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Augmentation of Antitumor T-Cell Responses by Increasing APC T-Cell C5a/C3a-C5aR/C3aR Interactions

PRINCIPAL INVESTIGATOR:

M Edward Medof, MD, PhD

CONTRACTING ORGANIZATION:

Case Western Reserve University
Cleveland, OH 44106

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14. ABSTRACT

While we initially found that T effector cell responses against breast cancers can be augmented by increasing C3a and C5a receptor (C3aR/C5aR) signaling by antigen presenting dendritic cell dendritic (DCs) by blocking the function of decay accelerating factor (DAF), we have found that in breast cancers themselves and vascular endothelial cells C3aR/C5aR signaling promotes breast cancer growth. In the past years, we have found that blockade of C3aR/C5aR signaling in $CD4^+$ cells enables endogenous TGF-b1 production and Foxp3 T regulatory cells induction.

We are developing ways to control these effects and have prepared specialized mice for testing the clinical efficacy of our findings.

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Cancer, Tumor, Breast Cancer, Complement, T cells, C3a receptor, C5a receptor

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Introduction

Decay accelerating factor (DAF or CD55) is a cell surface regulator which prevents activation of serum complement proteins on cell surfaces and thereby protects self-cells from complement mediated injury(1). Our project started with our unexpected observation (2) that induction of T cell effector cell responses with dendritic cells (DCs) devoid of DAF *ex vivo* can augment anti-tumor immunoreactivity. During the course of our studies, we surprising discovered that DAF not only controls immune cell proliferation associated with anti-cancer T effector response, but also controls cancer cell proliferation itself, as well as controls its associated angiogenesis. The underlying mechanism is that contrary to generally held concepts that complement resides exclusively in serum and derives solely from the liver, all cells (studied to date) locally synthesize alternative pathway (AP) complement components C3, factor B (fB), and factor D (fD) together with C5. These components spontaneously activate on the cell surface to produced C3a and C5a. The C3a/C5a anaphyotoxins feed-back to interact with C3a and C5a receptors (C3aR/C5aR) on the same cells and the resulting autocrine signaling drives cell growth and functions to prevent apoptosis. It is noteworthy that prior work by others (3) on cancer focused on using anti-DAF antibodies to inhibit DAF's serum complement shielding function, with the idea that this blockade would lead to cancer cell cytotoxicity. Counter intuitively, such blockade, in fact, functions to augment cancer call growth, as the antibodies generate C3a/C5a from serum C3/C5at the cancer cell surface which can amplify cancer cell C3aR/C5aR signaling. We later learned that the mitotic and anti-apoptotic activities of epidermal growth factor receptor (EGFR, Her-2) in cancer cells and vascular endothelial growth factor receptor 2 (VEGFR2) signaling in vascular endothelial cells (ECs) depend on C3aR/C5aR signaling. While one would hypothesize that blockade of C3aR/C5aR signaling would inhibit all of these cancer promoting activities that drive tumor progression, we have found that C3aR/C5aR blockade blocks the anti-cancer cell T effector cell response and concurrently induces Foxp3$^+$ T regulatory cells (Tregs). Consequently, for immune inhibition of cancers, one must deal with the tumor, and its microenvironment as well as the anti-tumor immune response. Studies extending our statements of work to focus on this key insight were approved in prior progress reports.

Body

1) Development of HER-2$^+$ E0771 murine breast cancer cells and related studies of *Daf1*$^{-/-}$, *Daf1*$^{-/-}$*Crry*$^{-/+}$ and wild-type mice : In the last year's progress report, we summarized our findings concerning the integral role that C3aR/C5aR signaling plays in EGFR/Her-2 growth induction of cancers and VEGF-A induction of angiogenesis. We presented *in vivo* data confirming decreased tumor mass and decreased tumor associated angiogenesis in mice devoid of C3aR/C5aR signaling. The mechanism had not yet been clarified. In the past year, we have found that IL-6gp130 signaling participates in this growth and anti-apoptotic signaling. As previously indicated, our experiments in ECs had shown that VEGF-A induced proliferation of ECs was blocked by pharmaceuticals against the receptors (C3aR-A/C5aR-A) or mAbs against the C3a/c5a ligands and that added C5a induces the cell cycle entry and growth of ECs comparably to added VEGF-A. Based on this, we examined VEGFR2 phosphorylation. We found that the added C5a, like added VEGF-A, induced VEGFR-2 phosphorylation in < 5 min, suggesting that C5aR and VEGFR-2 might be physically associated (**Fig 1**). In view of our prior connection of C5a generation with IL-6 receptor (IL-6R) signaling in immune cells, we tested whether IL-6R signaling might also participate in the interconnection of C5aR with VEGFR-2 in ECs. As indicated last year, we found that

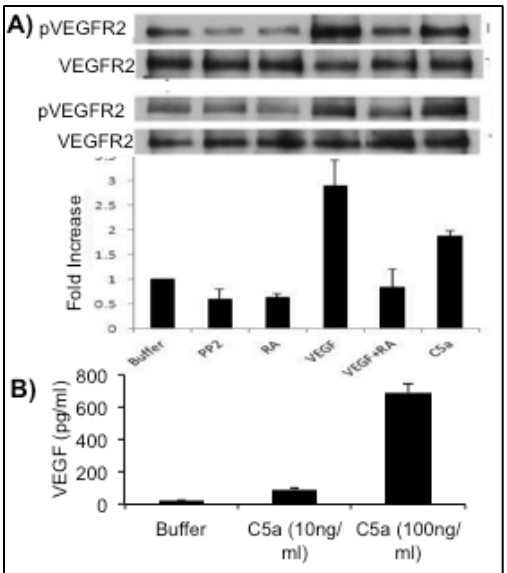

Fig 1. A) C5a or VEGF-A was added to serum starved primary cultures of WT murine ECs in the absence or presence of C3aR-A/C5aR-A and VEGFR2 phosphorylation assessed by immunoblotting with anti p-T1094/T1095 mAb. (2 representative exps shown). **B)** MS-1 cells were incubated with increasing concentrations of C5a for 24 hr and VEGF was assayed by ELISA.

both processes, which are known to activate STAT3 (4-7), were abolished by C3aR/C5aR antagonism (**Fig 2**). Based on these findings, we assayed culture supernatants of a) VEGF treated ECs, b) IL-6 treated ECs, and c) C5a treated ECs for VEGF, C5a, and IL-6 at increasing times. Remarkably, all three ligands were tonically produced by the ECs and each ligand stimulated 4-fold increases in VEGF, C5a, and IL-6 at 4 h (**Fig 3**). Based on this, we added VEGF-A to ECs for 5 min, and probed immunoblots of anti-C5aR, anti-gp-130 and, anti-VEGFR2 immunoprecipitates (IPs) with antibodies to each of the other components. This showed that all three co-immunoprecipitate (co-IP) and are associated (**Fig 4**). These findings implicate both C3aR/C5aR and IL-6Rgp130 signaling in VEGFR-2 induction of EC growth. Findings by others of a second phase of STAT3 phosphorylation at 4 h coincides with this. A corresponding set of data were obtained for EGFR, IL-6Rgp130 and p-STAT3 in breast cancer cells. The finding that C5a induces VEGFR-2 phosphorylation in <5 min time and the pull-down of C5aR, gp130, and VEGFR-2 by mAbs to each other implicate a signaling complex (signalosome) involving all three receptors. Mass Spec (MS) and liquid chromatography (LC)-MS-MS analyses are in progress to confirm this complex, identify potential additional partners, and determine phosphorylations. In addition to C3aR/C5aR antagonism in conjunction with IL-6 blockade, we expect that this analysis will open up new targets for clinical intervention. We have examined the effect of inhibiting C3aR/C5aR signaling on two cancer lines in which EGFR signaling is deregulated. The blockade in both cases inhibited their growth (**Fig 5**). In addition to characterizing the signalosome, we plan to assay EGF, C3a/C5a, and IL-6 production, and STAT3 phosphorylation kinetically in these and other breast cancer lines.

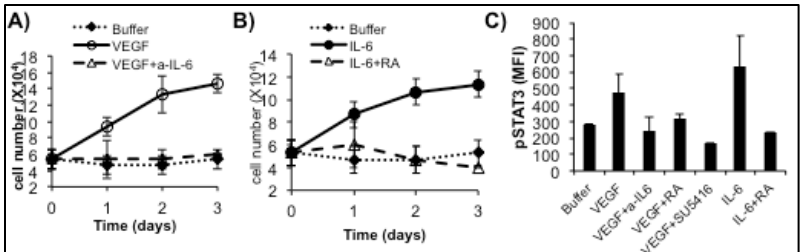

Fig 2. A) MS-1 cells were treated with VEGF± anti-IL-6 and their growth was monitored for 3 days. **B)** MS-1 cells were treated with IL-6 ± C3aR-A/C5aR-A and their growth was monitored for 3 days. **C)** Serum starved MS-1 cells were incubated for 5 min at 37°C as indicated and pStat3 was assayed by FACS.

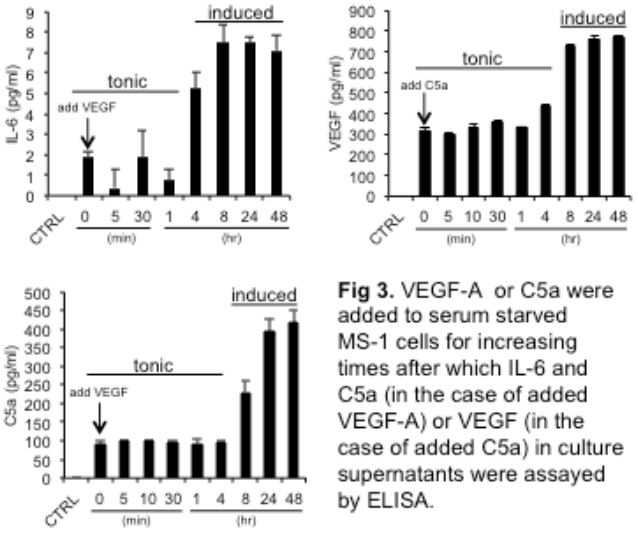

Fig 3. VEGF-A or C5a were added to serum starved MS-1 cells for increasing times after which IL-6 and C5a (in the case of added VEGF-A) or VEGF (in the case of added C5a) in culture supernatants were assayed by ELISA.

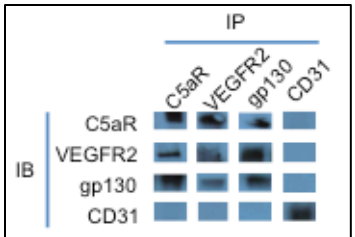

Fig 4. 1x10⁶ serum starved Bend.3 cells were extracted and C5aR, VEGFR2, gp130, and CD31 immunoprecipates were probed for C5aR, VEGFR2, gp130, and CD31.

2) Transfection of DCs and breast cancer cells with DAF, Crry and DAF/Crry and C3aR/C5aR siRNAs. In our prior report, we emphasized the importance of inhibiting DAF regulatory activity and consequently potentiating C3aR/C5aR signaling selectively immune cells while doing the reverse in cancer cells and tumor associated ECs. We presented data showing both siRNA and shRNA knockdown of C3aR and C5aR in NMuMG breast cancer cells and in DCs. While this methodology allows *in vitro* studies and short term *in vivo* studies of cancers silenced in these genes, it does not allow *in vivo* studies in which the role of DAF control of C3aR/C5aR signaling in cancer cell progression can be precisely defined. To make such studies feasible, we reported last year that we initiated efforts to prepare conditional *Daf1*$^{-/-}$ *C3aR*$^{-/-}$, and *C5aR*$^{-/-}$ mice from ES cells (obtained from Eucomm Switzerland) in which each gene is flanked by LoxP sites. The conditional *Daf1*$^{-/-}$ and *C5aR*$^{-/-}$ mice have been completed in our lab and the conditional *C3aR*$^{-/-}$, mice completed in our collaborator's lab. Mating these mice with mice expressing Cre under the control promoters specific for each cell type will allow analysis of the role that this signaling in each cell type in cancer progression and metastasis. Currently each conditional knockout

is now homozygous. Removal on the Neo cassette using Flp mice has been completed for one strain and is in progress for the other two. Once fully available, we will mate the conditional *C3aR*$^{-/-}$ and *C5aR*$^{-/-}$ mice with each other to prepare double knockouts. Inoculation of breast cancers which are shRNA silenced for DAF or C3aR/C5aR into mice with EC, DC, or CD4^{+} cell selective knockout of DAF, C3aR, C5aR or both C3aR/C5aR will allow analysis of the interaction of the cancer with its microenvironment. Our data indicate that the optimal approach would be to selectively increase C3a/C5a production in immune cells and disable C3aR/C5aR signaling elsewhere. This could be done by administering tumor cells silenced in C3aR/C5aR signaling to mice in which Daf1 is selectively knocked out in immune cells and C3aR/C5aR signaling selectively knocked out in ECs.

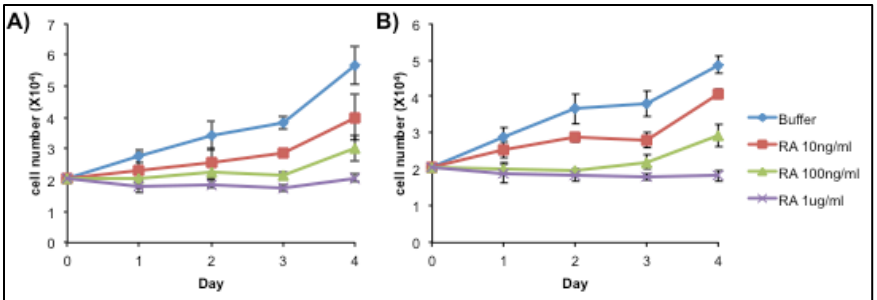

Fig 5. A) T1 and **(B)** PC-9 cells were incubated with increasing concentrations of C3aR-A/C5aR-A (RA) and cell growth determined daily.

3): Preparation of and studies with Adenoviruses expressing C5a, C3a and both anaphylatoxins. We provided evidence last year that both CD4^{+} cells and ECs locally produce thrombin which can cleave EC produced C5 to C5a. As discussed in 1) above, our data showed that added C5a transactivates to phosphorylate VEGFR2 and that VEGF-A induction of VEGFR2 phosphorylation is blocked by C3aR/C5aR antagonists. We have similar results for EGFR signaling. Consequently this is a second pathway that can drive cancer growth itself as well as its associated angiogenesis. These findings may explain the numerous reports connecting cancer growth with thrombin (8) and cancer cell thrombin with IL-6 (9, 10). We are analyzing breast cancers for production of thrombin and the effects of inhibiting thrombin on breast cancer C5a production on proliferation, clot formation, and circumvention of apoptosis.

4): Anti-breast tumor T cell responses in *Daf1*$^{-/-}$, *Daf1*$^{-/-}$*Crry*$^{-/+}$ and wild-type mice: In last years' report, we summarized our unique findings that absent C3aR/C5aR signaling in CD4^{+} cells enables their endogenous TGF-β1 production and their differentiation into Foxp3^{+} T regulatory cells, which are widely known to be responsible for the circumvention by breast (and other) cancers of anti-tumor cell T effector responses (11) (12) (13) This contrasts with potentiated C3aR/C5aR signaling which drives Th1/Th17 effector cell responses (14). While we described the connection of C3aR/C5aR signaling with the PI-3Kγ-AKT-mTOR pathway in our previous progress report, we discovered that absent C3aR/C5aR signaling is needed for PKA activation of p-CREB, that disabled AKT phosphorylation is needed to de-repress nuclear translocation of Foxo-1 and that absent C3aR/C5ar signaling is needed for phosphorylation STAT5, three transcription factors needed for

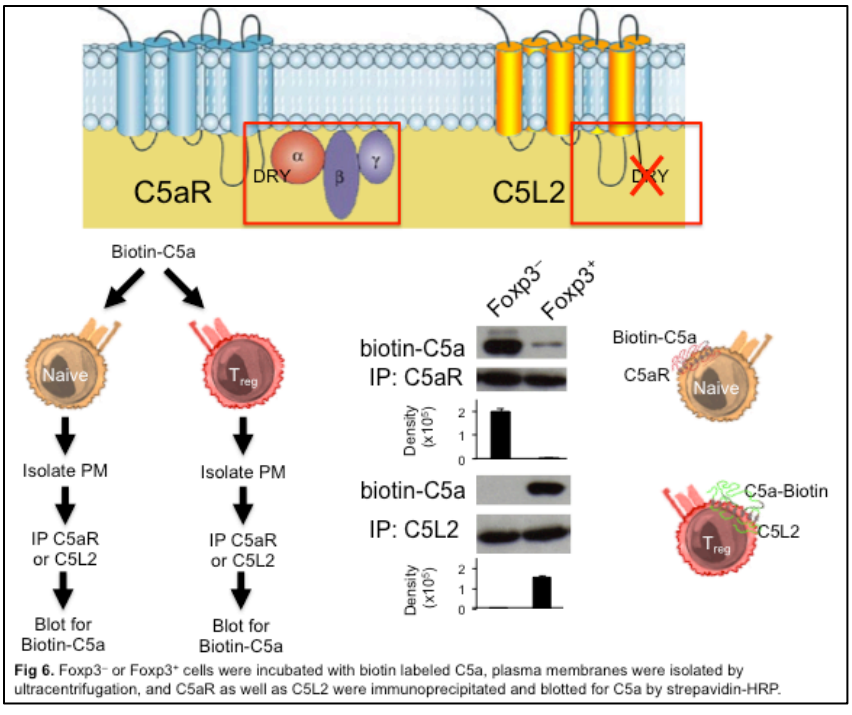

Fig 6. Foxp3⁻ or Foxp3⁺ cells were incubated with biotin labeled C5a, plasma membranes were isolated by ultracentrifugation, and C5aR as well as C5L2 were immunoprecipitated and blotted for C5a by strepavidin-HRP.

Foxp3 gene expression. We additionally found that absent C3aR/C5aR signaling causes up-regulation of C5L2, an alternative C5a receptor decoy receptor which scavenges C5a (and C3a) (15, 16) and prevents C3aR/C5aR receptor signaling. These paradigm shifting data were recently published in Nature Immunology (17). We are testing how this pathway is altered in breast cancers. Since all cells including cancers (tested to date) express C3aR/C5aR and endogenously produce C3a/C5a which enter into autocrine signaling loops which drive their growth, this must be taken into consideration for enhancing the T effector response. As indicated in last year's report, we plan studies with conditional knockouts and implanted cancers silenced in C3aR/C5aR signaling to test this.

5) *In vivo* studies with the transfectants and adoptive transfer studies: This was discussed in Statements of work 2 and 4.

6) Silencing of DAF and Crry in DCs: This was discussed in Statement of work 2:

7) Boosting local C5a/C3a production by DCs: This would be done with DCs from mice conditionally knocked out in Daf1 as was discussed in Statement of work 2.

Key Research Accomplishments

1): Finding that VEGFR2 and EGFR exist with C5aR and IL-6Rgp130 in a plasma membrane signaling complex, i.e. signalosome, that the C5a, IL-6, and VEGF-A or EGF ligands are endogenously produced by cells, and that autocrine signaling by each ligand drives the production of the other, and that this process underlies VEGF-A and EGF induced proliferation.

2): Finding that all three receptors transmit their signaling via activation of STAT3.

3): Finding that C5aR signaling is needed for VEGFR2 phosphorylation.

4): Finding that that suppression of C3aR/C5aR signaling by DAF downregulates VEGF-A and EGF mediated proliferation.

5): The preparation of conditional *Daf1*$^{-/-}$, *C5aR*$^{-/-}$ and *C3aR*$^{-/-}$ mice.

6): The finding that absent C3aR/C5aR signaling into CD4^{+} cells leads to Tregs which inhibit anti-tumor T cell responses.

7): The first description of a physiological function of the alternative C5a receptor C5L2.

Reportable Outcomes

Autocrine C3aR/C5aR signaling and its regulation by DAF are not only integrally involved in controlling the anti-tumor immune response, i.e. biasing between Th1/Th17 effector cell vs Treg commitment, but also directly involved in controlling the growth of cancer cells themselves as well as their associated angiogenesis.

Conclusion

C3aR/C5aR signaling must be augmented for enhancing the anti-breast cancer cell effector cell response and preventing cancer specific Tregs, but simultaneously suppressed in the cancer and in ECs to suppress breast cancer growth. To accomplish this, T effector must be primed with tumor antigen and DCs *ex vivo* and returned to patients under conditions in which C3aR/C5aR and IL-6 signaling is suppressed. For an optimal effect, methods are needed to target this suppression to cancers and endothelial cells.

Publications

1): Absence of signaling into CD4^{+} T cells via C3aR and C5aR enables auto-inductive TGF-β1 signaling and Foxp3^{+} T regulatory cell induction: Michael G. Strainic^{+}, Ethan M. Shevach$^{\#}$, Fengqi An^{+}, Feng Lin^{+} and M. Edward Medof^{+}: C5aR enables autoinductive TGF-beta1 signaling and induction of

Foxp3(+) regulatory T cells. Nature immunology. 2013;14(2):162-71. Epub 2012/12/25. PubMed PMID: 23263555

2): VEGF induction of angiogenesis depends on C3aR/C5aR and IL-6gp130 which exist with VEGFR2 in a signalosome. Ming-Shih Hwang[1], Hae Suk Kim[1], Elzbieta Pluskota[2], Edward F. Plow[2], Diana L. Ramirez-Bergeron[3], and M. Edward Medof[1]*, further data added, pending

3): Cancer cell growth is driven by constitutive and growth factor amplified C3a/C5a receptor signal transduction, Young Choi, Elliot S. Pohlmann, Michael J. Strainic, Sathyamangla Prasad[$], and M. Edward Medof, in preparation

4): Lowering DAF expression boosts anti-tumor T cell reactivity and extends survival: Young A Choi*, Brian Brannigan*: Gregory T. MacLennan[+], Gregory Plautz[#], Feng Lin*, and M. Edward Medof, in preparation*[&]

5): Autocrine C3aR/C5aR signal transduction participates in both the anti-apoptotic and mitotic activities of epidermal growth factor: Elliot Pohlmann, Hae-Suk Kim, M. Edward Medof, in preparation

NIH Grant Applications:

1): 1R01CA158342-01: Mechanism of TGF-beta Suppression of Tumorigenesis

2): 1RC4CA156475-01: Inflammatory autocrine and paracrine factors that regulate the response to cancer

Listed last year but not funded. Resubmission planned for 11/5/13. CTSC grant $10,000 for MS studies

Patents

1):Serial No. 13/350,402: COMPOSITIONS AND METHOD OF MODULATING GROWTH FACTOR FUNCTION; January 13, 2012

Others listed last year. Extended data on signalosome added this year.

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