Potential Role of Chemokines in Immune Therapy of Cancer

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This article was submitted in memory of Dr. Yaakov Matzner and Dr. Amiram Eldor. The hematology community in the United States is greatly saddened by the untimely loss of Dr. Matzner, an outstanding hematologist, dean, and a visionary leader of the medical community in Israel, as well as a wonderful gentleman, and of Dr. Eldor, a dedicated hematologist and internationally recognized expert on stem cell transplantation. Both will be sorely missed, in Israel and abroad. May their memory be blessed.

Chemokine biology

Chemokines (derived from *chemo*-attractant cyto*kines*) are a large family of small (8–14 kDa), low molecular weight molecules that regulate the trafficking of neutrophils, lymphocytes, monocytes, dendritic cells and other immune effector cells [1–4]. Chemokines are characterized by four conserved cysteines at the NH2 terminal forming two essential disulfide bonds (CYS1-3, CYS 2-4) and serving as a basis for their classification into four families – C, CC, CXC, CX_3C – according to the position of the first two cysteines [1–4]. They can be further subclassified on a functional basis, as either inflammatory (also known as inducible) or constitutive (also known as homeostatic or lymphoid), depending on whether their pattern of production is evoked in response to external antigenic and inflammatory stimuli, or tissue invasion, or is evoked to mediate constitutive migratory patterns required for cell differentiation [1–4]. Although most chemokines are secreted into the local environment, at least one chemokine, fractalkine, is actually tethered via a 'stalk' to underlying vasculature [5]. Most secreted chemokines will attach to sulfated proteoglycans on neighboring cells by virtue of their positive charge, creating a gradient that will guide the mobility of cells expressing receptors for that particular chemokine [6,7].

Chemokines mediate their activity through a family of seven transmembrane G-protein-coupled specific receptors [1–4]. Human chemokines currently number approximately 50, with at least 20 specific receptors identified so far, allowing for a remarkable degree of complexity as well as redundancy. Although chemokines were initially identified on the basis of their regulatory function of leukocyte migration, further work revealed a highly complex role of chemokines in regulation of lymphoid organ development and homeostasis, angiogenesis, antimicrobial defense, tumor metastasis, and lymphocyte activation [8–12].

Chemokines regulate leukocyte extravasation through endothelial barriers by means of a complex process initiated with leukocytes rolling on endothelial cells under the influence of selectins and their carbohydrate ligands [1–4]. This process allows chemokines originating from endothelial cells to interact with G-protein-coupled chemokine receptors and subsequently to activate integrins, which in turn will mediate leukocyte adhesion to the endothelial cells and subsequent trans-endothelial migration into underlying tissues. Thus, the process of leukocyte extravasation is a function of a combinatorial process involving selectins and their ligands, chemokines and their receptors, and activated integrins and their receptors. This process is highly sensitive to inhibition with pertussis toxins through inhibition of the G-protein-coupled chemokine receptors [1–4].

One unusual aspect of the chemokine evolution has been the functional clustering of chemokines in select chromosomal locations, likely due to gene duplication. For example, CXC chemokines for neutrophils tend to cluster on chromosome 4q12-13 [1–4]. CC chemokines, which interact extensively with monocytes and macrophages, are clustered on 17q11.2, and a variety of T cell chemokines including CXC 9, 10 and 11 are clustered on 4q21.21 [1–4,11]. Chemokine receptors may interact with a variety of chemokines with differing affinities, mediating a pleomorphic effect on migration depending on receptor expression and the local chemokine environment [Figure 1]. For example, CCR-1 can interact

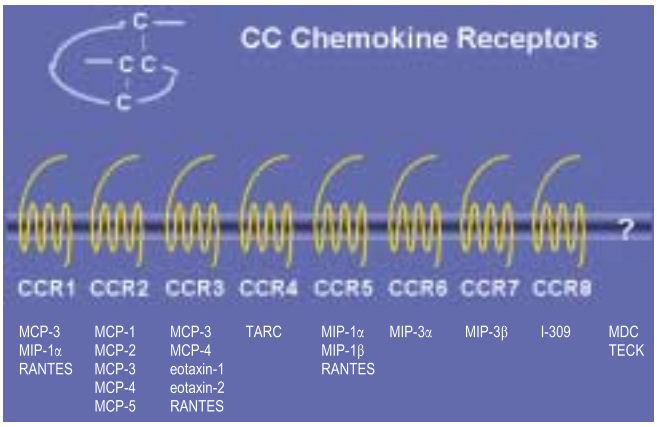


Figure 1. Promiscuous interactions with chemokine receptors. Alpha chemokine receptors CXCR1-CXR4 interact with a variety of chemokines at various affinities as shown. These include both inflammatory as well as constitutively produced chemokines. Beta chemokine receptors CCR1-CCR8 interact with a variety of inflammatory as well as constitutive chemokines allowing selective lymphocyte maturation and migration depending on local chemokine gradients and receptor expression.

with MCP-3, macrophage inflammatory protein-1α, and RANTES, while CCR5 can interact with MIP-1α , MIP-1β, and RANTES [Figure 1]. These interactions allow for both specificity and versatility in facilitating both migration and co-localization of cells that are required to interact to generate an immune response. Co-localization can be promoted due to the co-expression of shared chemokine receptors such as CCR1, CCR5 on Th1 cells and monocytes, CCR7 on naïve T cells and dendritic cells, or CCR3 on Th2 cells and basophils [1–4]. Immune cells in the periphery tend to express receptors for inflammatory chemokines, which following encounter with antigen may switch the expression of receptors for constitutively produced chemokines, enabling them to migrate to secondary lymphoid organs and undergo further differentiation as well as Th1 or Th2 polarization [Figure 2]. For example, immature dendritic cells released from the bone marrow may be attracted via inflammatory chemokines to the site of inflammation, via interactions with CCR1, CCR2, CCR5 and CCR6 receptors [Figure 2]. Following the capture of antigen and maturation due to the effects of local cytokines such as tumor necrosis factor alpha, interleukin-1 and lipopolysaccharide, dendritic cells may produce their own inflammatory chemokines, which cause down-regulation of their own receptors [13]. Following maturation, dendritic cells begin to express CCR4, CCR7 and CXCR4 and acquire the ability to respond to constitutively produced chemokines that direct migration to lymph nodes and/or other secondary lymphoid organs [14,15]. The receptor CCR7 can then mediate migration to the lymph nodes through the effects of the secondary lymphoid chemokine, which is secreted by the high endothelial venules on afferent lymphatics [15–17].

One interesting aspect of chemokine biology was recently reported in a seminal article by Poznansky et al. [18]. These

MIP = macrophage inflammatory protein

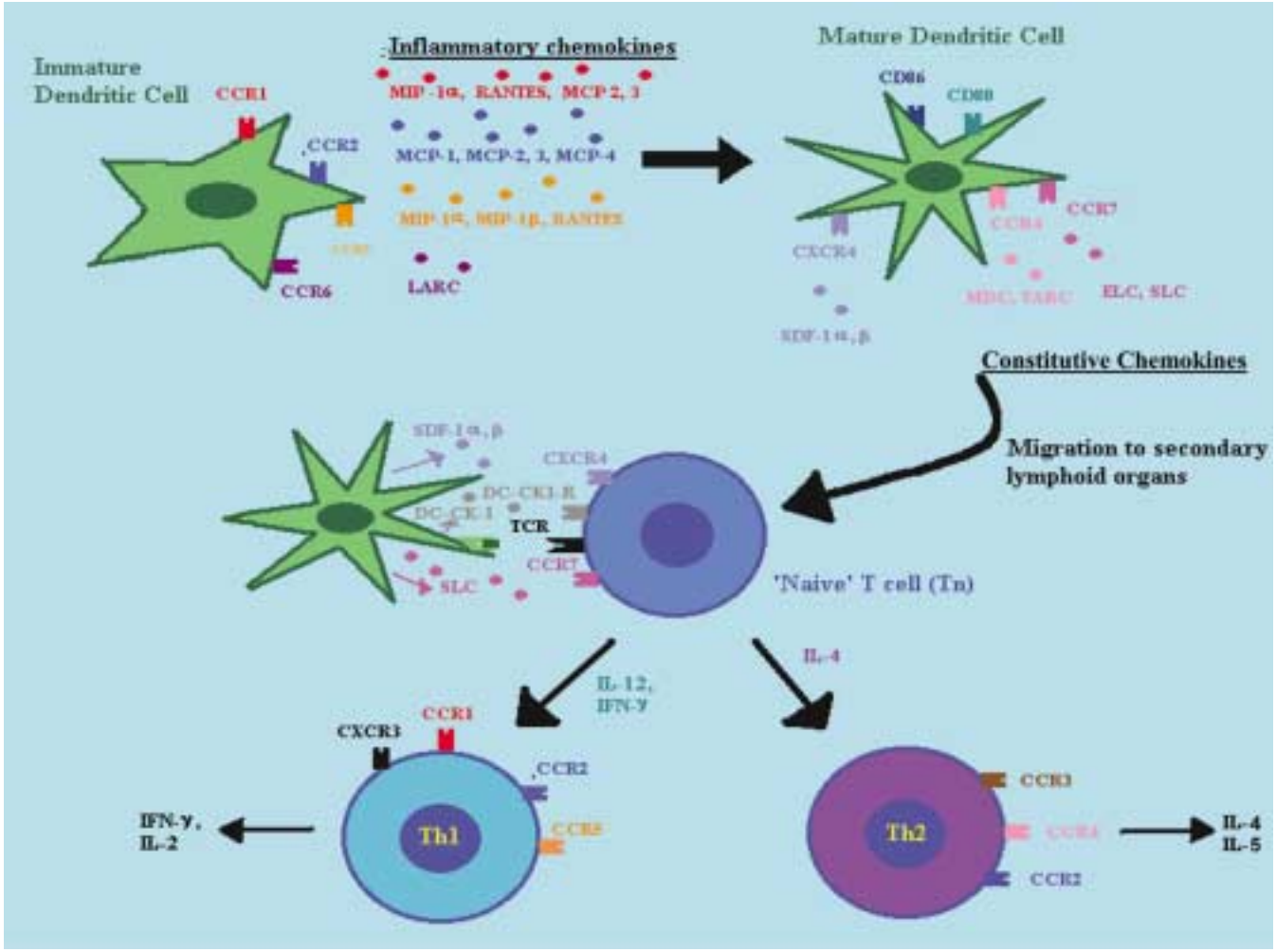


Figure 2. Role of chemokines in elaboration of a Th1, and Th2 response. Immature dendritic cell expressing CCR1, CCR2, CCR5 and CCR6 will respond to local inflammatory chemokines such as RANTES, MIP1α, and LARC, infiltrating tissue at the site of antigenic challenge. As antigen is captured and processed, antigen-presenting cells mature under the effects of local cytokines, and receptors for constitutively produced chemokines such as CXCR4 and CCR7 appear. These receptors mediate migration to secondary lymphoid organs such as the lymph nodes where encounters between mature dendritic cells and naïve T cells can take place. Naïve T cells share CXCR4 as well as CCR7 receptors allowing co-localization with dendritic cells. Following such encounters in the lymph nodes new chemokine receptors are acquired and further differentiation into Th1 and/or Th2 cells can proceed.

authors noticed the selective repulsion of subpopulations of T cells at high concentrations of recombinant SDF-1α mediated through the chemokine receptor CXCR4 [18]. Thus, migration away from a chemokine present at high concentrations is a newly recognized mechanism regulating the localization of mature T cells and other immune effector cells and may be involved in the egress and release of lymphoid cells following maturation in secondary lymphoid organs. Hence, chemokine biology is intimately linked to the process of antigen capture, dendritic cell maturation, and subsequent interactions between dendritic cells and lymphocytes in secondary lymphoid organs such as lymph nodes, and differentiation from naïve T cells into mature Th1 or Th2 effector cells. Naïve T cells express receptors for constitutively produced chemokines such as SDF-1α/β and SLC, as well as a receptor for a dendritic cell-produced chemokine known as DC-CK1 [19–21]. Following an encounter with dendritic cells in the lymph nodes, naïve T cells may differentiate further and acquire the ability to produce cytokines such as interferon gamma or IL-2 or for Th1 cells, and IL-4, IL-5 and IL-10 for Th2 type T cells.

SLC is a chemokine that plays a particularly important role in lymphoid differentiation. SLC is a CC chemokine that selectively attracts mature dendritic cells, naïve T cells, monocytes and natural killer cells [14–17]. SLC is strongly expressed in high endothelial venules and T cell-rich areas of the lymph node [14–17,21–23]. SLC binds to CCR7, which is expressed by mature dendritic cells and naïve T cells, and also to CXCR3, which is largely expressed on Th1 polarized T cells [14–17,21–23]. Disruption of the SLC-CCR7 interaction results in diminished dendritic cells and T cell recruitment to lymph nodes and Peyer's patches [24,25]. Loss of SLC secretion within lymph nodes is associated with a characteristic phenotype described as the PLT for "paucity of lymph node T cells" [25]. The PLT mouse lymph nodes are characterized by severe depletion of T cells [25].

Chemokine impact on tumor growth and metastasis

Like most normal tissues, several tumors are a rich source of chemokines while others express functional chemokine receptors [9–12,26]. Some of these chemokines will provide an autocrine growth signal such as IL-8 for cancer of the liver, pancreas [9] and ovary [26]. In ovarian cancer, the receptors for IL-8, CXCR1/2, interact with the EGFR receptor, a known oncogene that is currently being targeted in several epithelial tumors [26]. IL-8 also induces production of matrix metalloproteinases, necessary for transmigration through basement membranes and development of metastases [9,10]. CXC chemokines containing the ELR motif have been suggested to provide a pro-angiogenic signal necessary for the development of neovasculature in growing tumors [11]. The recent discovery of functional receptors for SDF-1 (CXCR4) and SLC/ELC (CCR7) on primary breast cancer and melanoma cells has provided an intriguing explanation for the propensity of these tumors as well as others to migrate into lymph nodes and other specific tissues [12]. Therefore, it is clear that chemokines may be involved in myriad aspects of tumor cell invasion, angiogenesis and metastatic behavior.

Chemokine delivery using gene transfer

Because of the central role played by chemokines and the considerable resources invested by the immune system in regulating migration, we and other investigators have postulated a potential role for chemokines in enhancing immune therapy strategies in cancer. In early experiments in the laboratory we utilized gene transfer to express inflammatory chemokines locally at the site of malignancy in an effort to stimulate the enhanced recruitment and local activation of T cells at the site of tumor [27]. We studied delivery of RANTES by using herpes simplex-derived amplicon vectors delivered alone or in combination with the T cell co-stimulatory ligand B7.1. Using pre-established murine lymphomas (EL4) and HSV amplicon vectors expressing B7.1 (HSV-B7.1) or RANTES (HSV-RANTES) we investigated the potential use of chemokines to evoke a response to established tumors [27]. Transduced cells expressed high levels of B7.1 or RANTES, as analyzed by flow cytometry and/or an enzyme-linked immunosorbent assay respectively. Direct injection of HSV-B7.1 or HSV-RANTES alone or in combination into established EL4 tumors led to complete tumor regression in both injected tumors as well as in the non-transduced contralaterally implanted tumors in about half of the mice, whereas control tumors or tumors injected with an HSV amplicon vector designed to express beta galactosidase did not regress in this model. We could achieve maximum protection (over 90% of mice) using a combination of HSV-B7.1 and HSV-RANTES, and animals achieving such protection were resistant to rechallenge with parental tumor cells [27]. All mice that rejected tumors demonstrated tumor cell-specific cytolytic T cell activity, suggesting that the local delivery of an inflammatory chemokine alone, or combined with local expression of a T cell co-stimulatory ligand in order to locally activate T cells, could result in the establishment of systemic immunity [27].

In a second series of experiments we explored the potential role of constitutive chemokine expression using gene transfer. Our candidate chemokine was the secondary lymphoid tissue chemokine, which promotes the co-localization of naïve non-polarized memory T cells and dendritic cells within lymph nodes and Peyer's patches [14–17,21–25]. As a co-stimulatory ligand we used CD40L. CD40L, a member of the tumor necrosis factor superfamily, is a type II transmembrane glycoprotein that is transiently expressed on antigen-activated CD4+ T cells [29]. CD40L plays a role in activating antigen-presenting cells, including dendritic cells, monocytes and B cells [28,29]. We explored the anti-tumor activity of SLC and CD40L used alone or in combination, once again using HSV amplicons as a vehicle for gene transfer. This was explored in two murine models: A20, a B cell lymphoma, and CT26, an adenocarcinoma. We hypothesized that the local elaboration of SLC could augment anti-tumor

SLC = secondary lymphoid chemokine
IL = interleukin

HSV = herpes simplex virus

immune responses in part through the increased recruitment of naïve T cells, and the co-localization of such T cells with mature antigen-presenting dendritic cells. Supernatant from HSV-SLC transduced tumors could mediate transmigration of T cells in a two-chamber assay, and production of SLC was further verified using an ELISA [30]. The administration of amplicons encoding SLC into established subcutaneous tumors resulted in very heavy infiltration with CD4+ as well as CD8+ T cells and dendritic cells. Significantly higher numbers of CD11c+ dendritic cells were observed in HSV-SLC transduced tumors compared to control transduced tumors. Approximately 80% of A20 tumors and about 40–50% of CT26 tumors transduced with HSV-SLC were eradicated, similar to the numbers eradicated using HSV-CD40L transduction [30]. In contrast to expression of SLC, the introduction of vectors encoding either SDF-1α or DC-CK-1 did not result in tumor rejection despite the ability to attract T cells to the tumor bed [Figure 3]. When bilateral tumors were implanted, the unilateral treatment of a single tumor with either HSV-SLC or HSV-CD40L resulted in eradication of approximately half the injected tumors [30]. A statistically significant improvement in eradication of the contralateral tumor resulting in 80–90% tumor eradication was seen using the combination of HSV-SLC and HSV-CD40L, with improvement in survival compared to either reagent alone [30]. The effect appeared to primarily depend on CD8+ T cells, whereas CD4 T cell depletion only modestly affected anti-tumor activity. CTL activity was detected in both the A20 and CT26 models. We also detected a substantial increase in local elaboration of IL-12 and IFNγ by infiltrating cells in tumors transduced with HSV-SLC or HSV-CD40L. Only tumors treated with HSV-CD40L or a combination of both amplicons had a detectable message for IFNγ, perforin and/or the inducible P40 subunit of IL-12 [30]. Hence this strategy appeared to result in the aggressive recruitment of T cells and an augmented Th1 response.

Since several different subsets of T cells expressed the CCR7 and/or CXCR3 receptors and are responsive to recruitment by SLC, the exact mechanism of rejection is not clearly known. Both naïve T cells and central memory T cells could be theoretically recruited and activated. Whether the anti-tumor activity of HSV-SLC is due to priming and expansion of the central memory T cell subset or to recruitment and priming of naïve T cells is still not known. However, we believe that by using a combination of HSV-SLC and HSV-CD40L we were able to foster local dendritic cell accumulation and T cell localization and reproduce, in part, lymph node conditions necessary for transition from naïve T cells into effector cells.

Several other groups have also documented the potential utility of SLC and other local chemokine expression in facilitating tumor regression [31–34]. In a mouse model for spontaneous bronchoalveolar carcinoma, the local injection of recombinant SLC into axillary nodes of mice led to enhanced infiltration of tumor sites by CD4+ and CD8+ T cells, as well as dendritic cells, enhanced local secretion of type 1 cytokines, and a reduction in overall tumor burden, as reported by Dubinett and co-workers [34]. Using adenoviral vectors expressing SLC Fushimi et al. [35] demonstrated the accumulation of dendritic cells and CD8+ T-cells at the site of tumors and reduced tumor growth in several murine tumor models, resulting in systemic immunity. In a clever strategy designed to mimic normal patterns of SLC expression and promote T cell-dendritic cell co-localization, SLC gene-modified dendritic cells were injected directly into pre-established B16 melanomas in mice, and were more effective than either control dendritic cells or injection of SLC alone at eliciting an anti-tumor response [33]. Hence, numerous laboratories have documented the ability of chemokines in general, and SLC in particular, to facilitate selective recruitment to the site of tumors and improve both local and systemic immune responses.

Chemokine delivery using antibody-chemokine fusion proteins

Our laboratory has also explored alternative delivery strategies for generation of local chemokine gradients. In collaboration with the laboratory of Dr. Sheri Morrison at UCLA we explored the potential linking of chemokine to an antibody for purposes of local delivery and chemokine gradient generation [36]. Using a humanized immunoglobulin G3 we were able to tether the amino acid backbone of RANTES to the amino terminus of an antibody heavy chain directed against a human tumor antigen. Our antibody sequences were directed against her2/neu, a tumor antigen prevalent on a subset of human breast, ovarian and other tumors. Using variable sequences directed at her2/neu, we were able to construct and purify an antibody fused to the RANTES chemokine [Figure 4]. Both antigen binding as well as chemokine function are preserved in this bi-functional molecule. Our proposed strategy would then involve the local recruitment of T cells to the site of tumors, by administering systemic antibody fusion protein followed by local co-stimulation with a separately designed antibody fusion protein capable of providing T cell co-stimulation. RANTES-HER2/IgG3 will bind selectively to her2/neu-expressing tumors as demonstrated by flow cytometry [36]. Experiments in the laboratory have demonstrated the ability of the RANTES-HER2/IgG3, a chemokine-antibody fusion protein, to localize selectively to tumors expressing HER2 in a xenograft model of severe combined immunodeficiency disease. This fusion protein retains the ability of RANTES to selectively engage the CCR5 chemokine receptor, as demonstrated by blocking the entry of human immunodeficiency virus-1, through interaction with the CCR5 co-receptor for HIV-1, similar to what is seen with RANTES [37]. In more recent experiments in the laboratory we have been able to demonstrate migration of a constitutively cytotoxic T cell line (TALL-104) in response to RANTES-HER2/IgG3, and we are currently testing the ability to selectively target T cell effector cells *in vivo* using either gene transfer or antibody-chemokine fusion proteins as a novel strategy [Figure 4].

ELISA = enzyme-linked immunosorbent assay

IFN = interferon

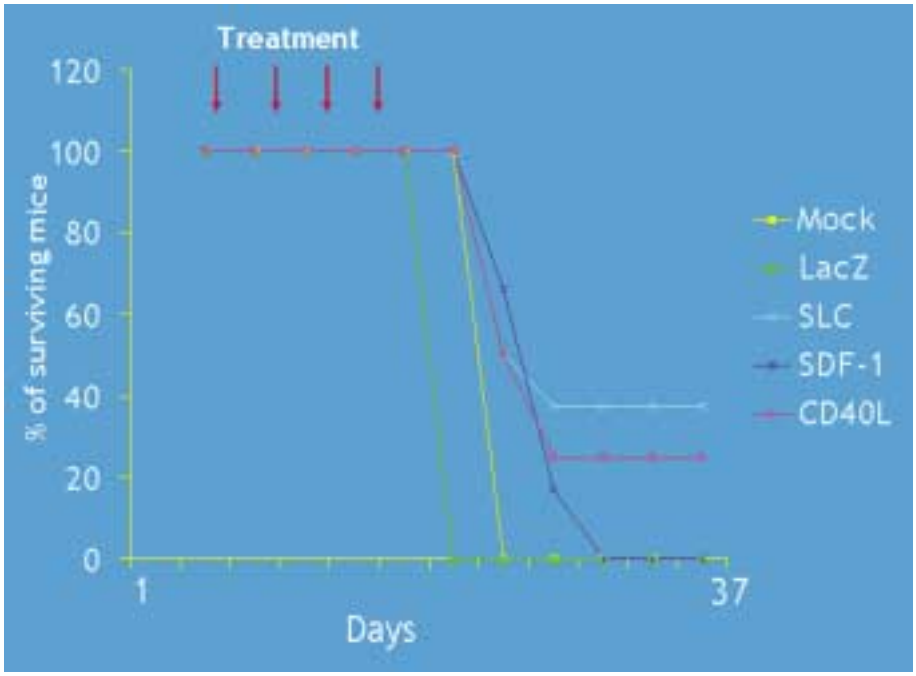
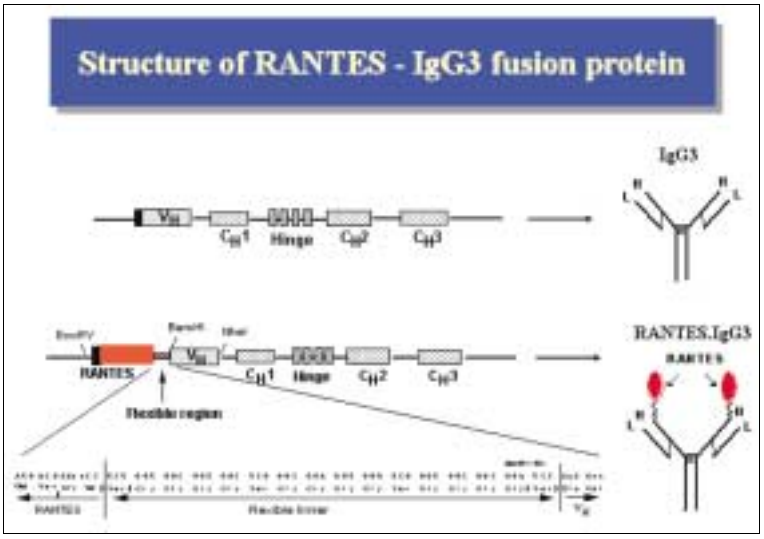


Figure 4. [A] Design of an antibody-chemokine fusion protein. A schematic representation of several anti-her2/neu proteins created in the laboratory. Anti-her2/neu variable sequences derived from the 4D5 parental antibody were subcloned into a humanized IgG3 backbone. Sequences encoding either the B7.1 T cell co-stimulatory ligand or the inflammatory chemokine RANTES were fused to the amino-terminus of the anti-her2/neu heavy chain via a flexible linker. The resultant antibody fusion proteins retained chemokine or co-stimulatory ligand function as well as antigen specificity.
[B] Migration of TALL-104 cells *in vitro* in response to RANTES -her2/IgG3. TALL-104 cells were placed in the upper chamber of a transwell migration chamber and either RANTES-her2/IgG3 or control anti-her2 IgG3 antibody at indicated concentrations was placed in the lower well. TALL-104 cells were allowed to migrate for 4 hours and migrating cells were counted. Migration was observed when either native RANTES or the RANTES-her2/IgG3 fusion protein was used to create a chemokine gradient between wells, but not in response to control antibody.

Conclusions

The versatility of chemokines and the evolving strategies for local chemokine delivery create myriad opportunities for potentially augmenting current immune strategies. Our experiments as well as those of others in the field demonstrate the potential utility of using chemokines to promote the recruitment and local engagement of T cells, dendritic cells, and other immune effector cells at the site of tumor for purposes of immune therapy. Our preliminary data suggest that both inflammatory as well as constitutively produced chemokines may be of utility. Furthermore, in our animal models it would appear that local elaboration of chemokines in conjunction with local expression of co-stimulatory molecules such as B7.1 or CD40L might engender systemic responses that are useful in eradicating distant tumors. Early experiments suggested that this can be readily accomplished by means of gene transfer and we are currently exploring whether engineered antibody fusion proteins may also be of use to create localized chemokine gradients. To date, immunotherapy has demonstrated efficacy in a small number of human tumors such as melanoma and renal cell carcinoma and utility



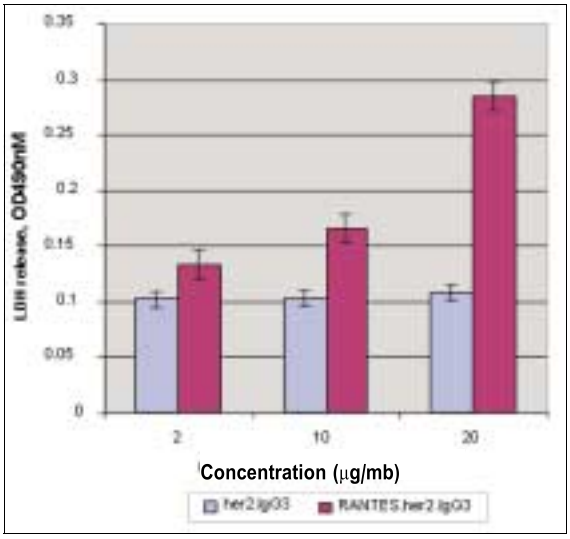


Figure 3. Effects of local elaboration of SLC, or SDF-1α on local tumor growth in mice. Coding sequences for the chemokines SLC, human DC-CK1, and SDF-1α and/or the co-stimulatory ligand CD40L were introduced into an HSV amplicon vector and packaged vector used to transduce 7–10 day old CT26 tumors previously established in mice. Tumor regression was observed in tumors transduced with HSV-SLC, and HSV-CD40L alone. Neither HSV-SDF-1α nor HSV-DC-CK1 (data not shown) used alone caused tumor regression. Tumor growth curves from a representative experiment are shown.

Ig = immunoglobulin

HIV = human immunodeficiency virus

has largely been limited to select patients. Nevertheless, immune responses are often long-lived and frequently complete. Further augmenting immune strategies through the local delivery of chemokines to the site of tumors may be a viable means by which to improve the immune response to human tumors.

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