in experimental allergic encephalomyelitis (EAE) models, two diseases manifested by autoreactive T cell responses. In EAE, the animal model of MS, the onset of the disease coincides with the mRNA expression of CCL2, CCL3 and other chemokines [14,15] and the accumulation of CXCL10 and CCL2 [16]. A model of human brain microvessel endothelial cells revealed that these endothelial cells were indeed the source and synthesized CCL2 and CCL3. CCL2 was expressed at constitutively high levels, but only CCL3 was dependent on cytokine and LPS activation for upregulation [17]. Altogether, both chemokines were shown to play a physiologic role in the regulation of inflammatory responses across the blood-brain barrier. This effect, which elicits immune effector cell recruitment and exacerbation of T-cell-reactivity, is highly relevant and necessary for robust immune response induction in cancer immunotherapy for CNS malignancies.

Marked expression of chemokines CCL5 (RANTES) and CXCL10 in colorectal tumors have played as main contributors of cytotoxic T lymphocyte (CTL) chemoattraction and indicators of prolonged survival [18]. CCL20, a potent attractant for DCs, has been utilized for superior intratumoral trafficking and antitumor immunity in established tumor and metastatic models. Intratumoral injection of CCL20 in four syngeneic tumor models demonstrated an increased survival advantage in mice with established tumors that was dependent on the presence of naive CD8⁺ T cells. Enhanced CTL activity by provision of CCL20 was long-lasting, as splenocyte transfer of these cells into immunotherapy-naïve tumor-bearing mice subsequently protected these mice from succumbing to tumor. Provision of CCL20 also inhibited the growth of distant tumors, suggesting that local delivery of this chemokine is a useful adjuvant for increased immune cell infiltration and anti-tumor immunity against local and metastatic tumors [19].

Chemokines, such as CCL21, that act as professional regulators of immune cell recruitment can be used within the tumor microenvironment itself to promote an antitumor milieu of cytokines and chemokines favoring Th1 antitumor responses, much reminiscent of that in the DLN. Using similar methodologies described above for chemokine-enhanced immune infiltration, investigators utilized intratumoral delivery of DCs to secrete secondary lymphoid tissue chemokine (CCL21/SLC) and demonstrated complete eradication of established tumor in 60% of mice. CCL21-expressing DC therapy also resulted in increased

infiltration of CD4⁺, CD8⁺, and CD3⁺CXCR3⁺ T-cells and endogenous CD11c⁺ DEC205⁺DCs that were activated and capable of efficient cross-presentation of antigen. Moreover, intratumoral T_{Regs} were markedly reduced after the CCL21-expressing DC therapy. These intratumoral accumulations subsequently showed elevated expression of GM-CSF, IFN- γ , MIG/CXCL9, IP-10/CXCL10, and IL-12 with diminished levels of the immunosuppressive mediators TGF- β and prostaglandin E₂ [20]. Thus, one advantage of this chemokine-enhanced immunotherapy is that it was effective at antitumor immune infiltration but also inducible of other chemokines involved in antitumor immunity.

Altogether, these studies have demonstrated that quite a few distinct chemokines can be used either for enhanced DLN homing or intratumoral recruitment of desired immune effector cells. Their utilization requires knowledge not only of endogenous selectivity for certain chemokine receptors but also a predilection for these receptors on distinct immune cell subsets. For instance, the chemokine CCL3 binds to receptors CCR1, CCR4, and CCR5, and each are predominantly expressed on distinct immune cell subsets, including immature myeloid cells and monocytes (CCR1), T helper cell type 2 (Th2) T-cells or T_{Regs} (CCR4), and monocytes, NK cells, Th1 T-cells, and plasmacytoid DCs (CCR5) [21]. The known cellular sources and downstream effects of human CCL3 under classical inflammatory conditions are summarized in Table 2. and Table 3. [22]. Next, we discuss the discovery of CCL3, its normal physiological role in various axes of the immune response, and lastly its potential as an adjuvant of chemokine-enhanced immunotherapy for the treatment of highly aggressive tumors.

2. Identified roles of CCL3 in inflammation

First characterized by Wolpe in 1988, CCL3 was purified from supernatant of endotoxin-stimulated murine macrophages [23]. Because of its inflammatory properties *in vitro* as well as *in vivo*, the protein mixture was denominated ‘macrophage inflammatory protein-1’ (MIP-1). Further biochemical separation and characterization of the protein doublet yielded two distinct, but highly related proteins, MIP-1 α and MIP-1 β that shared 68% identical amino acids [24]. CCL3 is regarded as a member of the CC family of chemokines, with no intervening amino acid between the first two cysteine residues. A variety of cells have been shown to secrete CCL3 including lymphocytes, fibroblasts, and epithelial cells, as well as both resident and recruited monocytes and macrophages [25,26].

A potent chemokine for lymphocytes and monocytes, CCL3 binds to CCR1, CCR4 and CCR5 receptors on T cells, DCs, B cells, and eosinophils. Preclinical studies have further demonstrated that CCL3 is released upon the induction of Th1 responses [27,28]. One of the most unique characteristics of CCL3 is its ability to coordinate the compartmentalization and mobilization of myeloid precursor cells (MPCs) [29,30]. Through the use of *Ccr1*^{-/-} mice, CCL3 has been shown to mediate the mobilization of MPCs from the bone marrow, as well as having regulatory effects on MPCs and acting to stimulate mature MPCs [31]. CCL3 has been reported to be chemotactic for both neutrophils and monocytes *in vitro* and *in vivo* in mice [24,32]. In fact, CCL3 production by these cells was enhanced during monocyte-endothelial cell interactions, and this upregulation was shown to be mediated by binding of the monocytes to intercellular adhesion molecule-1 (ICAM-1). Thus, the production of

CCL3 observed under endothelial cell-leukocyte interactions serves as an important mechanism in sustaining the recruitment of cells during inflammatory responses [33]. In humans and in primate models, predominantly monocytic cellular infiltrates have been observed to accumulate in response to direct injection of CCL3 [34]. In a number of model systems, CCL3 effectively recruits high amounts of mononuclear cells [35,36]. *Ccl3*^{-/-} mice were found to be partially protected from the accumulation of monocytes in myocarditis and to be impaired in the ability to control the viral infections of coxsackievirus and influenza [35]. Thus, given the extensive evidence of CCL3 as a key regulator of monocyte chemotaxis *in vivo*, an obvious utility of this chemokine would be its ability to direct immune cell trafficking and accumulation during the course of an immune response against tumors.

2.1 CCL3 links innate and adaptive immunity

Preclinical studies have classically characterized CCL3 to operate in the context of the “efferent” or recruitment phase of the inflammatory response, when leukocytes or immature monocytes are actively recruited to sites of inflammation. However, recent evidence challenges the role of CCL3 to be restricted to this phase, as Olszewski *et al.* revealed that CCL3 was involved in afferent inflammatory responses, where specific immune profiles are generated during initial priming and boosting phases within secondary lymphoid organs. Specifically in the case of *Cryptococcus neoformans* infection in mice, CCL3 was found to prevent the switch from a Th1 effector phenotype to a non-protective Th2 response during active infection [37].

In the setting of viral injection, chemokines released from CTLs are known to localize and amplify the immune response by further recruiting leukocytes to the site of viral replication. Viral antigens expressed on infected cells induce activation of CD8⁺ CTLs, which has been shown to result in the release of CCL3, CCL4, and CCL5 directly onto the target cell. The release of these chemokines at the site of infection also serves as a beacon to call in additional migrating cells, such as monocytes and macrophages, resulting in further amplification of the local immune response [38]. Preclinical studies have also suggested that, not only the antiviral, but the antimicrobial potential of CD8⁺ CTLs is also reflected in their ability to rapidly produce inflammatory cytokines such as IFN- γ , TNF- α , and CCL3, which all act in concert to control the growth of intracellular pathogens such as *Francisella tularensis*, *Leishmania major*, or *Listeria monocytogenes* [39]. The current dogma is that only classical T and B cells of the adaptive immune system are able to differentiate into long-lived memory cells exhibiting qualitatively improved functional properties. This notion has evolved to the current acceptance that immunological memory of these cells is gained through enhanced proliferation, the expression of several effector functions including secretion of specific cytokines and chemokines, and through cytotoxicity of infected cells. Recent work substantiated the role of memory cells in the secretion of certain chemokines. Investigators found that CCL3-secreting memory CD8⁺ T cell induced by *Listeria monocytogenes* infection were able to mediate “bystander” killing of an unrelated pathogen (wild-type bacteria) upon antigen-specific reactivation. This mechanism was observed to be dependent on CD8⁺ memory T cell-derived CCL3, which promoted TNF- α secretion from macrophages during a secondary infection to wild-type bacteria [40]. Such data reinforce the

concept that the innate immune response during a secondary antigenic encounter can be regulated via CCL3 in response to lymphocyte-derived cues. Bridging this concept into the use of chemokines with immunotherapy, these studies support their applicability to mediate lymphocyte activation and induce positive feedback mechanisms for priming and cytolytic phases of tumor-specific antigen responses.

2.2 CCL3 as a biomarker for negative outcomes

As previously mentioned, the vast utility of CCL3 unfortunately encompasses functions of this chemokine that may exacerbate certain disease states, whereby its hyperactivity has a directly detrimental effect on outcome. Specifically, CCL3 has been established as a primary inciter for progressive disease in multiple myeloma [41]. CCL3 produced by multiple myeloma cells in turn activate osteoclasts resulting in accelerated osteolytic bone disease. As such, the degree of CCL3 secretion by multiple myeloma cells in patients has correlated with the severity of osteolytic bone disease, bone resorption, and disease prognosis [42]. Interestingly, despite the already numerous studies describing the role of CCL3 in multiple myeloma and associated osteolytic bone disease, no therapies targeting CCL3 or its receptors have been evaluated clinically in the setting of this disease. This delay in clinical progress may stem from the historical difficulty in developing chemokine-targeted drugs [43,44] or may reflect early reports suggesting that concurrent inhibition of both receptors (CCR1 and CCR5) through which CCL3 signals might be required to completely neutralize its osteoclast-activating effects [45,46].

3. Expert Commentary: CCL3 in cancer immunotherapy

The past few decades of research on CCL3 comprise primarily the characterization of its chemotactic abilities and expand into its role in autoimmune inflammatory processes and cancers of the immune system such as multiple myeloma. As past studies have unveiled the dual roles for CCL3 acting as both a potent immune activator and instigator of inflammatory disease processes, so too should these roles be investigated in the context of cancer immunotherapy. The first section of this commentary will describe the utility of immunotherapy for cancer, with a further elaboration on how distinct functions of CCL3 binding can augment or hinder immune-activating strategies.

3.1 Cancer vaccines and tumor immunotherapy

The immune system is an accessible and auspicious treatment modality that can be harnessed to battle the most aggressive solid and hematologic malignancies. Due to its inherent specificity and safety profile, arming the effector cells of the immune system engenders the precise targeting of malignant host cells. Such an advantage of exquisite precision further offers the generation of antitumor memory responses should tumors recur, which is a feature that no current standard therapy can match.

Tumor immunotherapy refers to any approach that seeks to mobilize or manipulate the immune system for therapeutic benefit. In this regard, there are numerous strategies that have been employed to battle highly invasive tumors and can be classified into either of two main strategies: immune-activating or tolerance-reversing. Therapies under the immune-

activating domain entail either non-specific activation of the immune system with microbial components or cytokine adjuvants, antigen-specific adoptive immunotherapy with antibodies or T cells, or active immunotherapy via either direct vaccination against tumor antigens or overcoming immunosuppression with immune checkpoint blockade. Past investigations of active immunotherapy have utilized antigen-presenting cells (APCs) to educate effector T and B cells for the induction of cellular immunity.

One of the most attractive strategies is vaccination aimed at eliciting a specific *de novo* immune response against select tumor antigens. This form of therapy is expected to induce both therapeutic T-cell immunity (in the form of tumor-specific effector T-cells) [47] and protective T-cell immunity (in the form of tumor-specific memory T-cells that can control tumor recurrence) [48]. Various clinical trials are currently evaluating the safety and efficacy of direct vaccination with patient-specific dendritic cells (DCs) [49,50]. Additionally, vaccination with tumor-derived peptides that rely on recruitment and activation of APCs have demonstrated potent immune responses [51–55]. In our experience with the tumor specific epidermal growth factor receptor class III variant (EGFRvIII) antigen uniquely expressed in GBM tumors, vaccination against EGFRvIII has been shown to induce potently specific T-cell immunity as well as long-lasting humoral responses [56].

Adoptive immunotherapy (AIT), which comprises the *ex vivo* expansion of effector cells and returning tumor-specific T-cells back into tumor-bearing hosts, has been shown in recent clinical trials to be a potent modality for achieving markedly high and sustained levels of tumor-specific T-cells [57–59]. The effectiveness of adoptive T-cell therapy relies on the ability for adoptively transferred lymphocytes to localize to tumor sites and mediate the destruction of antigen expressing tumor cells [60–62].

Within the tolerance-reversing domain, immunotherapies have recently focused on disarming the regulatory receptor and ligand programmed death (PD)-1/PD-ligand-1 (PD-L1) and cytotoxic T-lymphocyte antigen-4 (CTLA-4). PD-1 is a co-inhibitory receptor that is inducibly expressed by T and B cells upon activation. PD-1/PD-L interactions have been implicated in the functional impairment of tumor antigen-reactive CD8⁺ T cells in solid tumors [63,64]. CTLA-4, a receptor constitutively expressed on T-regulatory cells (T_{Regs}) and upregulated during activation of effector T-cells, mediates downregulation of the immunologic response in activated lymphocytes [65,66]. Recent clinical studies employing monoclonal antibodies against CTLA-4 (ipilimumab, tremelimumab) and PD-1 (nivolumab, pembrolizumab, lambrolizumab, pidilizumab) are now FDA-approved for the treatment of metastatic melanoma, non-small cell lung cancer (NSCLC) and renal cell carcinoma and have shown dramatic responses in patients with advanced stage melanoma (unresectable stage III or IV) [67].

For malignant gliomas, however, current immunotherapeutic strategies tend to be short-lived and succumb to highly immunosuppressive mechanisms put forth by the gliomas themselves, including antigen escape [68], immunosuppressive cytokine secretion with IL-10 and TGF- β [69–73], and predominance of T_{Regs} that tolerize any antitumor responses [74,75]. As further understanding arose on requisites to achieving durable antitumor immune responses, it became clear that both DC and adoptive T-cell strategies share similar

shortcomings in effective cell migration to target sites upon transfer into the host [76]. One area of investigation is the use of repetitive chemokine signaling to perpetuate effective trafficking and ensure a more prolonged antitumor T helper cell type 1 (Th1) subtype. The benefits of employing a potent chemokine that can guide effector cells is just one means to target tumor-mediated immunosuppression, and the following section will discuss the advantages and current applications of chemokine delivery with the potent mediator CCL3.

3.2 Preclinical studies for CCL3 and antitumor immunity

CCL3, along with CCL4 (MIP-1 β) and its near-homologues CCL2 (MCP-1) and CCL5 (RANTES), have demonstrated the ability to regulate the trafficking of multiple immune cell subsets. Upon localization and binding to CCL3, immature DCs, monocytes, and memory or effector T cells will extravasate from vascular compartments into sites of peripheral inflammation. The very broad yet potent chemotactic signals of CCL3 and similar chemokines have made them a focus of adjuvant use in cancer immunotherapy protocols. Preclinical studies to date have implemented CCL3 as a monotherapy or combinatorial adjuvant to induce both tumor regression and immunity to tumor rechallenge [77] (Table 4.). One of the seminal studies using chemokine gene transfection demonstrated that tumor cells transfected with CCL3 could enhance T cell infiltration and macrophages within the tumor itself, which led to improved antitumor responses. This study also revealed for the first time that CCL3-mediated chemotaxis of neutrophils suppressed tumor growth, an axis that unveiled novel tumor killing mechanisms with provision of CCL3, including that of CCL3-induced TNF- α , oxygen radical damage and protease release killing off nearby tumor cells [78]. CCL3 gene transfer was then extended to use with adoptive T-cell strategies. Gough *et al.* found that a subpopulation of adoptively transferred activated OT-I T-cells expressed CCR5, a predominant CCL3 cognate chemokine receptor on T cells, which sufficiently promoted infiltration as a response to intratumorally injected CCL3. Importantly, this intratumoral immunotherapy also resulted in endogenous tumor-specific T-cell responses that were capable of rejecting subsequent tumor challenge [79]. The potency of CCL3 as a professional immune cell regulator has additionally been supported by studies using CCL3-recruited DCs [8,80,81]. One of the main obstacles of DC immunotherapy is generating sufficient DCs from the host for subsequent vaccination. Cao *et al.* first tackled this hurdle with CCL3 recruitment of DCs into the peripheral blood of patients. Hypothesizing that greater harvests of DCs could potentiate antitumor vaccination, they found that CCL3-recruited DCs exhibited superior antitumor effects, including increased lymphocyte proliferation, cytolytic capacity, survival, and decreased tumor growth, only when DCs were transduced with cDNA encoding the tumor antigen human melanoma-associated gene (MAGE)-1. These effects were not recapitulated when DCs were transfected with whole tumor lysate, stressing the need for antigen-specific vaccination [8]. The combinatorial use of CCL3 with an antigen-specific platform for MAGE-1 has also been used in the setting of gastric cancer [80]. CCL3 has also been shown to be a potent immunotherapy adjuvant for alternative non-vaccination cancer therapies including radiofrequency ablation. One study demonstrated that adjuvant use of a CCL3 active variant, ECI301, augmented tumor-specific responses after radiofrequency ablation, and this mechanism was dependent on the expression of the second predominant CCL3 chemokine receptor CCR1 [82]. Lastly, CCL3 as an immunotherapy adjuvant has been tested with

relative success in systemic cancers. Using the A20 leukemia/lymphoma vaccine model, authors vaccinated mice with CCL3 with concomitant interleukin-2 (IL-2) or granulocyte-macrophage colony-stimulating factor (GM-CSF) and demonstrated significantly improved survival against the blood-borne cancer in both combinations. However, suppression of lymphoblast proliferation by single-immunogen vaccines secreting either CCL3, GM-CSF, or IL-2 alone did not translate to improved survival, eluding to a key synergistic relationship between these immunomodulators. When detailing the required effector populations necessary for either combination, the authors found that the CCL3 + IL-2 group was mediated by CD8⁺ T cells and NK cells, whereas in the CCL3 + GM-CSF group, both CD4⁺ T cells and CD8⁺ cytotoxic T cells, underscoring that CCL3 acts in concert with other cytokines to armor immune effectors in a distinguished manner [83].

3.3 CCL3-mediated trafficking and activation of distinct immune phenotypes to intratumoral and metastatic sites

As regulators of cell migration, chemokine networks are frequently usurped by cancer cells to facilitate their growth and metastasis. Tumors have been characterized to seize normal chemokine axes in order to control lymphocyte tumor infiltration, exert immunosuppressive pathways, regulate angiogenesis, and facilitate metastatic spread [84]. Seminal studies have elucidated that provision of CCL3 is site-dependent in terms of the immune effectors it attracts and can be released from tolerizing or pro-tumorigenic lineages. In some models, CCL2 and CCL3 have been shown to increase the infiltration of T_{Regs}, myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs), contributing to suppressed antitumor immune responses and tumor tolerance mechanisms [85–87]. Intratumoral recruitment of these cell types, in particular TAMs, have correlated poorly with clinical outcomes [88]. Thus, careful consideration would be warranted to ensure that chemokine immunotherapy is not responsible for the activation and maintenance of these tolerogenic immune cells within the tumor microenvironment.

In cancers with high metastatic potential, such as lung, breast, and ovarian tumors, this balance between TAM recruitment and activated T-cell recruitment along the CCL3 axis has become critically important. In a murine model of metastasizing renal cell carcinoma, high numbers of CCL3⁺ infiltrating cells were observed within metastatic foci, and CCL3 or CCR5-deficient hosts markedly reduced the number of metastasis foci in the lung [89]. Others have identified a prometastatic chemokine cascade mediated by CCL3. Breast cancer metastases to the lung was first populated by CCL2-responding metastasis-associated macrophages that became activated to secrete CCL3, which was ultimately required for prolonged macrophage retention and maintenance of metastatic foci [90]. However, opposing studies seem to suggest that CCL3 within metastatic foci may not always translate to clinical decline. Investigators evaluated the chemokine profile of tumors as well as metastatic and non-metastatic lymph nodes of patients with oral squamous cell carcinoma and discovered a dual role for CCL3 with respect to its effects on the spread of malignant cells to lymph nodes versus its local control within the tumor microenvironment. In this study, indeed the density of CCL3-expressing cells was higher in metastatic compared to non-metastatic lymph nodes, yet patients with a higher percentage of CCL3-expressing cells within the tumor stroma itself showed significantly improved survival rates [91], eluding to

the importance of CCL3 production within the tumor microenvironment itself compared to metastatic sites (Figure 1). Additionally, investigators found that TAMs within Lewis lung carcinoma showed significantly elevated expression of IL-10 with concomitantly reduced levels of TNF- α , CCL3, CCL4, and IL-6 compared to peritoneal macrophages of tumor-naïve mice [92]. Subsequent studies described depressed CCL3 levels to be attributable to M2 TAMs carrying defective NF-KB activation [93,94] with transcriptional studies demonstrating severe down-regulation of the NF-KB-inducible gene *CCL3*. Therefore, continued investigations of using CCL3 as an immunotherapy warrants the understanding of its optimally delivered location, dosing, and timing for ensuring immune activation and mobilization while minimizing pro-tumorigenic or MDSC tolerance mechanisms.

3.4 CCL3 and migration of antigen-presenting cells to draining lymph nodes

The lymphoid chemokines CCL19 and CCL21 are two classically regarded chemokines that attract mature DCs from peripheral inflammatory sites into local draining lymph nodes by engagement with their chemokine receptor CCR7. Expression of CCR7 on mature DCs has been characterized in the literature to be obligatory for efficient DC-lymph node homing. It has been shown that CCR7-deficient DCs fail to migrate effectively from the skin into lymphatics [6,95], a biologic event that is critical for their interaction with and education of naïve T cells in the lymph node.

Recently, CCL3 has emerged as a pivotal player in regulating lymph node homing of DC subsets (Figure 1). A seminal study using mouse hepatitis viral infection in the CNS in mice demonstrated that host CCL3 was required for maturation, activation, and the migration of CD11c⁺CD11b⁺CD8a⁻ DCs to draining cervical lymph nodes. In the absence of CCL3, the diminished endogenous DC recruitment to LNs resulted in reduced IFN- γ expression by MHV-specific T-cells as well as increased levels of IL-10 [96].

CCL3 has been utilized as an adjuvant to DNA vaccines for the specialized recruitment of local DCs to encapsulate antigen and proceed to engender enhanced cytotoxic CD8⁺ responses. Using a model of HIV Gag DNA vaccination, investigators found that transfection with CCL3 could markedly increase the local infiltration of CD11c⁺ DCs with resultant responses in the spleen by IFN- γ single cell ELISpot. Inoculation with the CCL3 and Gag plasmid also resulted in strong protection upon viral challenge, with a nearly 200-fold reduction in virus PFUs. In contrast, injection of pGag with another chemokine plasmid such as CCL19 (MIP-3 β) did not increase protection compared with injection of the Gag plasmid alone [97].

Recent work from our laboratory introduced a new role for CCL3 in mediating the migration of peripherally injected mRNA-pulsed DCs in the form of a tumor antigen-specific vaccine to vaccine site-draining lymph nodes. The study encompassed both a randomized clinical trial in patients with newly diagnosed glioblastoma treated with Cytomegalovirus pp65 mRNA-pulsed DCs and supportive mouse studies using OVA mRNA-pulsed DCs. Prior to the tumor antigen-specific DC vaccine, the injection site was pre-conditioned with a booster dose of tetanus-diphtheria (Td) toxoid at a single side. Thereafter, a bilateral DC vaccine was given intradermally at the groin site, and migration and tumor growth were measured. Uptake of human and murine DCs in vaccine site-draining lymph nodes demonstrated that

migration of DCs given bilaterally did not preferentially migrate to lymph nodes ipsilateral to the side the Td was given, supporting a systemic effect for increased DC numbers reaching the draining lymph nodes. Analysis of the serum from patients and mice following the Td skin pre-conditioning prior to DC vaccination showed significant fold increase in CCL3 compared to non-Td control mice and patients. Subsequent investigations in *Ccl3*^{-/-} mice revealed the requirement for CCL3 in increasing DC homing to bilateral lymph nodes as well as in ensuring protection in a subcutaneous treatment model with aggressive OVA-expressing melanoma tumors [98].

3.5 CCL3 and the induction of antigen-specific T cell responses

Looking beyond DC infiltration and migration in the context of CCL3, this chemokine has additionally been documented to have profound effects on induced T-cell responses. During the early course of a *de novo* immune response, CD4⁺ helper T cells often engage with incoming DCs carrying the antigen of interest and coordinate engagement of these antigen-specific DCs with incoming naïve CD8⁺ T cells for effective priming (Figure 1). A highly relevant study demonstrated that, prior to antigen recognition in draining lymph nodes, naïve CD8⁺ T cells entering from the circulation upregulate the chemokine receptor CCR5, which allows their ability to respond to sites of antigen-specific DC-CD4⁺ encounter. Investigators were able to detect that at these CD4-DC sites of engagement, the chemokines CCL3 and CCL4 were produced in high amounts. Interfering with the secretion of these chemokines severely abrogated the ability of helper CD4⁺ T cells to promote memory CD8⁺ T cell populations, indicating that CCL3 and its near homologue played vital roles in the local draining lymph nodes for the generation and maintenance of CD8⁺ antigen-specific responses [12]. The aforementioned effects of CCL3 on induced immune response are highly relevant for enhancement of immunotherapeutic strategies and are reviewed in Figure 1.

4. Five-year view: CCL3 as a biomarker for tumor response and adjuvant for immunotherapy

A considerable number of potent effects of CCL3 on immune cell recruitment and induction of Th1 immune responses have been discussed. Only recently, investigators of clinical trials have focused on CCL3 induction as a biomarker for antitumor immune responses. There are a few recent clinical studies evaluating CCL3 in the context of outcomes with immunotherapy, which are delineated in Table 5. The first is a single arm Phase II study for metastatic breast, ovarian, endometrial, and cervical cancers for which patients are administered a TLR7 agonist. Secondary endpoints for this study are evaluating the mean differences in CCL3 levels as a surrogate of immune activation (NCT00319748). Another single arm Phase II study is evaluating the level of CCL3 induction following administration of a potent TLR9 agonist with trastuzumab for the treatment of locally advanced or metastatic breast cancer (NCT00824733). A fully randomized Phase II study recently evaluated the efficacy of bevacizumab with one of two chemotherapy regimens and secondary endpoint objectives for level of NK, T, and B cell activation with correlative analyses for percent changes in systemic CCL3 following these therapies, though results are not yet available (NCT00626405). Ultimately, the translational approach of utilizing this

chemokine for patients with aggressive cancers will rely heavily on ensuring that the therapy is stable and safe. Towards this end, we envision a delivery system for CCL3 either through controlled-release or multiple infusions systems. Because protein delivery brings forward its own feasibility issues with respect to short-lived stability [99–102], the future use of CCL3 in conjunction with cancer vaccines may very well be centered on inducible systems at the gene expression level. Such a controllable method of delivery provides its own advantages for specificity, thereby avoiding the induction of detrimental tolerance mechanisms such as MDSC recruitment and T_{Reg} activation. As these methods are tested more thoroughly in preclinical models and Phase I studies with patients, primary endpoints should focus on how CCL3 dosing and frequency affects immune responses as a whole: activation of lymph nodes, migration of DC populations, saturation of T cell entry into LNs or tumors, induction of single cytokine versus polyfunctional T cell populations, and undesirable recruitment of T_{Regs} or MDSCs to sites of T cell priming and killing.

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Key Issues

- Therapies that awaken the immune system can be categorized into the following domains: non-specific activation of the immune system with microbial components or cytokines, antigen-specific adoptive immunotherapy with antibodies or T cells, or active immunotherapy, which is direct vaccination against tumor antigens.
- Certain limitations to realizing the full potential of immunotherapy originate from suboptimal immune cell trafficking to sites of antigen-specific priming and effective killing at the tumor site; however, these shortcomings are currently being challenged by arming several axes of the chemokine network.
- Some of the most promising chemokine axes for cancer immunotherapy are CCL19/CCL21-CCR7; CCL2, CCL3, CCL5 and their cognate receptors (CCR1, CCR2, CCR4, and CCR5); CCL16 (liver-expressed chemokine [LEC]); and CXCL12-CXCR4 and CXCR7.
- The potent functions of CCL3 encompass the following: 1) compartmentalization and mobilization of myeloid precursor cells from the bone marrow into the periphery, 2) enhanced leukocyte infiltration to sites of acute inflammation, 3) memory CD8⁺ T cell control of secondary infection to different pathogens, 4) regulation of lymph node homing of dendritic cell subsets, and 5) generation and maintenance of CD8⁺ antigen-specific responses within draining lymph nodes.
- One of the rare instances by which permutations in chemokine expression and function dictate disease severity exists with CCL3. Copy number variations in *CCL3* and *CCL3L*, along with single nucleotide polymorphisms, have impacted both HIV-1 susceptibility and its disease progression. As such, it is critical to investigate the extent of such permutations in the context of available responses to immunotherapeutic strategies using CCL3.
- As CCL3 delivery methods are tested more thoroughly in preclinical models, Phase I studies, and randomized trials, the translational approach of utilizing this chemokine for patients with aggressive cancers will rely heavily on ensuring that the therapy is primarily stable, specific, and safe.

Figure 1. CCL3 participates in the induction and maintenance of antitumor immune responses *in vivo*

CCL3 is a chemokine with various functions of interest to immunotherapeutic platforms. Beginning in the periphery, CCL3 has been shown to be a potent mobilizer of DCs into the blood and into sites of inflammation (1.) [81,106]. Subsequently, systemic CCL3 has also been implicated in mediating DC trafficking into draining lymph nodes from sites of peripheral inflammation (2.) [98]. Within the draining lymph node itself, CCL3 is induced as a result of antigen-specific CD4⁺ T cell engagement with DCs, guiding CCR5⁺ naïve CD8⁺ T cells for antigen-specific activation (3.) [12]. In the final phase, CCL3 has been shown to enhance immune cell trafficking to various tumors (4.) [79,89,91]. DC, dendritic cell.

Table 1

Candidate chemokines for immunotherapy

Chemokine	Peripheral Inflammatory Sites	Draining Lymph Nodes	Intratumoral Sites
CCL2, CCL7	++ [17]	[11]	+ [104,105]
CCL3,CCL4	+++ [106]	++ [12]	+++ [89]
CCL5	++ [107]	+ [108]	++ [18,109]
CCL19, CCL21	+ (afferent lymph channels) [110]	+++ [10]	+ (construct) [20]
CCL20	+ [111]	+ (gut-associated lymphoid tissue) [112]	+ (construct) [19]
CXCL9, CXCL10	+++ [5]	+ [113]	+ [114]

+, ++, and +++ indicate increasing degree of chemoattraction relevant to host compartment

Table 2

Cellular sources and regulators of human MIP-1 α .
Reproduced with permission from [22].

Cell type	Inducer (+)/inhibitor (–)
Monocytes	(+) LPS; IL-1 β ; PHA; IFN- γ (+) IFN- α ; lipoteichoic acid; ICAM-1 (–) IL-4; IL-10; IL-13; dexamethasone
Macrophages	(+) HIV-1 infection; LPS; IL-1 β (–) IL-4; IL-10; (–) dexamethasone; NOS inhibitors ^d
T lymphocytes	(+) HIV-1 infection; PMA + PHA ^b ; (+) ICAM-1 + LFA-3 ^c (–) IL-4; IL-10; IL-18; IFN- γ ; (–) NOS inhibitors ^d
B lymphocytes	(+) BCR triggering
NK cells	(+) IL-12 + IL-15; (+) Physiological activation signals
Dendritic cells	(+) LPS; TNF- α ; CD40 ligand
Neutrophils	(+) LPS; LPS + GM-CSF; (+) Meningococcal outer membrane vesicles (–) IL-10 (+) Anti-IgE
Basophils	(+) RSV infection ^d
Eosinophils	Constitutively
Platelets	Constitutively
Bone marrow CD34 ⁺ cells	Active bone remodeling ^b
Osteoblasts	(+) IL-1 β
Astrocytes	(+) LPS; TNF- α ; IL-1 β
Microglia (fetal)	(+) RSV infection ^d
Epithelial cells (lower airway)	(+) <i>Salmonella dublin</i> ; IL-1 α ; TNF- α
Epithelial cells (colon)	

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Cell type	Inducer (+)/inhibitor (-)
Mesangial cells	(+) TNF- α + IFN- γ
Fibroblasts	(+) IFN- α + IL-1; IL-1
Vascular smooth muscle cells	(+) IL-4; IFN- γ ; IL-10; IL-1 β ; TNF

^a NOS inhibitors nitric oxide synthase inhibitors.

^b Only the induction of MIP-1 α mRNA, but not of MIP-1 α protein was investigated.

^c LFA: lymphocyte function associated.

^d RSV: respiratory syncytial virus.

Table 3

In vitro inflammatory activities of human MIP-1
Reproduced with permission from [22].

Cell type	Activity	M.E.C. ^a (ng/ml)	
		LD78 ^{a,b}	MIP-1 ^β
Monocytes	Chemotaxis	0.1	10–100
	Transendothelial migration	1–10	100
	[Ca ²⁺] _i increase	<20	<50
	<i>N</i> -Acetyl-β-D-glucosaminidase release ^c	10	300
T lymphocytes	Arachidonic acid release	<50	ND ^d
	Chemotaxis	10	10
	Transendothelial migration	0.1–10	10
Neutrophils	Chemotaxis	100 ^e	– ^f
	[Ca ²⁺] _i increase	10–30	–
	Transendothelial migration	–	–
	Elastase release	–	–
Eosinophils	Actin polymerization	–	–
	Chemotaxis	3–10	–
	[Ca ²⁺] _i increase	3	–
	Eosinophil cationic protein release	<100	–
Basophils	Chemotaxis	10	–
	[Ca ²⁺] _i increase	<500	–
	Histamine and LTC ₄ release	–	–
	Histamine and LTC ₄ release	10–100	–
Basophils (IL-3-treated)	Chemotaxis	1–10	1–10
Dendritic cells (immature)	Transendothelial migration	10	10
Natural killer cells	Chemotaxis	0.1	10
	Increase of NK cytolytic activity	1	1
	BLT ^g esterase release	1	1

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Cell type	Activity	M.E.C. ^a (ng/ml)	
		LD78a ^b	MIP-1β
NK cell clone (ERNK57)	Chemotaxis	3	10
	[Ca ²⁺] _i increase	3	10
Vascular smooth muscle cells	Granzyme A and N-acetyl-β-D-glucosaminidase release ^c	10	10
	Increase in tissue factor activity ([Ca ²⁺] _i dependent)	ND	10
Coronary endothelial cells	Chemotaxis	25	25
Platelets	[Ca ²⁺] _i increase	100–300	ND

^aM.E.C.: minimal effective concentration

^bIn most studies rLD78a(5-70) was used

^cCytochalasim B-treated cells.

^dND: not determined

^eAfter IFN-γ stimulation.

^fNo effect

^gBLT: N-α-benzylloxycarbonyl-L-lysine thiobenzyl ester

Table 4

CCL3 in experimental tumor models

Species CCL3	Experimental Model	Preclinical Strain	Cellular-Dependent Response	Treatment Outcome	Reference
Human	Ovarian carcinoma (CHO)	BALB/c	Neutrophils, granulocytes	Reduced tumorigenicity	[78]
Human, Mouse	Colon adenocarcinoma (C26)	BALB/c	Macrophages, granulocytes	Reduced tumorigenicity	[115]
Mouse	Melanoma (B16F10-OVA, B16-CCL3)	C57BL/6	Adoptively transferred antigen-specific CD8 ⁺ T cell (OT-1), endogenous CD8 ⁺ T cells	Suppressed tumor growth and increased survival upon rechallenge	[79]
Mouse	Melanoma (B16-MAGE -1)	C57BL/6	CD4 ⁺ , CD8 ⁺ T cells	Reduced tumorigenicity and increased survival with pulmonary metastasis	[8]
Mouse (EC1301)	Hepatocellular carcinoma (BNL IME A, 7R, 1)	BALB/c	CD4 ⁺ , CD8 ⁺ , CD11c ⁺ , F4/80 ⁺	Suppressed tumor growth	[82]
Mouse	Prelymphoblastic leukemia/lymphoma (A20)	BALB/cBYJ	CD8 ⁺ T cell, NK cells (CCL3 + IL-2 vaccine); CD4 ⁺ , CD8 ⁺ T cells (CCL3+ GM-CSF vaccine)	Suppressed tumor growth and extended survival	[83]
mCCL3	Gastric carcinoma (MFC)	BALB/c and C57BL/6	CCL3-recruited F4/80 ⁺ B220 ⁺ CD11c ⁺ cells, effector T cells	Suppressed tumor growth, reduced lung metastatic foci and extended survival	[80]

OVA, ovalbumin; MAGE-1, melanoma-associated antigen 1; GM-CSF, granulocyte-macrophage colony-stimulating factor

Table 5

Recent clinical studies evaluating CCL3 in the context of outcomes with immunotherapy

NCT Identifier	Phase	Patient Population	Intervention	Endpoint [Relevance to CCL3]	Status (opened)
NCT 00319748	II non-randomized; single group assignment	Metastatic refractory breast, ovarian, endometrial, and cervical cancer	852A (TLR7 agonist) subcutaneous injection twice weekly for 12 weeks with provisions for dose escalation	<u>Primary:</u> Time to progression (RECIST criteria) <u>Secondary:</u> mean difference values for CCL3	Completed, detailed results pending (2006)
NCT 00824733	II non-randomized; single group assignment	Locally advanced or metastatic HER2+ breast cancer	PF03512676 (24mer CpG TLR9 agonist) subcutaneous injection in combination with trastuzumab	<u>Primary:</u> Antibody-dependent cell-mediated cytotoxicity in context of PF03512676 <u>Secondary:</u> CCL3 induction following PF03512676 + trastuzumab delivery	Completed, detailed results pending (2009)
NCT 00626405	II randomized; two arms	Stage IV melanoma	Arm I: TMZ + bevacizumab Arm II: Bevacizumab + carboplatin + paclitaxel albumin-stabilized nanoparticle formulation	<u>Primary:</u> 6-month PFS and CTC toxicity <u>Secondary:</u> Tumor response rate, OS, % change in NK, T and B cell populations from baseline, % change in systemic CCL3	Completed, results not yet available (2008)
NCT 00088855	II non-randomized; single group assignment	State I, II, III plasma cell myeloma	Bortezomib and pegylated liposomal doxorubicin hydrochloride	<u>Primary:</u> Complete response rate and toxicity <u>Secondary:</u> Change in serum CCL3	Ongoing (2004)
NCT 02943473	II non-randomized; single group assignment	High risk smoldering multiple myeloma	Ibrutinib (kinase inhibitor) given daily orally	<u>Primary:</u> Number of patients without symptomatic myeloma <u>Secondary:</u> Change in serum CCL3	Ongoing (2017)

NCT, national clinical trial; TLR, toll-like receptor; RECIST, response evaluation criteria in solid tumors; HER2, human epidermal growth factor receptor 2; PFS, progression-free survival; CTC, common toxicity criteria; NK, natural killer