Published in final edited form as:

Cancer Discov. 2016 September ; 6(9): 1022–1035. doi:10.1158/2159-8290.CD-15-1412.

Autocrine Complement Inhibits IL10-Dependent T-Cell Mediated Antitumor Immunity to Promote Tumor Progression

Yu Wang¹, Sheng-Nan Sun², Qing Liu², Yang-Yang Yu³, Jian Guo¹, Kun Wang⁴, Bao-Cai Xing⁴, Qing-Feng Zheng⁵, Michael J. Campa⁶, Edward F. Patz Jr.^{6,7}, Shi-You Li², and You-Wen He¹

¹Department of Immunology, Duke University Medical Center, Durham, NC 27710, USA.

²Key Laboratory of Genomic and Precision medicine, Beijing Institute of Genomics, Chinese Academy of Sciences, No. 1 Beichen West Road, Chaoyang District, Beijing 100101, China.

³Key Laboratory of TCM-information Engineer of State Administration of TCM, School of Chinese Materia Medica, Beijing University of Chinese Medicine, No. 6 Wangjing Zhong Huan South Road, Chaoyang District, Beijing 100102, China.

⁴Hepatopancreatobiliary Surgery Department, Key Laboratory of Carcinogenesis and Translational Research, Ministry of Education, Peking University School of Oncology, Beijing Cancer Hospital and Institute, Beijing, China. No. 1 Beichen West Road, Chaoyang District, Beijing 100101, China.

⁵Thoracic Surgery Department, Peking University School of Oncology, Beijing Cancer Hospital and Institute, Beijing, China. No. 1 Beichen West Road, Chaoyang District, Beijing 100101, China.

⁶Department of Radiology, Duke University Medical Center, Durham, NC 27710, USA.

⁷Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710, USA

Abstract

In contrast to its inhibitory effects on many cells, IL-10 activates CD8⁺ tumor infiltrating lymphocytes (TILs) and enhances their antitumor activity. However, CD8⁺ TILs do not routinely express IL-10 as autocrine complement C3 inhibits IL-10 production through complement receptors C3aR and C5aR. CD8⁺ TILs from C3-deficient mice, however, express IL-10 and

Address correspondence to Dr. You-Wen He, Box 3010, Department of Immunology, DUMC, Durham, NC 27710. Phone: 919-613-7870. Fax: 919-684-8982. youwen.he@duke.edu.

Accession Number

The referenced microarray data in the NCBI GEO public database are under accession number GEO: GSE25846 and GSE79127.

Authors' Contributions

Conception and design: Y. Wang, Y-W. He

Development of methodology: Y. Wang

Acquisition of data: Y. Wang, S-N. Sun, Q. Liu, Y-Y. Yu, K. Wang, B-C Xing, Q-F. Zheng, S-Y. Li, M. Campa, E. Patz

Analysis and interpretation of data: Y. Wang, Y-W. He

Writing, review and/or revision of the manuscript: Y. Wang, Y-W. He

Administrative, technical, or material support: J. Guo

Study supervision: Y-W. He

The authors have declared that no conflict of interest exists.

exhibit enhanced effector function. C3-deficient mice are resistant to tumor development in a T cell- and IL-10-dependent manner; human TILs expanded with IL-2 plus IL-10 increase the killing of primary tumors *in vitro* compared to IL-2 treated TILs. Complement-mediated inhibition of antitumor immunity is independent of the PD-1/PD-L1 immune checkpoint pathway. Our findings suggest that complement receptors C3aR and C5aR expressed on CD8⁺ TILs represent a novel class of immune checkpoints that could be targeted for tumor immunotherapy. Moreover, incorporation of IL-10 in the expansion of TILs and in gene-engineered T cells for adoptive cell therapy enhances their antitumor efficacy.

Keywords

CD8⁺ tumor infiltrating lymphocytes; IL-10; complement; antitumor immunity; complement receptors C3aR and C5aR

INTRODUCTION

The complement system is a major component of innate immunity but also regulates many aspects of the adaptive immune response (1). Complement receptors are expressed on T lymphocytes (2-5) and locally produced C3a and C5a bind to their receptors on CD4⁺ T cells to promote differentiation and cell survival, and regulate IFN γ and IL-17 production(2, 6). Intracellular generation of C3a is required for human CD4⁺ T cell survival (7), although the role of complement on regulatory CD4⁺ T cells (Tregs) is complex. Complement has been shown to decrease the immunosuppressive capacity mediated by Tregs while others suggest that complement promotes Treg generation (3-5, 8).

Given its important role in innate and adaptive immunity, complement has long been assumed to play an active role in tumor immune surveillance. However, mounting evidence suggests that complement signaling may inhibit antitumor immunity in humans (9). Clinical studies have found that complement levels in patients' plasma or tumors positively correlate with tumor size and poor outcome in lung cancer, colorectal cancer, neuroblastoma in children, ovarian cancer, carcinomas of the digestive tract, brain tumors, and chronic lymphocytic leukemia (9). Furthermore, recent experimental results show that mice lacking complement components (C3, C4, or C5aR) or treated with complement inhibitors exhibit tumor resistance or suppressed metastasis (8, 10-12). Complement may also inhibit antitumor immunity by recruiting myeloid-derived suppressor cells (MDSCs) (8, 10, 12) or by inhibiting NK cell activation(13).

Despite the well-known inhibitory function on many types of cells (14), IL-10 enhances the effector function of activated human and mouse CD8⁺ T cells by increasing cytolytic activity (15-17), and has been used to boost antitumor immunity (18-22). These studies demonstrate that IL-10 activates and expands CD8⁺ tumor infiltrating lymphocytes (TILs) and enhances their antitumor activity *in situ*. However, conflicting reports suggest that IL-10 may promote tumor growth and progression (23, 24). Given its pleiotropic function, the source of IL-10 may be critical in promoting CD8⁺ TIL antitumor activity. Generating IL-10 producing CD8⁺ TILs within the tumors and autocrine IL-10/IL-10R interaction are a preferable approach for IL-10-based tumor immunotherapy, as this avoids the inhibitory effect of IL-10

on other cell types. However, CD8⁺ TILs do not produce IL-10 under normal conditions in tumor models (24, 25) and it is not clear whether IL-10 can be induced or how induction would be achieved. Furthermore, the antitumor effect of IL-10 producing CD8⁺ T cells is also not known. In this study, we aimed to identify the molecular regulator of IL-10 production in CD8⁺ TILs and characterize the roles of complement and IL-10 in the regulation of CD8⁺ TIL antitumor T cell responses.

RESULTS

Regulation of IL-10 Expression in CD8⁺ T Cells by Complement

A previous report by Tandem et al. found that a fraction of the viral-specific CD8⁺ T cells in the central nerve system expresses IL-10 at the peak of coronavirus infection (26). These IL-10⁺CD8⁺ T cells express higher levels of IFN γ and TNF α compared to IL-10⁻CD8⁺ T cells and protect mice from chronic encephalomyelitis. The authors analyzed the gene expression profiles in sorted IL-10 eGFP⁺ or eGFP⁻ effector CD8⁺ T cells. To investigate the molecular regulation of IL-10 production in effector CD8⁺ T cells, we analyzed the gene expression profiles of IL-10⁺CD8⁺ T cells, based on the data by Tandem et al. (26). IL-10⁺CD8⁺ T cells had a very distinct gene expression pattern compared to that in IL-10⁻CD8⁺ T cells (Fig. 1A) (26). The higher *Il10* mRNA level in GFP⁺CD8⁺ T cells than that in GFP⁻CD8⁺ T cells indicates that GFP expression faithfully reflected IL-10 expression (Supplementary Fig. S1A). These differentially expressed genes are likely comprised of regulators and/or effectors of IL-10. Pathway analysis revealed that genes involved in the complement pathway were highly enriched among the genes differentially expressed between IL-10⁺ and IL-10⁻CD8⁺ T cells (Fig. 1B). The mRNA expression levels of several complement components and their receptors were upregulated in the IL-10⁺CD8⁺ T cells (Fig. 1C, D and Supplementary Fig. S1B). These data suggest that complement signaling pathways may be involved in the regulation of IL-10 expression in CD8⁺ T cells.

We then tested whether complement signaling could regulate IL-10 production in effector CD8⁺ T cells during tumor development. We crossed *Il10* reporter mice (termed Tiger mice), in which an IRES-GFP cassette was inserted between the stop codon and polyadenylation signal of the *Il10* gene (27), with C3-deficient mice (28) and inoculated wild type and *C3*^{-/-} *Il10* reporter mice with B16 melanoma. We examined IL-10 production in CD8⁺ TILs. Interestingly, approximately 10% of CD8⁺ TILs expressed high levels of IL-10 in *C3*^{-/-} mice, whereas, as expected, no IL-10 producing CD8⁺ TILs were detected in the wild type Tiger mice (Fig. 1E). Importantly, CD8⁺ T cells in the draining lymph nodes (dLNs) of either wild type Tiger or *C3*^{-/-} Tiger mice did not produce IL-10 (Supplementary Fig. S1C). These data demonstrate that complement signaling inhibits IL-10 production in CD8⁺ TILs and its removal is sufficient to promote IL-10 production in the tumor microenvironment but not in the periphery.

Suppression of T cell-mediated Antitumor Immunity by Complement

Exogenous IL-10 activates CD8⁺ TILs and promotes their antitumor activity (21, 22). Given that CD8⁺ TILs produced IL-10 in the absence of complement, complement-deficient mice may be resistant to tumor development due to an enhanced antitumor activity of CD8⁺ TILs.

Indeed, B16 melanoma growth was dramatically slower in C3-deficient mice than that in wild type mice (Fig. 2A-C). $C3^{-/-}$ mice were also resistant to E0771 breast cancer development (Fig. 2D-F). These results are consistent with previous reports showing resistance to tumor development or metastasis when complement signaling is disrupted *in vivo* (8, 10-12).

Since CD8⁺ TILs from $C3^{-/-}$ mice produce IL-10, the tumor-resistance by $C3^{-/-}$ mice may be due to an IL-10 induced effector function. Indeed, CD8⁺ T cells were the dominant cell population in TILs from $C3^{-/-}$ mice, representing an approximate 4-fold increase in their numbers compared to those from wild type mice (Fig. 2G). These CD8⁺ TILs exhibited multi-potency as demonstrated by a simultaneous increase in their IFN γ and TNF α expression (Fig. 2H). To further determine whether the enhanced antitumor response in $C3^{-/-}$ mice is T cell-mediated, we crossed $C3^{-/-}$ mice with $TCRa^{-/-}$ mice (29) and investigated tumor growth in the $C3^{-/-}TCRa^{-/-}$ mice. As expected, $TCRa^{-/-}$ mice had impaired antitumor immunity (Fig. 2I). Removal of mature T cells from $C3^{-/-}$ mice resulted in a complete loss of their tumor resistance (Fig. 2I). These results suggest that the enhanced antitumor immunity in $C3^{-/-}$ mice was likely mediated through the enhanced CD8⁺ CTL-mediated killing.

Non-CD8⁺ T Cell Responses in Tumor Bearing Mice

Previous studies suggest that complement inhibits antitumor immunity by recruiting myeloid-derived suppressor cells (MDSCs) or preventing NK activation in different tumor types or different animal models (8, 10, 12, 13). We quantified MDSCs in B16 melanoma from tumor bearing mice. Comparable numbers of myeloid cells in the dLNs of both types of mice were observed (Supplementary Fig. S2A). The percentages of CD11b⁺GR1⁺ cells, which contain both CD11b^{high}GR1^{high} neutrophils and CD11b⁺GR1^{dim} MDSCs, were comparable in tumor infiltrating leukocytes from the tumors growing in either wild type or $C3^{-/-}$ mice (Fig. 3A). Most of these cells were CD11b^{high}GR1^{high} neutrophils (Fig. 3A). These data suggest that MDSC-mediated immunosuppression might not be a major cellular mechanism in B16 melanoma in $C3^{-/-}$ mice.

Expanded Tregs in dLNs and TILs are associated with tumor immunosuppression. Complement signaling regulates Treg differentiation through C3a and C5a receptors in CD4⁺ T cells (3). However, no difference was found in the percentage of Tregs in dLNs or TILs from wild type and $C3^{-/-}$ mice (Supplementary Fig. S2B and Fig. 3B). Although the percentage of CD4⁺ TILs was comparable between wild type and $C3^{-/-}$ mice (data not shown), more CD4⁺ TILs from $C3^{-/-}$ mice expressed effector cytokines (Supplementary Fig. S2C). To test the direct impact of IL-10 on CD4⁺ T cell function, we purified human and mouse CD4⁺ T cells and activated them by anti-CD3/CD28 antibodies *in vitro* with or without IL-10. IL-10 did not obviously alter the effector status of either human or mouse CD4⁺ T cells *in vitro* (Supplementary Fig. S2D and E). These results suggest that the enhanced effector phenotype in CD4⁺ TILs is likely due to an indirect effect in the tumors from $C3^{-/-}$ mice. Furthermore, the percentage of total or activated NK cells in the TILs from $C3^{-/-}$ mice did not change compared to wild type mice (Fig. 3C). In addition, we observed

no difference in the cell populations of NKT cells, Th17 cells, macrophages, or dendritic cells in the tumor infiltrating leukocytes between wild type and $C3^{-/-}$ mice (data not shown).

Essential Role for IL-10 in Antitumor Response in $C3^{-/-}$ Mice

To test whether IL-10 is essential for the enhanced antitumor immunity in $C3^{-/-}$ mice, we crossed $C3^{-/-}$ mice with $Il10^{-/-}$ mice (30) to generate double mutant mice and examined tumor development in these mice. Deletion of *Il10* in $C3^{-/-}$ mice completely abolished their enhanced tumor resistance to B16 melanoma (Fig. 4A-C) as well as E0771 breast cancer (Fig. 4D-F). However, *Il10* deletion in the wild type background did not result in altered antitumor immunity compared to wild type mice (Fig. 4A-C), suggesting that IL-10 may not be involved in antitumor immunity in these tumor models when complement signaling is intact, as the complement signaling prevents IL-10 production in CD8⁺ T cells (Fig. 1E).

Enhanced Human TIL Function by IL-10

We tested whether recombinant human IL-10 could enhance the function of TILs from cancer patients. TILs isolated from human lung tumors started to expand and enter logarithmic growth phase after two weeks of culture in the presence of IL-2 (Fig. 5A). IL-10 robustly enhanced the proliferative capacity of TILs when added with IL-2, whereas IL-10 alone did not drive TILs into the cell cycle (Fig. 5A). To test the tumor killing of the expanded TILs directly, we co-cultured the *in vitro*-expanded TILs with autologous primary tumors. Compared to IL-2 expanded TILs, TILs expanded by IL-2 plus IL-10 induced a rapid and more effective killing of primary tumor cells (Fig. 5B). Furthermore, CD8⁺ TILs expanded *in vitro* with IL-2 plus IL-10 had a much enhanced expression of IFN γ and TNF α (Fig. 5C and D). However, the expression of T cell exhaustion markers, such as PD-1, LAG3, and TIM-3 were not altered by IL-10 (Supplementary Fig. S3). Together, these results suggest that IL-10 may be applied as a T cell growth factor for *in vitro* expansion of human TILs. To further understand how IL-10 promotes human CD8⁺ TIL function, we performed a mRNA expression profiling assay in IL-2-expanded CD8⁺ TILs from human lung tumors with or without IL-10. IL-10/IL-2 cultured CD8⁺ TILs displayed a different gene expression pattern compared to that in IL-2 cultured CD8⁺ TILs (Fig. 5E). IL-10 upregulated genes related to several signaling pathways, including TCR signaling (Fig. 5F), Notch signaling (Fig. 5G), cell cycle (Fig. 5H), and killing activity (Fig. 5I). The effect of IL-10 on the expression of chemokine genes was variable depending on the specific chemokine (Fig. 5J). These results are consistent with published mouse data (21), which also suggests that IL-10 expands CD8⁺ TILs and promotes their killing activity by upregulation of perforin and granzymes *in vivo* and *in vitro*. Together, these data indicate that IL-10 plays a similar role in human and mouse CD8⁺ T cells that will facilitate the translation of mouse studies to human clinical trials.

Suppression of IL-10 Production by Autocrine C3

We next determined whether endogenous C3 expressed by CD8⁺ T cells inhibits their IL-10 production. We transplanted CD4⁺ and CD8⁺ T cells from wild type or $C3^{-/-}$ naive mice into $C3^{-/-}$ *TCRA*^{-/-} recipients and inoculated the mice with B16 melanomas (Fig. 6A). Tumors in mice receiving $C3^{-/-}$ CD8⁺ T cell developed slower than those in mice receiving wild type CD8⁺ T cells (Fig. 6B), indicating that complement C3 expressed by CD8⁺ T cells

inhibits their antitumor activity through an autocrine mechanism. In addition, C3aR and C5aR transcripts were upregulated in IL-10 producing effector CD8⁺ T cells (Fig. 1D). We then examined the surface expression of C3aR and C5aR on CD8⁺ TILs. Peritoneal macrophages and splenic neutrophils, which express high levels of C3aR and C5aR (Supplementary Fig. S4A and B) were used as controls. CD8⁺ T cells from dLNs of naïve mice or tumor bearing mice did not express C3aR or C5aR (Fig. 6C and D, left 2 panels). However, ~20% of the CD8⁺ TILs from melanomas expressed C3aR and C5aR (Fig. 6D, right 2 panels). A similar percentage of CD8⁺ TILs from an E0771 breast cancer model also expressed C3aR and C5aR (Fig. 6E and F). Moreover, CD8⁺ TILs isolated from human liver tumors expressed high levels of C3aR and C5aR (Fig. 6G).

It has been reported that complement signaling is activated and anaphylatoxins are generated in the tumor microenvironment(10). We found that anaphylatoxins C3a and C5a were detected in freshly isolated B16 tumors from wild type mice (Supplementary Fig. S4C). To test the role of locally produced C3a and C5a in regulating IL-10 expression in CD8⁺ TILs, we overexpressed both subunits of carboxypeptidase N (CPN) 1 and 2 in B16 melanoma cells (Supplementary Fig. S4D and E). Local overexpression of CPN inactivated anaphylatoxins (C3a and C5a) in the tumors from wild type mice (Supplementary Fig. S4C) and restored IL-10 production in CD8⁺ TILs to an extent similar to that in CD8⁺ TILs from tumors of C3^{-/-} hosts (Fig. 6H and I). In addition, blockade of C3aR and C5aR using antagonists also restored IL-10 production *in vivo* (Fig. 6I), indicating that locally produced C3a and C5a inhibit IL-10 production in CD8⁺ TILs. To determine whether anaphylatoxins inhibited IL-10 expression through C3aR and C5aR on CD8⁺ T cells, we purified and activated CD8⁺ T cells *in vitro* and determined their C3aR and C5aR expression. Interestingly, C3aR and C5aR were detected on activated CD8⁺ T cells only after long-term *in vitro* culture (Supplementary Fig. S4F and G). C3aR or C5aR *in vitro* blockade alone using antagonist slightly increased IL-10 expression in activated CD8⁺ T cells while combined blockade of both receptors further enhanced IL-10 expression in these cells (Fig. 6J). Taken together, the *in vivo* and *in vitro* results demonstrate that C3aR and C5aR have redundant suppressive functions in IL-10 production by CD8⁺ T cells.

We further investigated whether blockade of the engagement of anaphylatoxins to their receptors could inhibit the development of established tumors using complement receptor antagonists. SB 290157 is a non-peptide small compound that was developed as a selective antagonist of C3aR (31). PMX205, the cyclic hexapeptide hydrocinnamate-(L-ornithine-proline-D-cyclohexylalanine-tryptophan-arginine), is a well-defined C5aR antagonist (32). Pharmacological blockade of C3aR and C5aR by SB 290157 and PMX205 suppressed tumor growth in wild type mice and the efficacy was IL-10-dependant (Fig. 6K, Supplementary Fig. S4H). These data demonstrate that C3aR and C5aR play important roles in complement mediated suppression of IL-10 production and antitumor immunity. The results suggest that C3aR and C5aR expressed on CD8⁺ TILs may serve as novel immune checkpoint receptors that may be targeted for further immunotherapy of cancers.

Complement inhibits antitumor immunity through a PD-1 independent pathway

Immune checkpoint blockade with monoclonal antibodies targeting PD-1 expressed on CD8⁺ TILs results in an objective antitumor response in select patients (33). We investigated the roles of the PD-1/PD-L1 pathway in complement/IL-10 mediated antitumor immunity. CD8⁺ TILs from *C3*^{-/-} mice expressed comparable levels of PD-1 to those from wild type mice (Fig. 7A). IL-10 did not modulate PD-1 expression on activated CD8⁺ T cells *in vitro* (Fig. 7B). Tumor cells from *C3*^{-/-} mice expressed similar levels of PD-L1 compared to that from wild type mice (Fig. 7C and D). In addition, PD-L1 expression on tumor cells was not changed upon culture in IL-10 containing medium (Fig. 7E). These results demonstrate that IL-10 does not obviously regulate PD-1/PD-L1 expression in CD8⁺ T and tumor cells. It has been reported that PD-L1 stimulates activated T cells to produce IL-10 (34). We generated a *PD-L1* deficient B16 melanoma cell line using CRISPR/CAS9-mediated gene editing (Fig. 7F). IL-10 expression in CD8⁺ TILs from PD-L1-deficient B16 tumors resembled that in CD8⁺ TILs from unmanipulated B16 tumors (Fig. 7G and 1E), suggesting that PD-1 signaling does not regulate IL-10 production in CD8⁺ T cells *in vivo*. Based on these findings, we tested the effect of combined blockade of complement signaling and PD-L1/PD-1 signaling pathways. *PD-L1* deficient tumors developed slower than the wild type tumors in *C3*^{-/-} mice (Fig. 7H). To further investigate the therapeutic effect of combined blockade of PD-1 and complement receptors on established tumors, we implanted B16 melanoma cells into a cohort of wild type B6 mice and randomized the tumor bearing mice into four groups after six days of tumor development. We then treated with control antibody, anti-PD-1 blocking antibody, C3aR and C5aR antagonists, and a combination of anti-PD-1 blocking antibody with C3aR and C5aR antagonists. In line with published data (35), the anti-PD-1 blocking antibody alone did not show anti-tumor effects in the B16 melanoma model (Fig. 7I). However, combined treatment with anti-PD-1 blocking antibody plus C3aR and C5aR antagonists further enhanced the antitumor effect mediated by C3aR and C5aR blockade (Fig. 7I).

DISCUSSION

This work has identified complement signaling as an important regulator of endogenous IL-10 production in CD8⁺ TILs. Although IL-10 is largely considered as an inhibitory cytokine, extensive evidence also supports its role in enhancing antitumor immunity by activating and expanding CD8⁺ TILs *in situ* (21, 22). Based on the findings from animal studies, a phase I clinical trial is underway to test the therapeutic role of recombinant human IL-10 in different types of cancers (NCT02009449). Given its pleiotropic functions, however, systemic use of exogenous IL-10 may cause adverse effects. Indeed, injection of pegylated IL-10 causes lymphocyte and monocyte infiltration and apoptosis of epithelial cells in the liver and pancreas in mice (21). Therefore, promoting endogenous IL-10 production in CD8⁺ TILs may be an effective approach to boost host antitumor immunity. However, CD8⁺ TILs do not produce endogenous IL-10 under normal conditions in different tumor models (24). We demonstrate that CD8⁺ TILs produce endogenous IL-10 when complement signaling is removed.

Our data suggest that complement proteins are activated locally, producing C3a and C5a. C3a and C5a then interact with their receptors on CD8⁺ TILs, which inhibits the expression of IL-10. Several lines of evidence support this pathway. First, IL-10 is produced by CD8⁺ TILs in mice lacking C3 expression or in tumors expressing CPN for local inactivation of anaphylatoxins. Second, complement receptors C3aR and C5aR are expressed on CD8⁺ TILs. Consistent with a previous genetic tracing study (36), we found that C3aR and C5aR are not expressed on peripheral CD8⁺ T cells. The observation that IL-10⁺CD8⁺ T cells express genes related to the complement signaling pathway suggests a feedback regulatory loop between IL-10 and complement gene expression. The fact that co-expression of C3aR and C5aR on CD8⁺ TILs and C3aR blockade alone is not sufficient to inhibit tumor development suggest that C3aR and C5aR play a redundant role in suppressing IL-10 expression. Our *in vitro* data also indicate that blockade of both receptors results in a further enhanced IL-10 expression than blockade of single receptor. Although it was reported that some cancer cells produce complement proteins (37), we did not detect C3a and C5a from *ex vivo*-cultured B16 melanomas. Thus, C3a and C5a are primarily provided by the host. C3 is the central mediator of both classical and alternative complement activation pathways. C3b is an essential component of C5 convertase in both classical (C4b2a3b) and alternative (C3bBbC3b) pathways. C3 deficiency will impede the cleavage of C5 to C5a in both classical and alternative complement activation pathways. Although it has been shown that C5 activation can occur in the absence of C3 under certain conditions (38), our results demonstrate that C5 is not activated in the tumor microenvironment in the absence of C3. Indeed, C5a was barely detected in the tumor from *C3*^{-/-} mice.

Our results have also established a mechanistic link between two major types of experimental and clinical observations. The first concerns the role of complement in host antitumor immunity. As discussed in the introduction, extensive experimental studies and clinical findings suggest that complement inhibits host antitumor immunity. The second relates to the role of IL-10 in host antitumor immunity. It was observed that IL-10- or IL-10R-deficient mice are susceptible to tumor development (39, 40) and exogenous IL-10 can boost host antitumor immunity (18-22, 41). Our data suggest that complement signaling inhibits IL-10 production in CD8⁺ TILs and prevents the CD8⁺ TILs from becoming potent effectors. Autocrine production of complement by CD8⁺ T cells is sufficient to exert this effect. Previous studies also demonstrate that complement inhibits antitumor immunity by recruiting MDSCs and preventing NK cell activation in different tumor models (8, 10, 12, 13). Thus, complement signaling-mediated inhibition of host antitumor immunity is multifactorial. We did not observe MDSC infiltration in a B16 melanoma model in either wild type or *C3*^{-/-} mice. Furthermore, there was no increase in the total or activated NK cells from *C3*^{-/-} mice. These data suggest that MDSC-mediated immunosuppression may be tumor-type specific. The inhibition of NK function by complement through MDSCs may thus also depend on the tumor type. Previous publications have demonstrated conflicting roles of complement on Tregs (3-5, 8). However, the percent of Tregs in dLNs or TILs from wild type and *C3*^{-/-} mice were comparable. Therefore, the effect of complement on Tregs appears to be context dependent (12).

Our study suggests several novel strategies for cancer immunotherapy. First, a combined blockade of complement signaling by antagonists to C3aR, C5aR, and anti-PD-1 may

enhance the clinical efficacy of anti-PD-1 mAb therapy. It is worth noting that the C5aR antagonist PMX205 has been successfully used in a phase I clinical trial for the treatment of inflammatory disorder (42). Second, a targeted strategy to deliver IL-10 to CD8⁺ TILs by generating an anti-PD1-IL-10 or anti-CTLA4-IL-10 fusion protein may improve the efficacy of anti-PD-1 antibody by blocking PD-1 expressed on TILs and PD-L1 expressed on APCs and tumor cells (43) and promoting the differentiated CD8⁺ TILs into more potent effectors. And finally, addition of IL-10 in the expansion protocol of TILs for adaptive cellular therapy may enhance the number and potency of the effector T cells needed for a long-term durable response (44).

METHODS

Mice and Murine Cell Lines

C57BL/6J (wild type) mice (stock number: 000664), *C3^{-/-}* mice (N7, Stock No: 003641), *TCRa^{-/-}* mice (N13, Stock No: 002116), *Il10^{-/-}* mice (N13, Stock No: 002251), *Il10 GFP* reporter (Tiger) mice (N10, stock number: 008379) were purchased from The Jackson Laboratory (Bar Harbor, ME). *C3^{-/-}* mice were further backcrossed to C57BL/6J for 5 generations (N12). N indicates the number of backcrossed generations. Six to eight week old mice were used for all experiments. All mice strains are of the C57BL/6 genetic background. Mice were housed in a specific pathogen-free facility in the Duke University Medical Center and used according to protocols approved by the Duke University Institutional Animal Care and Use Committee. The B16-F10 murine melanoma was purchased from ATCC in 2011. E0771, a murine mammary adenocarcinoma, was a gift from Dr. Scott A. Gerber (University of Rochester) in 2013. The mouse tumor cell lines used in this manuscript have not been authenticated by us.

Tumor Models

5×10^4 - 4×10^5 B16F10 or 1×10^6 E0771 tumor cells were s.c. inoculated into six to eight week old mice in 100 μ l PBS. Tumor development was monitored daily or every other day. The mice were sacrificed when their tumor volumes reached 2000 mm³.

Mouse Ex vivo and In vitro Experiments

Tumors were cut into small (< 3mm) pieces and incubated in 5 ml dissociation solution (RPMI medium supplemented with Collagenase type I (200 U/ml) and DNase I (100 μ g/ml)) for 30 min at 37 °C. Samples were mixed by pipetting and vortexing every 10 minutes during the incubation. Collagenase (C0130) and DNase I (ND25) were purchased from Sigma-Aldrich. CD45 was used to distinguish tumor infiltrating leukocytes from other cells, and different antibodies targeting surface markers were used for flow cytometry analysis. For intracellular cytokine staining, cells were cultured in complete RPMI for 4-6 hrs with PMA, ionomycin, and Brefeldin A (Cell Activation Cocktail, Biolegend).

Microarray Data Analysis

TILs from human lung tumors were *in vitro*-expanded as described in the Supplementary methods for 20 days with 6000 u/ml rIL-2 and/or 100 u/ml rIL-10. The expanded TILs were activated by incubation in 3 μ g/ml anti-CD3/CD28 antibodies for 6 hrs. The CD8⁺ TILs

were enriched by negative selection using a kit (Stem cell). RNA was extracted using Trizol reagent (Life technologies) according to the instructions. Microarray analyses were performed using human U133A 2.0 arrays by the Duke University microarray facility. Raw intensities from the CEL files were analyzed using Partek Genomics Suite (Partek Incorporated, St. Louis, MO) with standard background correction to generate an RMA (robust multi-array average intensity) on a log₂ scale for each probe set. Probe sets were filtered for “present” detection in two or more arrays, and the interquartile intensity range was >0.5. The filtered RMA intensities were then analyzed for differential expression using the advanced analysis of variance (ANOVA), and differentially expressed genes were generated with/without FDR-adjusted P values using the Benjamini-Hochberg method.

RT-PCR

RNA was isolated with Direct-Zol (Zymo Research) according to the manufacturer’s protocol. Complementary DNA was synthesized with SuperScript III Reverse Transcriptase (Life Technologies). Quantitative realtime PCR was performed using a SYBR green-based assay (Applied Biosystems). For mRNA expression in tumor cells, β -actin mRNA was used for normalization across samples.

Complement Receptor Antagonists and PD-1 antibody Treatment

C3a receptor antagonist SB 290157 was purchased from EMD Millipore and C5a receptor antagonist PMX205 was purchased from Selleck Chemicals LLC. For E0771 breast tumors, mice were treated intraperitoneally with SB 290157 at 10 mg/kg and PMX205 at 1 mg/kg (both in 5% DMSO: 5% ethanol: 90% PBS) twice a day from day 9 after tumor implantation. Control mice were treated with 5% DMSO: 5% ethanol: 90% PBS. For B16 melanoma, mice were i.p. injected with anti-PD-1 antibody (Clone: RMP1-14, Bioxcell) at 200 μ g/mouse twice a week, or SB 290157 and PMX205 as described in the E0771 model, or the combination of anti-PD-1 antibody with SB 290157 and PMX205.

Real-time Impedance Assay

The human TILs *in vitro* killing assay was performed using a real-time impedance assay using an RTCA S16 (ACEA Bio). 1.5×10^4 primary autologous cancer cells / well were seeded in an E-plate 16, and cultured for 2 days. The TILs were *in vitro*-expanded for 21 days and stimulated with anti-CD3/CD28 antibodies for 24 hours. Effector cells were added into each well cultured with tumor cells at a ratio of 20:1 (effector cell / cancer cell). Cancer cells and effector cells were co-cultured in a 37°C CO₂ incubator, and real-time monitored by RTCA S16.

Adoptive Cell Transfer

CD4⁺ and CD8⁺ T cells were negatively enriched using EasySep mouse CD4⁺ T cell and EasySep mouse CD8⁺ T Enrichment kits (Stemcell). One million mixed CD4⁺ and CD8⁺ T cells (2:1) were transferred by i.v. injection into 6-8 week old gender matched recipient mice. After 14 days of T cell transfer, recipient mice were implanted s.c. with B16F10 melanoma and tumor growth was monitored daily.

Generation of PD-L1 deficient stable cell line using CRISPR/CAS9-mediated gene editing

B16 cells were cultured in complete DMEM medium. Cells were passaged every 2-3 days with a ratio of 1:6 to 1:8. pLentiCRISPR V1 plasmid was a gift from Feng Zhang (Addgene plasmid # 49535. Current version is pLentiCRISPR V2, plasmid# 52961). Cas9 guide sequence for mouse *PD-L1* (NCBI Accession number: GQ904196) was designed as 5' AGCCTGCTGCTCACTTGCTAC 3' by the online program (Reference 45). The two oligos were synthesized from IDT as 5' **CACCGAGCCTGCTGCTCACTTGCTAC** 3' and 5' **AAACGTAGCAAGTGACAGCAGGCTC** 3' (Bold indicates the BsmBI restriction site). The pLentiCRISPR V1 was digested by the BsmBI and the annealed oligos were cloned into pLentiCRISPR V1 according to the protocol from Zhang laboratory. To make the lentivirus, pLentiCRISPR (with cloned sgRNA) were co-transfected into HEK293(F)T cells with the packaging plasmids pVSVg (AddGene #8454) and psPAX2 (AddGene #12260). For PD-L1 silencing, CRISPR control or CRISPR sgRNA targeting PD-L1 viruses were transduced into B16F10 cells according to the protocol. The transduced B16F10 cells were selected in complete medium containing 1 µg/ml puromycin (Invivogen) 48 hrs after transduction. The culture medium was replaced every 48 hrs. The expression of PD-L1 was determined by flow cytometry. More detailed information regarding guide RNA design and construct cloning can be found at Reference 46.

Primer List

Mouse *CPN1* (NM_030703)

Forward: 5' GGTGGACCTGAACCGCAACTTC 3'

Reverse: 5' CGTTGGTGATGCCGTCTGGAA 3'

Mouse *β-Actin* (NM_007393)

Forward: 5' ACCTTCTACAATGAGCTGCG 3'

Reverse: 5' CTGGATGGCTACGTACATGG 3'

C3a and C5a ELISA

Complement C3a and C5a mouse ELISA kits (cat# ABIN415413 and ABIN415613) were purchased from Antibodies-online. Tumors were carefully dissected from each mouse to keep them intact. The tumors were rinsed in cold PBS to remove blood thoroughly and weighed before homogenization. The tumors were then minced to small pieces and homogenized in PBS. The homogenates were centrifuged to obtain a cell free supernatant. The quantities of C3a and C5a present in the supernatants, and in the medium from B16 cell culture, were determined by ELISA.

C3aR and C5aR antagonists in vitro blockade

Mouse CD8⁺ T cells from lymph nodes were enriched using a negative selection kit (Stemcell, Cat# 19853) and activated by plate-bound anti-CD3 (2 µg/ml) and anti-CD28 (1 µg/ml) antibodies for 72 hrs. The activated CD8⁺ T cells were then cultured in complete medium alone (control), 10 µM SB290157 (C3aR antagonist, C3aRA), 10 µM PMX205

(C5aR antagonist, C5aRA), or a combination of 10 μ M SB290157 and 10 μ M PMX205 for 6 days. The IL-10 expression was determined by intracellular staining.

Human Samples

Freshly isolated human hepatocarcinoma and lung cancer biopsies as well as peripheral blood were provided by Hepatopancreatobiliary and Thoracic Surgery Departments, Beijing Cancer Hospital and Institute. The study was conducted in accordance with the Declaration of Helsinki and approved by the ethical committee of the Beijing Cancer Hospital and Institute. Patients signed an informed consent.

Statistical Analysis

Data are presented as mean $\pm$ SEM. Results were analyzed by 2-tailed Student's *t* test, or 1-way ANOVA when multiple comparisons were made. Statistical significance was defined as *P* < 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Dr. Scott A. Gerber for the gift of E0771 breast cancer cell line. We would like to thank the Duke Microarray Core facility (a Duke National Cancer Institute and a Duke Genomic and Computational Biology shared resource facility) for their technical support, microarray data management and feedback on the generation of the microarray data reported in this manuscript.

Grant Support

This project was supported by the US National Institutes of Health AI074944 to You-Wen He.

References

1. Heeger PS, Kemper C. Novel roles of complement in T effector cell regulation. *Immunobiology*. 2012; 217:216–24. [PubMed: 21742404]
2. Strainic MG, Liu J, Huang D, An F, Lalli PN, Muqim N, et al. Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4⁺ T cells. *Immunity*. 2008; 28:425–35. [PubMed: 18328742]
3. Strainic MG, Shevach EM, An F, Lin F, Medof ME. Absence of signaling into CD4(+) cells via C3aR and C5aR enables autoinductive TGF-beta1 signaling and induction of Foxp3(+) regulatory T cells. *Nat Immunol*. 2013; 14:162–71. [PubMed: 23263555]
4. van der Touw W, Cravedi P, Kwan WH, Paz-Artal E, Merad M, Heeger PS. Cutting edge: Receptors for C3a and C5a modulate stability of alloantigen-reactive induced regulatory T cells. *J Immunol*. 2013; 190:5921–5. [PubMed: 23690475]
5. Kwan WH, van der Touw W, Paz-Artal E, Li MO, Heeger PS. Signaling through C5a receptor and C3a receptor diminishes function of murine natural regulatory T cells. *J Exp Med*. 2013; 210:257–68. [PubMed: 23382542]
6. Lalli PN, Strainic MG, Yang M, Lin F, Medof ME, Heeger PS. Locally produced C5a binds to T cell-expressed C5aR to enhance effector T-cell expansion by limiting antigen-induced apoptosis. *Blood*. 2008; 112:1759–66. [PubMed: 18567839]
7. Liszewski MK, Kolev M, Le Friec G, Leung M, Bertram PG, Fara AF, et al. Intracellular complement activation sustains T cell homeostasis and mediates effector differentiation. *Immunity*. 2013; 39:1143–57. [PubMed: 24315997]

8. Vadrevu SK, Chintala NK, Sharma SK, Sharma P, Cleveland C, Riediger L, et al. Complement c5a receptor facilitates cancer metastasis by altering T-cell responses in the metastatic niche. *Cancer Res.* 2014; 74:3454–65. [PubMed: 24786787]
9. Pio R, Corrales L, Lambris JD. The role of complement in tumor growth. *Adv Exp Med Biol.* 2014; 772:229–62. [PubMed: 24272362]
10. Markiewski MM, DeAngelis RA, Benencia F, Ricklin-Lichtsteiner SK, Koutoulaki A, Gerard C, et al. Modulation of the antitumor immune response by complement. *Nat Immunol.* 2008; 9:1225–35. [PubMed: 18820683]
11. Corrales L, Ajona D, Rafail S, Lasarte JJ, Riezu-Boj JI, Lambris JD, et al. Anaphylatoxin C5a creates a favorable microenvironment for lung cancer progression. *J Immunol.* 2012; 189:4674–83. [PubMed: 23028051]
12. Gunn L, Ding C, Liu M, Ma Y, Qi C, Cai Y, et al. Opposing roles for complement component C5a in tumor progression and the tumor microenvironment. *J Immunol.* 2012; 189:2985–94. [PubMed: 22914051]
13. Janelle V, Lamarre A. Role of the complement system in NK cell-mediated antitumor T-cell responses. *Oncoimmunology.* 2014; 3:e27897. [PubMed: 24800174]
14. Ouyang W, Rutz S, Crellin NK, Valdez PA, Hymowitz SG. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annual review of immunology.* 2011; 29:71–109.
15. Chen WF, Zlotnik A. IL-10: a novel cytotoxic T cell differentiation factor. *J Immunol.* 1991; 147:528–34. [PubMed: 1906502]
16. Groux H, Bigler M, de Vries JE, Roncarolo MG. Inhibitory and stimulatory effects of IL-10 on human CD8+ T cells. *J Immunol.* 1998; 160:3188–93. [PubMed: 9531274]
17. Santin AD, Hermonat PL, Ravaggi A, Bellone S, Pecorelli S, Roman JJ, et al. Interleukin-10 increases Th1 cytokine production and cytotoxic potential in human papillomavirus-specific CD8(+) cytotoxic T lymphocytes. *J Virol.* 2000; 74:4729–37. [PubMed: 10775611]
18. Yang G, Hellstrom KE, Mizuno MT, Chen L. In vitro priming of tumor-reactive cytolytic T lymphocytes by combining IL-10 with B7-CD28 costimulation. *J Immunol.* 1995; 155:3897–903. [PubMed: 7561096]
19. Berman RM, Suzuki T, Tahara H, Robbins PD, Narula SK, Lotze MT. Systemic administration of cellular IL-10 induces an effective, specific, and long-lived immune response against established tumors in mice. *J Immunol.* 1996; 157:231–8. [PubMed: 8683120]
20. Fujii S, Shimizu K, Shimizu T, Lotze MT. Interleukin-10 promotes the maintenance of antitumor CD8(+) T-cell effector function in situ. *Blood.* 2001; 98:2143–51. [PubMed: 11568001]
21. Mumm JB, Emmerich J, Zhang X, Chan I, Wu L, Mauze S, et al. IL-10 elicits IFN γ -dependent tumor immune surveillance. *Cancer Cell.* 2011; 20:781–96. [PubMed: 22172723]
22. Emmerich J, Mumm JB, Chan IH, LaFace D, Truong H, McClanahan T, et al. IL-10 directly activates and expands tumor-resident CD8(+) T cells without de novo infiltration from secondary lymphoid organs. *Cancer Res.* 2012; 72:3570–81. [PubMed: 22581824]
23. Hattori E, Okumoto K, Adachi T, Takeda T, Ito J, Sugahara K, et al. Possible contribution of circulating interleukin-10 (IL-10) to anti-tumor immunity and prognosis in patients with unresectable hepatocellular carcinoma. *Hepatology research : the official journal of the Japan Society of Hepatology.* 2003; 27:309–14. [PubMed: 14662119]
24. Stewart CA, Metheny H, Iida N, Smith L, Hanson M, Steinhagen F, et al. Interferon-dependent IL-10 production by Tregs limits tumor Th17 inflammation. *J Clin Invest.* 2013; 123:4859–74. [PubMed: 24216477]
25. Ruffell B, Chang-Strachan D, Chan V, Rosenbusch A, Ho CM, Pryer N, et al. Macrophage IL-10 blocks CD8+ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells. *Cancer Cell.* 2014; 26:623–37. [PubMed: 25446896]
26. Trandem K, Zhao J, Fleming E, Perlman S. Highly activated cytotoxic CD8 T cells express protective IL-10 at the peak of coronavirus-induced encephalitis. *J Immunol.* 2011; 186:3642–52. [PubMed: 21317392]
27. Kamanaka M, Kim ST, Wan YY, Sutterwala FS, Lara-Tejero M, Galan JE, et al. Expression of interleukin-10 in intestinal lymphocytes detected by an interleukin-10 reporter knockin tiger mouse. *Immunity.* 2006; 25:941–52. [PubMed: 17137799]

28. Wessels MR, Butko P, Ma M, Warren HB, Lage AL, Carroll MC. Studies of group B streptococcal infection in mice deficient in complement component C3 or C4 demonstrate an essential role for complement in both innate and acquired immunity. *Proc Natl Acad Sci U S A*. 1995; 92:11490–4. [PubMed: 8524789]
29. Mombaerts P, Clarke AR, Rudnicki MA, Iacomini J, Itohara S, Lafaille JJ, et al. Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages. *Nature*. 1992; 360:225–31. [PubMed: 1359428]
30. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*. 1993; 75:263–74. [PubMed: 8402911]
31. Ames RS, Lee D, Foley JJ, Jurewicz AJ, Tornetta MA, Bautsch W, et al. Identification of a selective nonpeptide antagonist of the anaphylatoxin C3a receptor that demonstrates antiinflammatory activity in animal models. *J Immunol*. 2001; 166:6341–8. [PubMed: 11342658]
32. Reid RC, Abbenante G, Taylor SM, Fairlie DP. A convergent solution-phase synthesis of the macrocycle Ac-Phe-[Orn-Pro-D-Cha-Trp-Arg], a potent new antiinflammatory drug. *J Org Chem*. 2003; 68:4464–71. [PubMed: 12762752]
33. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med*. 2012; 366:2443–54. [PubMed: 22658127]
34. Dong H, Zhu G, Tamada K, Chen L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med*. 1999; 5:1365–9. [PubMed: 10581077]
35. Peng W, Liu C, Xu C, Lou Y, Chen J, Yang Y, et al. PD-1 blockade enhances T-cell migration to tumors by elevating IFN-gamma inducible chemokines. *Cancer Res*. 2012; 72:5209–18. [PubMed: 22915761]
36. Dunkelberger J, Zhou L, Miwa T, Song WC. C5aR expression in a novel GFP reporter gene knockin mouse: implications for the mechanism of action of C5aR signaling in T cell immunity. *J Immunol*. 2012; 188:4032–42. [PubMed: 22430734]
37. Cho MS, Vasquez HG, Rupaimoole R, Pradeep S, Wu S, Zand B, et al. Autocrine effects of tumor-derived complement. *Cell Rep*. 2014; 6:1085–95. [PubMed: 24613353]
38. Huber-Lang M, Sarma JV, Zetoune FS, Rittirsch D, Neff TA, McGuire SR, et al. Generation of C5a in the absence of C3: a new complement activation pathway. *Nat Med*. 2006; 12:682–7. [PubMed: 16715088]
39. Berg DJ, Davidson N, Kuhn R, Muller W, Menon S, Holland G, et al. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J Clin Invest*. 1996; 98:1010–20. [PubMed: 8770874]
40. Tanikawa T, Wilke CM, Kryczek I, Chen GY, Kao J, Nunez G, et al. Interleukin-10 ablation promotes tumor development, growth, and metastasis. *Cancer Res*. 2012; 72:420–9. [PubMed: 22123924]
41. Wang L, Liu JQ, Talebian F, Liu Z, Yu L, Bai XF. IL-10 enhances CTL-mediated tumor rejection by inhibiting highly suppressive CD4 T cells and promoting CTL persistence in a murine model of plasmacytoma. *Oncoimmunology*. 2015; 4:e1014232. [PubMed: 26140236]
42. Flight MH. Avoiding bad complement in Alzheimer's disease. *Nature reviews Neuroscience*. 2009; 8:316.
43. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer*. 2012; 12:252–64. [PubMed: 22437870]
44. Rosenberg SA. IL-2: the first effective immunotherapy for human cancer. *Journal of immunology*. 2014; 192:5451–8.
45. <http://crispr.mit.edu/>
46. <http://crispr.genome-engineering.org>

SIGNIFICANCE

Our data suggest novel strategies to enhance immunotherapies: a combined blockade of complement signaling by antagonists to C3aR, C5aR, and anti-PD-1 to enhance anti-PD1 efficacy; a targeted IL-10 delivery to CD8⁺ TILs by using anti-PD1-IL-10 or anti-CTLA-4-IL-10 fusion proteins; addition of IL-10 in TIL expansion for adoptive cellular therapy.

Figure 1.

Regulation of IL-10 expression in CD8⁺ T cells by complement. **A**, heat map of the differentially-expressed genes in IL-10⁺ and IL-10⁻ CD8⁺ T cells. **B**, pathway analysis of differentially expressed genes as shown in (A). Shown are the top 10 pathways that are highly enriched in IL-10⁺CD8⁺ T cells. **C** and **D**, mRNA expression of complement (C) and complement receptors (D) in IL-10⁺CD8⁺ (GFP⁺) and IL-10⁻CD8⁺ (GFP⁻) T cells. Plots show relative expression levels of mRNAs for each indicated gene based on gene chip data. Shown are the mean $\pm$ SEM from data deposited by Trandem et al (Reference 26). **E**, expression of IL-10 in CD8⁺ TILs from WT and C3^{-/-} mice. IL-10 reporter (Tiger) mice were crossed with C3^{-/-} mice and inoculated with B16 melanoma cells. TILs were analyzed by flow cytometry from day 12 to 13. The percentages of IL-10⁺CD8⁺ TILs from six C3^{-/-} Tiger mice are shown in the right panel. Error bars indicate SEM. Significance was determined in all panels by Student's *t* test (*p < 0.05, **p < 0.01, ***p < 0.001).

Figure 2.

Suppression of T cell-mediated antitumor immunity by complement. **A-C**, melanoma development in *C3*^{-/-} mice. B16F10 melanoma cells (2×10^5 /mouse) were subcutaneously (s.c.) inoculated into WT and *C3*^{-/-} mice. Tumor growth was monitored daily starting from day 7. Shown are tumor volume, size, and weight in these mice (n=9 mice per group). **D-F**, breast cancer development in *C3*^{-/-} mice. E0771 breast cancer cells (1×10^6 /mouse) were s.c. inoculated into WT and *C3*^{-/-} mice. Tumor growth was monitored every other day starting from day 7. Shown are tumor volume, size, and weight in these mice (n=9 mice per group). **G** and **H**, phenotypes of CD8⁺ TILs from *C3*^{-/-} mice. WT and *C3*^{-/-} mice were s.c. inoculated with B16F10 cells (2×10^5 /mouse). Total, IFNγ⁺, and TNFα⁺-producing CD8⁺ TILs were analyzed by flow cytometry (n=5 mice per group) at day 12 after tumor inoculation. **I**, B16F10 tumor development in WT, *C3*^{-/-}, *TCRα*^{-/-}, and *C3*^{-/-} *TCRα*^{-/-} mice (n=6 mice per group). All experiments shown are representative of at least three independent experiments. Bars and error bars indicate mean $\pm$ SEM. Significance was determined in all panels by Student's *t* test (ns, $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 3.

Non-CD8⁺ T cell responses in tumor-bearing mice. B16F10 melanoma cells (2×10^5 /mouse) were s.c. inoculated into WT and *C3*^{-/-} mice. The draining lymph nodes (dLNs) and tumors were treated with collagenase and DNase to generate a single-cell suspension. Leukocytes were pre-gated on CD45⁺ cells. **A**, CD11b and GR1 expression in leukocytes from tumor-infiltrating leukocytes (TILs) were analyzed by flow cytometry (n=4 mice per group). **B**, regulatory CD4⁺ T cell population in leukocytes from TILs was analyzed by flow cytometry using CD4 and Foxp3 as markers (n=4 mice per group). **C**, NK population in leukocytes from TILs was analyzed by flow cytometry using NK1.1 as a marker (n=4 mice per group). Experiments shown are representative of three independent experiments. Bars and error bars indicate mean $\pm$ SEM. Significance was determined in all panels by Student's *t* test (ns, $p > 0.05$).

Figure 4.

Essential role for IL-10 in the antitumor response in $C3^{-/-}$ mice. **A-C**, melanoma development in $C3^{-/-}$ mice. B16F10 melanoma cells (2×10^5 /mouse) were s.c. inoculated into WT, $Il10^{-/-}$, $C3^{-/-}$, and $Il10^{-/-}C3^{-/-}$ mice. Tumor growth was monitored daily starting from day 7. Shown are tumor volume, size, and weight in these mice (n=8 mice per group). **D-F**, breast cancer development in $C3^{-/-}$ mice. E0771 breast cancer cells (1×10^6 /mouse) were s.c. inoculated into WT, $C3^{-/-}$, and $Il10^{-/-}C3^{-/-}$ mice. Tumor growth was monitored every other day starting from day 7. Shown are tumor volume, size, and weight in these mice (n=8 mice per group). All experiments shown are representative of three independent experiments. Bars and error bars indicate mean $\pm$ SEM. Significance was determined by Student's *t* test in panels (A) and (D), and by ANOVA in panels (C) and (F) (ns, $p > 0.05$, * $p < 0.05$, ** $p < 0.01$).

Figure 5.

IL-10 enhances the function of TILs from cancer patients. **A**, cell number of *in vitro*-expanded TILs from lung cancer patients. TILs were isolated and cultured in the presence of 6000 U/ml rIL-2, 100 U/ml rIL-10, or 6000 U/ml rIL-2 plus 100 U/ml rIL-10. The numbers of live TILs counted are shown (y-axis). The inserted panel shows the ratio of TILs from two types of culture from 3 patients. **B**, killing activity of *in vitro*-expanded TILs. The expanded TILs in (A) were activated by anti-CD3/CD28 antibodies for 24 hrs and tested for their ability to kill autologous primary tumor cells at an E:T ratio of 20:1. The killing activity was measured at 15 minute intervals by Impedance assay. **C-D**, IFN γ and TNF α expression in CD8⁺ TILs expanded *in vitro*. TILs from lung cancers were expanded in complete culture medium with 6000 U/ml rIL-2 alone or combined with 100 U/ml rIL-10 for 20 days. IFN γ and TNF α expression in CD8⁺ TILs were analyzed by flow cytometry. A-D show results representative of three to six patients. **E**, heat map of the differentially-expressed genes in IL-10-treated human lung tumor CD8⁺ TILs. **F-J**, mRNA expression of different pathways as indicated in IL-10/IL-2-treated human CD8⁺ TILs. Plotted are relative expression levels of mRNAs compared to those from IL-2-treated cells for each indicated gene based on gene chip data. Shown are the mean $\pm$ SEM. Significance was determined by ANOVA in panels (F-J) *p 0.05, **p 0.01.

Figure 6.

Suppression of IL-10 production by autocrine C3. **A**, schematic of T cell transfer to C3^{-/-} TCR^{-/-} mice and tumor development. **B**, melanoma development in chimeric mice. B16F10 melanoma cells were inoculated into T cell-reconstituted C3^{-/-} TCR^{-/-} mice and tumor development was monitored daily (n=5 mice per group). **C**, FACS profiles of C3aR and C5aR expression on CD8⁺ T cells from dLNs of naïve mice. **D**, FACS profiles of C3aR and C5aR expression on CD8⁺ T cells from dLNs and melanomas. B16F10 melanoma cells (2×10⁵/mouse) were s.c. inoculated into WT mice, and TILs were isolated at day 13 and analyzed by flow cytometry. **E**, FACS profiles of C3aR and C5aR expression on CD8⁺ T cells from breast cancer. E0771 cells (1×10⁶/mouse) were s.c. inoculated into WT mice. The expression of C3aR and C5aR on CD8⁺ TILs was analyzed at day 19 by flow cytometry. **F**, summary of results from (D) and (E). **G**, FACS profiles of C3aR and C5aR expression on CD8⁺ T cells from PBMCs and TILs from liver cancer (n=5). **H**, effect of carboxypeptidase N (CPN) expression in tumor cells on IL-10 production in CD8⁺ TILs. Control and CPN-expressing B16F10 cells (4×10⁵/mouse) were s.c. inoculated into WT Tiger mice. IL-10-reporter eGFP expression in CD8⁺ TILs was assayed at day 13. Right panel shows the percentages of IL-10⁺CD8⁺ TILs from 5 mice. **I**, effect of C3aR and C5aR antagonists on

IL-10 expression in CD8⁺ TILs. B16 tumor-bearing WT Tiger mice were treated with control or C3aR and C5aR antagonists (n=3 per group). **J**, effect of C3aR and C5aR antagonists on IL-10 expression in *in vitro*-activated CD8⁺ T cells (n=4). **K**, effect of complement signaling blockade on breast cancer development. E0771 breast cancer cells (1×10⁶/mouse) were s.c. inoculated into WT, *C3*^{-/-}, and *II10*^{-/-} mice. WT and *II10*^{-/-} mice were treated with C3aR and C5aR antagonists or control solution every 12 hrs starting from day 9 after tumor implantation. Tumor volume was monitored every other day (n=8 mice per group). (B and K) show results representative of three independent experiments. Bars and error bars indicate mean ± SEM. Significance was determined by Student's *t* test in panel (B) and by ANOVA in panels (J) and (K) (ns, p>0.05, **p 0.01, ***p 0.001).

Figure 7.

Complement inhibits antitumor immunity through a PD-1-independent pathway. **A**, B16F10 melanoma cells (2×10^5 /mouse) were s.c. inoculated into WT and $C3^{-/-}$ mice. Expression levels of PD-1 on CD8⁺ TILs were analyzed by flow cytometry at day 12 after tumor implantation. **B**, expression level of PD-1 on CD8⁺ T cells activated with anti-CD3/CD28 antibodies in the presence of IL-10. T cells from LNs of naïve mice were activated by incubation with 3 μg/ml anti-CD3/CD28 antibodies for 48 hrs with or without 500 U/ml IL-10 and analyzed for PD-1 expression. **C-D**, PD-L1 expression on tumor cells developed in WT and $C3^{-/-}$ mice. B16F10 melanoma cells (2×10^5 /mouse) or E0771 breast cancer cells (1×10^6 /mouse) were s.c. inoculated into WT and $C3^{-/-}$ mice. PD-L1 expression in CD45⁻ B16 tumor cells (C) and CD45⁻ E0771 cells (D) were measured by flow cytometry at day 12 and day 19, respectively. **E**, PD-L1 expression in tumor cells after IL-10 stimulation. B16F10 cells were cultured with or without 500 U/ml IL-10 for 5 days. PD-L1 expression was analyzed by flow cytometry. **F**, PD-L1 expression on gene-engineered B16F10 cells. B16F10 cells were transduced with control virus or sg-RNA-targeting PD-L1 virus and selected with puromycin to generate a stable PD-L1-silenced polyclonal cell line. **G**, IL-10 expression in CD8⁺ TILs from PD-L1-silenced B16F10 tumors. Control or PD-L1-silenced B16F10 melanoma cells (4×10^5 /mouse) were s.c. inoculated into $C3^{-/-}$ and $C3^{-/-}$ Tiger

mice. GFP expression in CD3⁺CD8⁺ TILs was analyzed by flow cytometry. Right panel shows the percentages of IL-10⁺CD8⁺ TILs from 4 individual mice. **H**, effect of blockade of PD-1/PD-L1 and complement signaling pathways on tumor development. Control or PD-L1-silenced B16F10 melanoma cells (4×10^5 /mouse) were s.c. inoculated into WT and *C3*^{-/-} mice. Tumor development was monitored every day starting from day 7 after implantation (n=7, 7, 9, and 8 mice respectively). **I**, B16F10 melanoma cells (5×10^4 /mouse) were s.c. inoculated into WT mice. Mice were randomized into 4 groups 6 days after implantation. Each group of mice received control antibody, anti-PD-1 antibody, C3aR and C5aR antagonists, or anti-PD-1 antibody plus C3aR and C5aR antagonists. (n=8 mice per group). (A-F) Solid gray color indicates isotype control staining. (A-E) Data represent a pool of 6-8 mice in each group. Significance was determined in panels (H) and (I) by Student's *t* test. * p 0.05, **p 0.01.