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14. ABSTRACT

Our studies suggest that a unique RGDKGE collagen fragment is generated within ovarian tumors. Given these and other current observations, the proposal was designed to test the central hypothesis that targeting the soluble RGDKGE collagen fragment may re-activate immune control of ovarian cancer and enhance the efficacy of immune checkpoint inhibitors by selectively disrupting a novel integrin-dependent signaling cascade. Data generated from our current studies suggest that integrin alpha-V beta-3 plays a role in regulating the expression of the immune checkpoint molecules LAG-3 and PD-L1. In particular, we provide evidence that the protein kinase FAK, but not Src plays an important role in regulating PD-L1 and LAG-3 expression in T-cells. In addition, our new studies provide the first evidence that selective targeting of the RGDKGE collagen fragment may differentially alter the accumulation of distinct subsets of immune and stromal cells within specific tissue compartments in mice during tumor growth. Collectively, these novel observations may lead to the development of new treatment strategies for ovarian cancer and may lead to a better molecular understanding of how mechano-transduction signaling pathways regulate immune suppression.

15. SUBJECT TERMS
Tumor Associated Macrophages Lymphocyte Activation Gene Programmed Cell Death Ligand 1. Extracellular Matrix Immune Checkpoint Inhibitors Integrins Ovarian Cancer T-Cells Collagen Monoclonal Antibodies

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1). Introduction:

It is estimated that over 21,000 new cases of ovarian cancer were diagnosed in the US in 2015 and this disease continues to be a leading cause of cancer death among women with gynecologic malignancies. While progress has been made in developing new treatments strategies such as immunotherapies, these clinical approaches have had only minimal if any impact on ovarian cancer. Thus, uncovering novel mechanisms that limit the efficacy of immunotherapy in ovarian cancer may lead to more effective treatment paradigms. To this end, we made the surprising discovery that a highly conserved RGDKGE containing collagen fragment that binds to integrin receptor alpha-V beta-3, was generated and secreted by a subset of macrophages. A soluble form of this RGDKGE collagen fragment potently induced angiogenesis and inflammation in vivo. An antibody (Mab XL313) that selectively binds the RGDKGE collagen fragment, but not other RGD containing collagen fragments, nor to intact triple helical collagen, blocks binding of this collagen fragment to integrin alpha-V beta-3 expressing cells and inhibited angiogenesis and inflammation in vivo. Importantly, our exciting preliminary data suggested that this unique RGDKGE collagen fragment may be generated within ovarian tumors. In addition, cellular interactions with this endogenously generated collagen fragment may control expression of immune checkpoint molecules. Given these observations our current proposal was designed to test the central hypothesis that targeting the soluble RGDKGE collagen fragment may re-activate immune control of ovarian cancer and enhance the efficacy of immune checkpoint inhibitors by selectively disrupting a novel integrin-dependent signaling cascade.

2). Key Words:

1). Tumor Associated Macrophages
2). Lymphocyte Activation Gene 3
3). Programmed Cell Death Ligand 1.
4). Extracellular Matrix
5). Immune Checkpoint Inhibitors
6). Integrins
7) Ovarian Cancer
8) T-cells
9). Collagen
10). Monoclonal Antibodies

3). Accomplishments:

Summary of major goals of project:

A). As outlined in aim-1, tasks 1 and 2, we have proposed to test the effects of blocking cellular interactions with the soluble RGDKGE collagen fragment on ovarian tumor growth. In addition, we will determine whether this novel treatment strategy selectively enhances the therapeutic response of ovarian cancer to immune checkpoint inhibitors.

B). As outlined in aim-2, tasks 3 through 5, we have proposed to determine how the soluble RGDKGE collagen fragment stimulates immunosuppression by activating a unique integrin signaling pathway in T-cells.

Specific Accomplishments for reporting period as they relate to the major goals described above.

We have made significant progress towards the overall goals of our project during this initial funding period (April 2017 through April 2018). Specifically, we have made substantial progress on both aims 1 and 2 and their associated tasks. In this regard, a detailed summary of our current research accomplishments as they pertain to the tasks outlined in the statement of work is provided below.

Effects of Mab XL313 on subcutaneous murine ID8 tumor growth in vivo. Our previous studies suggested that the RGDKGE collagen fragment could be detected within ovarian tumors growing in vivo, and a significant proportion of this collagen fragment was closely associated with F4/80 positive-tumor associated macrophages. To begin to examine the effects of targeting the RGDKGE collagen fragment on ID8 ovarian tumor growth in vivo, we first carried out a pilot experiment to examine the effects of Mab XL313 specifically directed to the RGDKGE collagen fragment on ID8 tumor growth in immune competent C57BL/6 mice. Briefly, ID8 tumor cells were injected subcutaneously in mice and 5 days later following development of well-defined solid tumors, mice were treated with either control non-specific antibody or anti-RGDKGE collagen fragment antibody Mab XL313. Tumor volumes were estimated by caliper measurements. As shown in figure 1, at the end of a 14-day incubation period, tumors from mice treated with Mab XL313 were on average smaller than tumors from control antibody treated mice with approximately 35% inhibition observed. However, do to outliers in the Mab XL313 treated group, this reduction in tumor volume did not reach statistical significance. Surprisingly, observations from this initial pilot experiment indicated that while a reduction in tumor size was observed following treatment with Mab XL313, tumors from control treated mice also regressed from their initial size. These observations suggested that while Mab XL313 may have exhibited anti-tumor activity, ID8 tumors appeared to exhibit spontaneously regression on their own, suggesting that Mab XL313 might accelerated the spontaneous regression under these experimental conditions. However, it is clear that additional in-depth experiments will be needed to test this possibility.

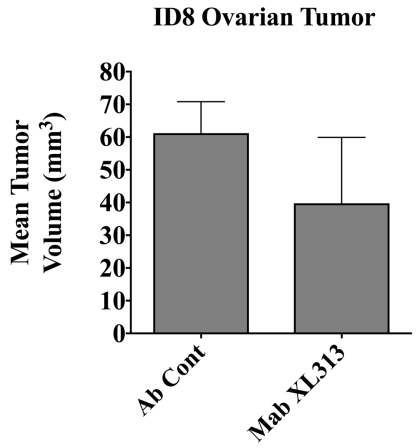


Figure 1. Effects of Mab XL313 on subcutaneous ID8 ovarian tumor growth in vivo. Murine ID8 ovarian carcinoma cells ($10x10^6$/mouse) were injected subcutaneously in C57BL/6 mice (N=8 per group). Five days later mice were treated i.p. with non-specific control antibody (Ab Cont) or Mab XL313 (100ug/mouse 3X week). Data bars represent mean tumor volumes $\pm$ standard errors from 8 mice per condition.

Analysis of immune cell infiltration of ID8 ovarian tumors over a 14-day growth period in vivo. Given our surprising preliminary observations suggesting that ID8 ovarian tumors appear to exhibit some degree of spontaneous regression under our experimental conditions, we sought to assess whether this spontaneous regression was associated with any time dependent changes in the relative levels of immune cell infiltration. Using similar experimental approaches, ID8 ovarian tumor cells were injected subcutaneously into immune competent C57BL/6 mice and the representative tumors were harvested on days 2 through 14. As shown in figure 2A, ID8 ovarian tumor cells rapidly formed well-defined solid ID8 subcutaneous tumors but began to regress over time without any specific treatment. Importantly, the lesions that formed exhibited well established tumors that were associated with stromal and immune cell infiltrates as indicated by H&E stain (figure 2B). To analysis these tumors in more detail, representative examples from each time point (N=2 to 3 per time point) were dissected and single cell suspension were prepared from whole tumors. Single cell suspension from each group were analyzed by flow cytometry by staining with antibodies directed to the indicated antigen markers including CD8, CD3, CD4, CTLA-4. As shown in figure 2C and D, the relative levels of both CD3 and CD8 expressing T-cells increased in a time dependent manner within the ID8 tumors from day-3 to day-14 while the levels of CD4 expressing cells appeared to peak on day-7 showing little if any increase between day-7 to day-14 (figure 2E). Interestingly, the relative levels of CD4+/CTLA-4 double positive cells which may help mark immune suppressive T-Reg like cells significantly increased in a time

dependent manner (figure 2F). These data are consistent with a time dependent differential accumulation of distinct sub sets of immune cells into ID8 tumors under these experimental conditions.

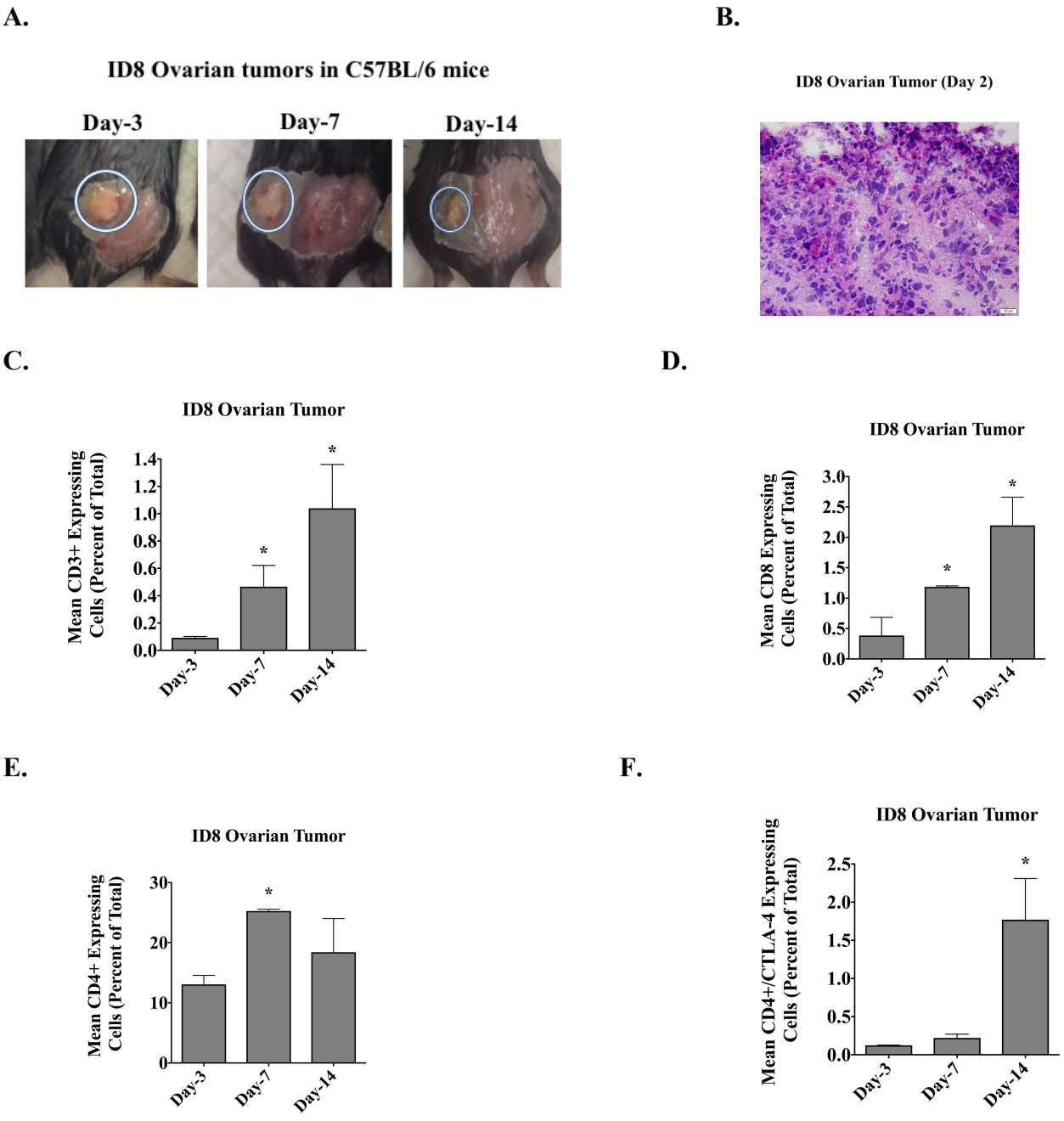


Figure 2. Time dependent differential accumulation of immune cells in subcutaneous ID8 ovarian tumor growth in vivo. Murine ID8 ovarian carcinoma cells ($10x10^6$/mouse) were injected subcutaneously in C57BL/6 mice. A). Examples of ID8 subcutaneous tumors growing in C57BL/6 mice at each time point. B). Example of ID8 tumor stained by H&E method. C-F). Single cell suspensions were prepared for individual tumors (N=2 to 3 per treatment group)**.** Samples were analyzed by flow cytometry by staining with the indicated antibodies directed to markers of distinct subsets of cells including CD8, CD3, CD4 and CTLA-4. Data bars indicate mean percent positive cells per time point quantified as the percentage of the total cells collected. Bars indicate mean $\pm$ S.E from 2 to 3 individual tumors. * $P<0.05$.

Analysis of stromal cell infiltration of ID8 ovarian tumors over a 14-day growth period in vivo. Given the time dependent differential infiltration of immune cells into the ID8 ovarian tumors, we sought to carry out similar experiments to examine potential differential infiltration of additional stromal cells by flow cytometry using antibodies directed to specific markers including LAG-3, PDL-1, Gr1, CD11b, F4/80 and PDGFRa. Single cell suspensions from each group were analyzed by flow cytometry by staining with antibodies directed to the indicated antigens. As shown in figure 3A, while a time dependent increase in the relative number of LAG-3 expressing cells were observed by day-14, the number of PD-L1 expressing cells was significantly enhanced by day-7 then surprisingly returned to lower levels, similar to that observed on day-3 (figure 3B). Moreover, the relative levels of Gr1/CD11b double positive cells with characteristics of myeloid derived suppressor cells (MSDS) significantly decreased by day-14 as compared to day-3 (figure 3C). Finally, while relatively minimal changes were observed with F4/80 expressing macrophages (figure 3D), a sharp increase in the relative levels of PDGFRa expressing cells were observed by day-14 (figure 3F), however this increase did not meet statistical significance. These data are again consistent with a time dependent differential accumulation of distinct sub sets of stromal cells into ID8 tumors under these experimental conditions.

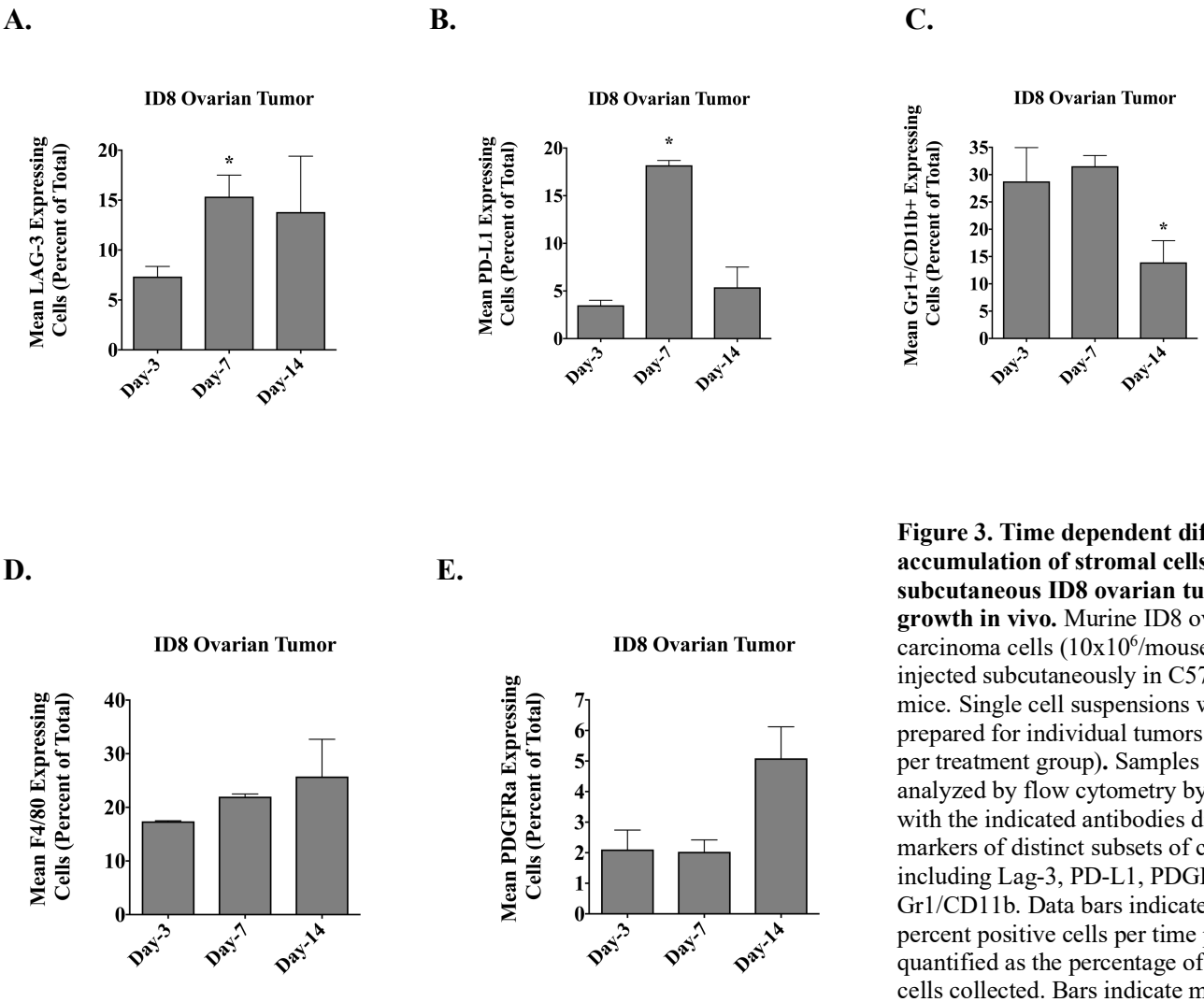


Figure 3. Time dependent differential accumulation of stromal cells in subcutaneous ID8 ovarian tumor growth in vivo. Murine ID8 ovarian carcinoma cells ($10x10^6$/mouse) were injected subcutaneously in C57BL/6 mice. Single cell suspensions were prepared for individual tumors (N=2 to 3 per treatment group)**.** Samples were analyzed by flow cytometry by staining with the indicated antibodies directed to markers of distinct subsets of cells including Lag-3, PD-L1, PDGFRa and Gr1/CD11b. Data bars indicate mean percent positive cells per time point quantified as the percentage of the total cells collected. Bars indicate mean $\pm$ S.E from 2 to 3 individual tumors. * $P<0.05$.

Analysis of immune cell content of spleens from ID8 ovarian tumor bearing mice over a 14-day growth period in vivo. Given the time dependent differential infiltration of immune and stromal cell into the ID8 tumors, we sought to carry out similar experiments to examine potential differential composition of the spleens from mice injected with ID8 tumor cells by flow cytometry using antibodies directed to specific markers including CD8, CD3, CD4, CTLA-4, Gr1/CD11b and Lag-3. Single cell suspension from each group were analyzed by flow cytometry by staining with antibodies directed to the indicated antigens. As shown in figure 4A-C, no significant changes in the relative levels of CD8, CD3 or CD4 expressing T-cells were observed in the spleen of ID8 ovarian tumor bearing mice between day-3 and day-14. Interestingly, a time dependent reduction in the relative levels of CD4+/CTLA-4 double positive cells with characteristics of immune suppressive T-Regs was observed in the spleens of mice between day 3 and day 14 (figure 4D), however, do to the small sample size this change did not meet statistical significance. Interestingly, the relative levels of immune suppressing cells including Gr1/CD11b double positive cells with characteristics of myeloid derived suppressor cells (MSDS) and LAG-3 expressing cells were reduced on day-7 as compared to either day-3 or day-14 (figure 4E and F).

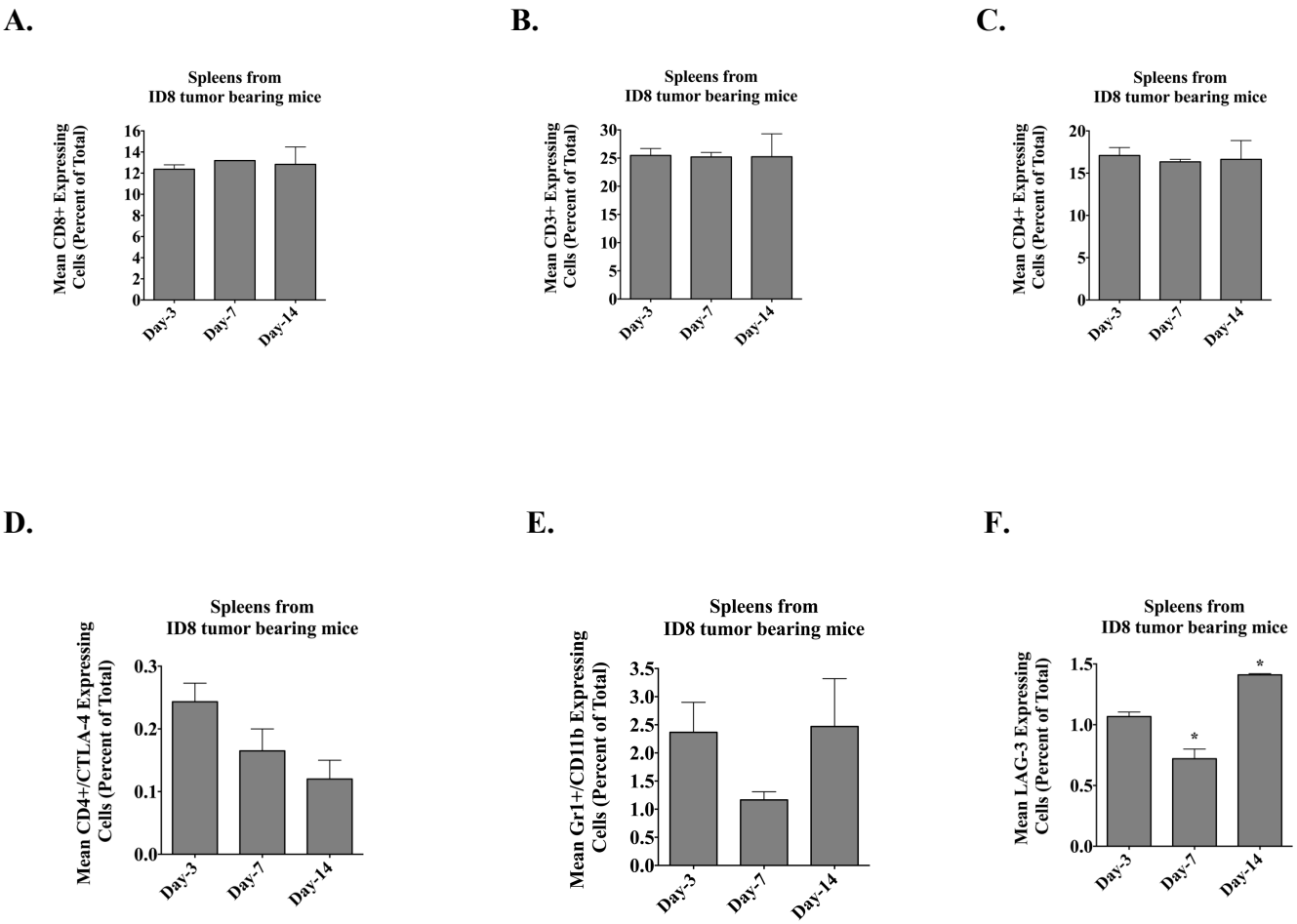


Figure 4. Analysis of immune cell content of spleens from ID8 ovarian tumor bearing mice over a 14-day growth period in vivo. Murine ID8 ovarian carcinoma cells ($10x10^6$/mouse) were injected subcutaneously in C57BL/6 mice. Single cell suspensions were prepared for individual spleens from each mouse (N=2 to 3 per treatment group)**.** Samples were analyzed by flow cytometry by staining with the indicated antibodies directed to markers of distinct subsets of cells including CD8, CD3, CD4, CTLA-4, Gr1, CD11b and LAG-3. Data bars indicate mean ($\pm$ S.E from 2 to 3 individual tumors) percent positive cells per time point quantified as the percentage of the total cells collected. * $P<0.05$.

Analysis of stromal cell content of spleens from ID8 ovarian tumor bearing mice over a 14-day growth period in vivo. Given the time dependent differential composition of immune cells in the spleens of ID8 ovarian tumor bearing mice, we sought to expand our analysis to examine potential differential composition of the spleens from mice injected with ID8 tumor cells by flow cytometry using antibodies directed to specific markers including PD-L1, F4/80 and PDGFRa. Single cell suspensions from the spleens from each group were analyzed by flow cytometry by staining with antibodies directed to the indicated antigens. As shown in figure 5A and B, no significant changes were observed in the relative levels of PD-L1 expressing cells or F4/80 expressing macrophages in the spleens of ID8 tumor bearing mice between day 3 and day-14. While a small increase in the relative levels of PDGFRa expressing cells was observed in the spleens of ID8 tumor earing mice this change did not meet statistical significance (figure 5C).

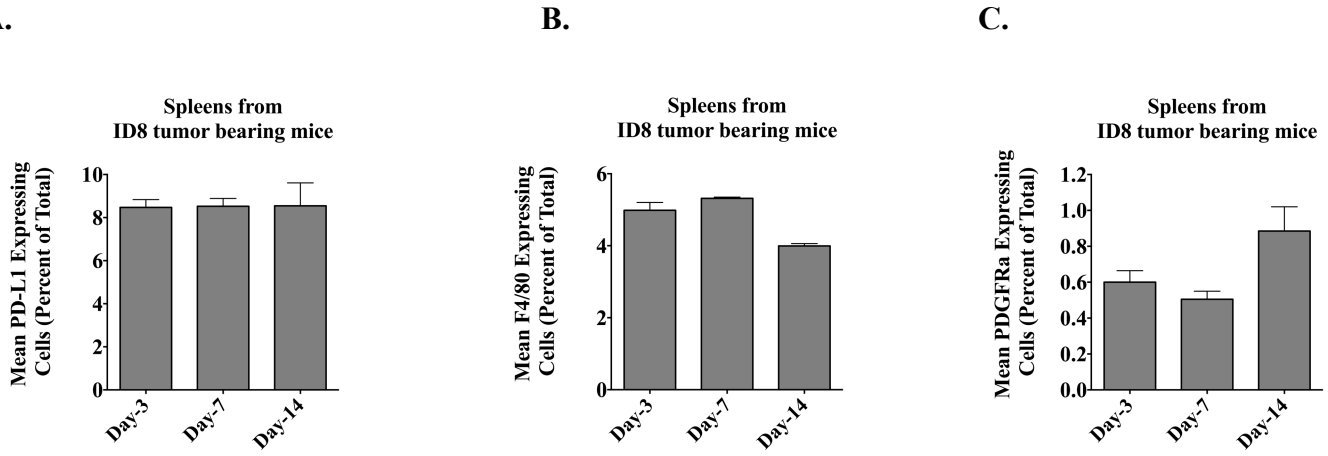
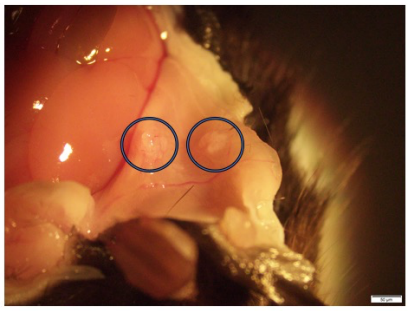


Figure 5. Analysis of immune cell content of spleens from ID8 ovarian tumor bearing mice over a 14-day growth period in vivo. Murine ID8 ovarian carcinoma cells ($10x10^6$/mouse) were injected subcutaneously in C57BL/6 mice. Single cell suspensions were prepared for individual spleens from each mouse (N=2 to 3 per treatment group)**.** Samples were analyzed by flow cytometry by staining with the indicated antibodies directed to markers of distinct subsets of cells including PD-L1, F4/80, and PDGFRa. Data bars indicate mean (± S.E from 2 to 3 individual tumors) percent positive cells per time point quantified as the percentage of the total cells collected. * $P<0.05$.

Effects of Mab XL313 on intraperitoneal ID8 tumor growth in vivo. Our previous studies suggested specific targeting of the RGDKGE collagen fragment may inhibit the subcutaneous growth of ID8 ovarian tumors in mice. However, as described above, while ID8 cells did form subcutaneous tumors they exhibited some degree of spontaneous regression in the absence of Mab XL313 treatment. Given these results we sought to examine the effects of Mab XL313 on the growth of intraperitoneal ID8 tumors. Briefly, ID8 tumor cells were injected intraperitoneally (i.p.) in C57BL/6 mice and allowed to form i.p tumors for 4 weeks prior to treatment. After the 4-week growth period, mice were treated with either non-specific control antibody (Ab Cont) or Mab XL313 once per week for a total of 7 additional weeks. Mice were sacrificed and examined for the presence of peritoneal ovarian tumors. ID8 tumor cells formed multiple tumor lesions throughout the peritoneal cavity (figure 5A) and these tumors ranged from 1 to 4 individual tumor foci per mouse with sizes ranging from 2mgs to 37mgs. The ID8 ovarian tumors were found to be of various sizes scattered throughout the peritoneal cavity of the majority (7 out of 8) of the control treated mice. In contrast, only 5 out of 8 mice treated with Mab XL313 exhibited evidence of tumors and the tumors that were detected were smaller. Quantification of the total tumor burden (wet tumor weight per mouse) indicated a significant ($P<0.05$) inhibition of approximate 80% reduction in total tumor burden in mice treated with Mab XL313 as compared to control treated mice (Figure 6B).

A.



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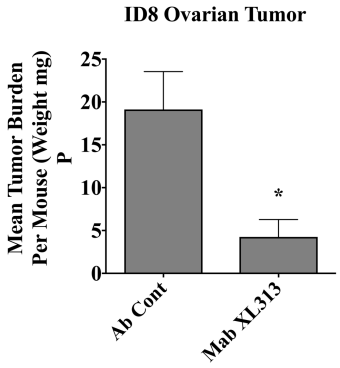
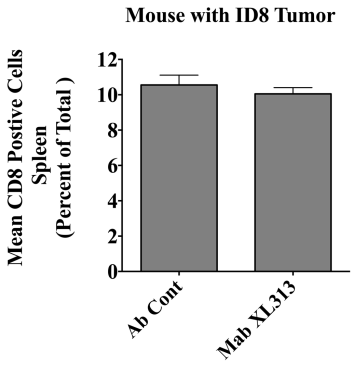


Figure 6. Effects of Mab XL313 on intraperitoneal ID8 ovarian tumor growth in vivo. Murine ID8 ovarian tumor cells ($10x10^6$/mouse) were injected i.p in C57BL/6 mice (N=8 per group). Four weeks later mice were treated i.p with non-specific control antibody (Ab Cont) or Mab XL313 (100ug/mouse 1X week) for 7 additional weeks. A). Examples of i.p tumors detected scattered throughout the intraperitoneal cavity. B). Quantification of total tumor burden per mouse. Data bars represent mean tumor weights ± standard errors from 8 mice per condition. *P<0.05

Analysis of differential levels of immune cells within spleens from peritoneal ID8 tumor bearing mice treated with Mab XL313. Given our observations suggesting differential changes in immune and stromal cell composition within the spleens of subcutaneous ID8 tumor bearing mice, we used a similar experimental approach to examine potential alterations in immune and stromal cell composition of spleens from peritoneal ID8 tumor bearing mice treated with either control non-specific antibody (Ab control) or Mab XL313. Single cell suspensions from the spleens from each group were analyzed by flow cytometry by staining with antibodies directed to the indicated antigens including CD8, CD4 and LAG-3. As shown in figure 7A, no significant changes were observed in the relative levels of CD8 cells in the spleens from each condition. Interestingly, a small reduction in CD4 expressing T-cells was observed (figure 7B) in the spleens. While a small reduction in the relative levels of LAG-3 expressing cells was also observed following treatment of Mab XL313 this reduction did not meet statistical significance (Figure 7C).

A.



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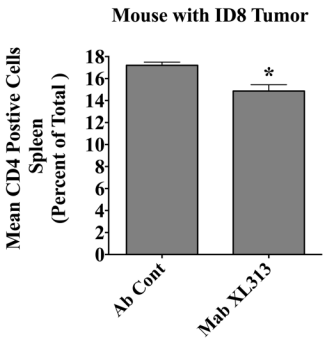

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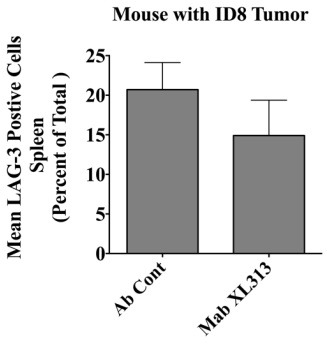

Figure 7. Analysis of immune cell content of spleens from peritoneal ID8 ovarian tumor bearing mice treated with Mab XL313. Murine ID8 ovarian carcinoma cells ($10x10^6$/mouse) were injected i.p. in C57BL/6 mice. Four weeks later mice were treated i.p with non-specific control antibody (Ab Cont) or Mab XL313 (100ug/mouse 1X week) for 7 additional weeks. Single cell suspensions were prepared for individual spleens from each mouse (N=3 to 4 per treatment group)**.** Samples were analyzed by flow cytometry by staining with the indicated antibodies directed to markers of distinct subsets of cells including CD8, CD4 and LAG-3. Data bars indicate mean (± S.E from 3 to 4 individual spleens) percent positive cells per condition quantified as the percentage of the total cells collected. *P<0.05.

Analysis of differential levels of stromal cells within spleens from peritoneal ID8 tumor bearing mice treated with Mab XL313. We used similar experimental approaches to examine any alterations in stromal cell composition of spleens from peritoneal ID8 tumor bearing mice treated with either control non-specific antibody (Ab control) or Mab XL313. Single cell suspensions from the spleens from each group were analyzed by flow cytometry by staining with antibodies directed to the indicated antigens including PDGFRa and F4/80. As shown in figure 8A, while a small reduction in the relative levels of F4/80 expressing macrophages were detected in the spleens from mice treated with Mab XL313, a large statistically significant reduction in the overall levels of PDGFRa expressing cells were observed following treatment with Mab XL313 (figure 8B).

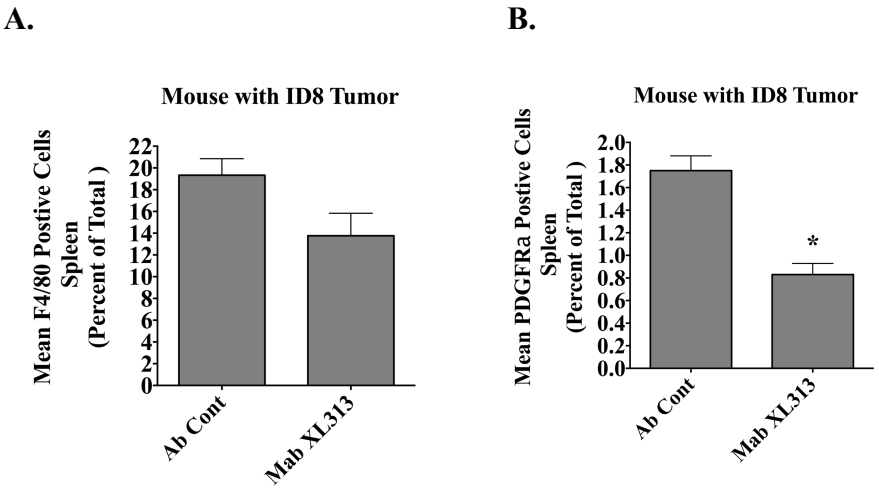

Figure 8. Analysis of stromal cell content of spleens from peritoneal ID8 ovarian tumor bearing mice treated with Mab XL313. Murine ID8 ovarian tumor cells ($10x10^6$/mouse) were injected i.p. in C57BL/6 mice. Four weeks later mice were treated i.p with non-specific control antibody (Ab Cont) or Mab XL313 (100ug/mouse 1X week) for 7 additional weeks. Single cell suspensions were prepared for individual spleens from each mouse (N=3 to 4 per treatment group). Samples were analyzed by flow cytometry by staining with the indicated antibodies directed to markers of distinct subsets of cells including F4/80 and PDGFRa expressing cells. Data bars indicate mean percent positive cells per condition point quantified as the percentage of the total cells collected. *P<0.05.

Analysis of differential levels of stromal cells within bone marrow from peritoneal ID8 tumor bearing mice treated with Mab XL313. We used a similar experimental approach to examine potential alterations in stromal cell composition of bone marrow from peritoneal ID8 tumor bearing mice treated with either control non-specific antibody (Ab control) or Mab XL313. Single cell suspensions from the bone marrow samples from each group were analyzed by flow cytometry by staining with antibodies directed to the indicated antigens including PDGFRa and F4/80. As shown in figure 9A and B, a small but statistically significant reduction in the relative levels of both F4/80 expressing macrophages and PDGFRa expressing cells was observed following treatment with Mab XL313 in these mice.

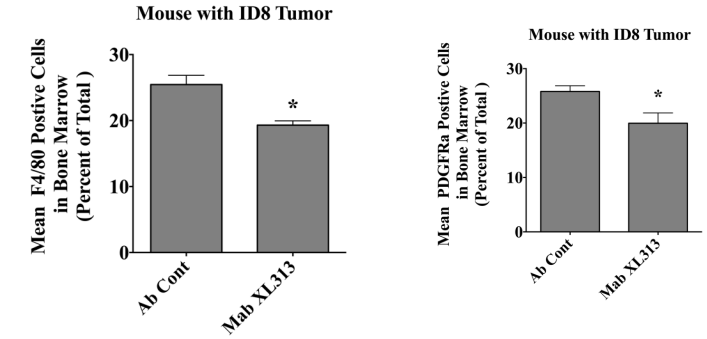

Figure 9. Analysis of stromal cell content of bone marrow from peritoneal ID8 ovarian tumor bearing mice treated with Mab XL313. Murine ID8 ovarian tumor cells ($10x10^6$/mouse) were injected i.p. in C57BL/6 mice. Four weeks later mice were treated i.p with non-specific control antibody (Ab Cont) or Mab XL313 (100ug/mouse 1X week) for 7 additional weeks. Single cell suspensions were prepared for individual bone marrow from each mouse (N=3 to 4 per treatment group). Samples were analyzed by flow cytometry by staining with the indicated antibodies directed to markers of distinct subsets of cells including F4/80 and PDGFRa expressing cells. Data bars indicate mean percent positive cells per condition quantified as the percentage of the total cells collected. *P<0.05.

Analysis of differential levels of stromal cells within peripheral blood from peritoneal ID8 tumor bearing mice treated with Mab XL313. We used a similar experimental approach to examine potential alterations in stromal cell composition of peripheral blood from peritoneal ID8 tumor bearing mice treated with either control non-specific antibody (Ab control) or Mab XL313. Blood from each group were analyzed by flow cytometry by staining with antibodies directed to the indicated antigens including PDGFRa and F4/80. As shown in figure 10A, while a statistically significant reduction in the relative levels of F4/80 monocytes were detected within blood from ID8 ovarian tumor bearing mice treated with Mab XL313, no change was observed in the relative levels of CD8 expressing T-cells.

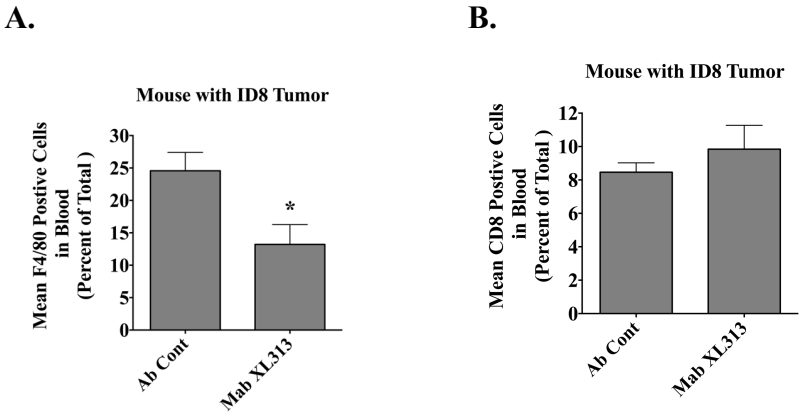

Figure 10. Analysis of immune cell content of peripheral blood from ID8 ovarian tumor bearing mice treated with Mab XL313. Murine ID8 ovarian carcinoma cells ($10x10^6$/mouse) were injected i.p. in C57BL/6 mice. Four weeks later mice were treated i.p with non-specific control antibody (Ab Cont) or Mab XL313 (100ug/mouse 1X week) for 7 additional weeks. Peripheral blood was collected for individual mice (N=3 to 4 per treatment group)**.** Samples were analyzed by flow cytometry by staining with the indicated antibodies directed to markers of distinct subsets of cells including F4/80 and CD8 expressing T-cells. Data bars indicate mean percent positive cells per condition quantified as the percentage of the total cells collected. *P<0.05.

Expression of PD-L1 within subcutaneous ID8 ovarian tumors growing in C57Bl/6 mice. Given our previous experimental findings indicating differential levels of immune cells and stromal cells in ID8 ovarian tumors over a time course as well as following treatment with Mab XL313, we examined the base line distribution and co-localization of PD-L1 expressing cells in these tumors with different subsets of stromal cells including CD31 expressing endothelial cells and alpha-SMA expressing stromal fibroblasts-like cells. As shown in figure 11A, PD-L1 (green) was widely distributed throughout these vascularized tumors but exhibited little if any co-localization with CD31 (Red) expressing blood vessels. In contrast, PD-L1 (Green) expression strongly co-localized with alpha-SMA expressing (Red) stromal fibroblasts-like cells (Figure 11B). These data suggest that a large proportion of the PD-L1 was closely associated with alpha-SMA expressing stromal cells.

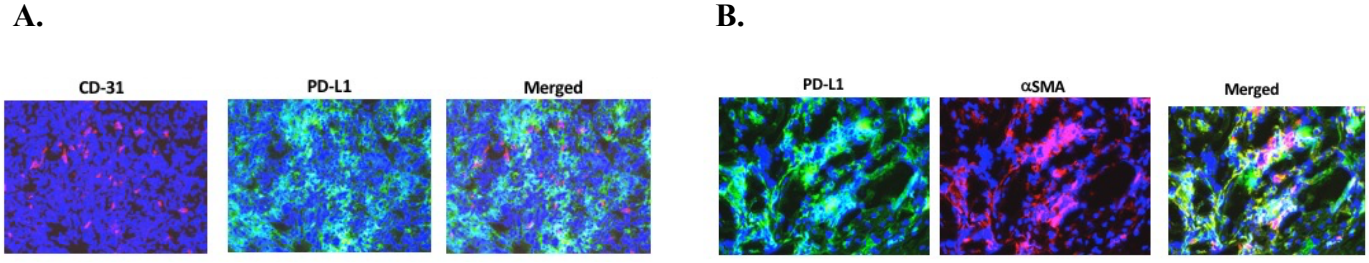

Figure 11. Expression of PD-L1 within subcutaneous ID8 ovarian tumors growing in C57Bl/6 mice. Murine ID8 ovarian carcinoma cells ($10x10^6$/mouse) were injected subcutaneously in C57BL/6 mice. ID8 ovarian tumors were dissected and frozen sections of tumor tissues were co-stained with antibodies directed to PD-L1, CD-31 and alpha-SMA. **A**). Examples of co-staining of PD-L1 (Green) and CD-31(Red) in ID8 ovarian tumors. **B**). Examples of co-staining of PD-L1(Green) and alpha-SMA (Red) in ID8 ovarian tumors.

Integrin beta-3 regulates expression of c-Myc in Jurkat T-cells. Our previous studies indicate that the RGDKGE collagen fragment recognized by Mab XL313 can bind to integrin alpha-V beta-3. Given our studies suggesting that the RGDKGE collagen fragment can regulate expression of c-Myc, and c-Myc has been suggested to regulate PD-L1 in some cell types, we sought to determine whether signaling through integrin alpha-V beta-3 may control expression of c-Myc in T-cells. To examine this possibility, we first knocked down expression of beta 3 integrin in Jurkat T-cells. As shown in figure 12A, transfection of anti-beta-3 integrin specific shRNA readily reduced the expression of beta-3 integrin in Jurkat cells (b3-KD) as compared to non-targeting control transfected cells (NT-Cont). Importantly, as shown in figure 12B, knock down of beta-3 integrin was associated with a reduction in expression of c-Myc in these Jurkat T-cells. These data provide evidence that integrin beta-3 may contribute to the control of c-Myc in these T-cells.

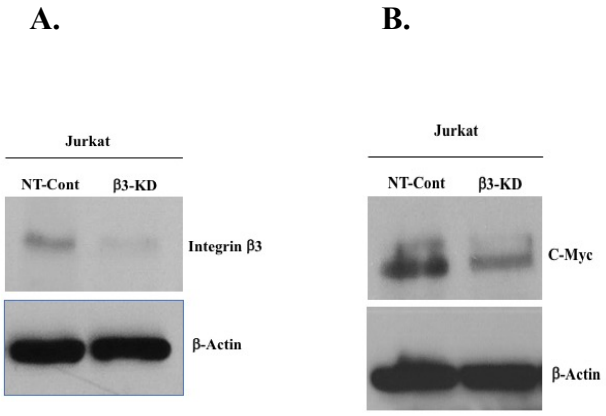

Figure 12. Integrin beta-3 regulates expression of c-Myc in Jurkat T-cells. Human T-cells (Jurkat) were transfected with either non-targeting control shRNA or beta-3 integrin specific shRNA. Whole cell lysates were examined for the relative expression of beta-3 integrin and c-Myc. A). Western blot analysis of Jurkat cell variants for expression of beta-3 integrin. B). Western blot analysis of Jurkat cell variants for expression of c-Myc.

Integrin beta-3 regulates expression of PD-L1 in Jurkat T-cells. Given our studies indicating that beta-3 integrin may regulate expression of c-Myc in Jurkat cells and c-Myc has been suggested to regulate PD-L1 in some cell types, we next examined whether beta-3 integrin may regulate PD-L1 in Jurkat T-cells. As shown in figure 13, expression of PD-L1 was reduced in integrin beta-3 knock down cells as compared to non-targeting control-transfected Jurkat cells. These data are consistent with a role for integrin beta-3 in regulating expression of PD-L1 in these human T-cells.

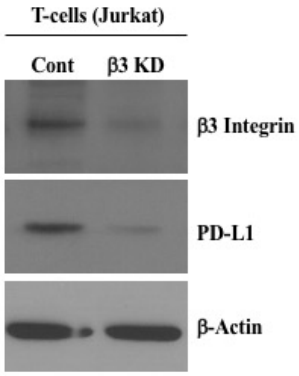

Figure 13. Integrin beta-3 regulates expression of PD-L1 in Jurkat T-cells. Human T-cells (Jurkat) were transfected with either non-targeting control shRNA or beta-3 integrin specific shRNA. Whole cell lysates were examined for the relative expression of PD-L1. Western blot analysis of Jurkat cell variants for expression of PD-L1.

Focal Adhesion Kinase (Fak) regulates expression of PD-L1 and LAG-3 T-cells. Given our studies indicating that beta-3 integrin may regulate expression of c-Myc, and PD-L1 and that down-stream signaling from beta-3 integrin can be mediated in part by protein kinases, we examined the effects of blocking protein kinases Src and Fak on the expression of PD-L1 and LAG-3 in T-cells. Jurkat T-cells were incubated with either control (DMSO), or inhibitors of either Src or Fak. As shown in figure 14A and B, while blocking Src had little effect of expression of either PD-L1 or Lag-3, inhibition of Fak readily reduced expression of PD-L1 and LAG-3 in Jurkat cells. These data are consistent with a role for Fak in regulating expression of the immune suppressing molecules PD-L1 and LAG-3 in these human T-cells.

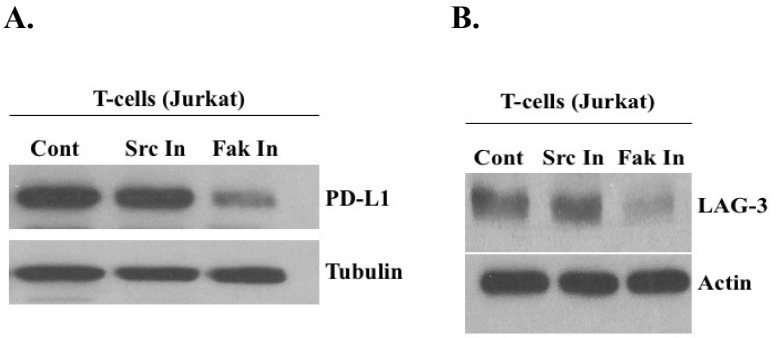

Figure 14. Focal Adhesion Kinase (Fak) regulates expression of PD-L1 and LAG-3 in T-cells. Human T-cells (Jurkat) were treated with inhibitors of the protein kinases Src (Src In) or Fak (Fak In) and whole cell lysates prepared. A). Western blot analysis of Jurkat cells for expression of PD-L1. B). Western blot analysis of Jurkat cells for expression of LAG-3.

What opportunities for training and professional development has the project provided? Nothing to report.

How were the results disseminated to communities of interest? Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals? We plan to complete the remaining studies proposed in our original proposal. Briefly, we expect to complete our studies on examining the effects of Mab XL313 on the efficacy of immune checkpoint point inhibitor anti-CTLA-4 using ID8 ovarian tumor cells in C57BL/6 mice as well as using the SKOV-3 ovarian tumor cells in humanized mice. In addition, we expect to complete our analysis of the effects of Mab XL313 alone and in combination with anti-CTLA-4 on the functional activity of T-cells. In further studies, we plan to complete our analysis of the effects of beta-3 integrin and cMyc on the regulation of PD-L1 and LAG-3 in T-cells. Finally, we expect to finish our analysis of the roles of mir-34a and FAK on PD-L1 and LAG-3 in T-cells. We hope to incorporate these novel findings into a new manuscript in the future. When taken together, these studies should complete the overall goals of our pilot project.

4). Impact

A). What was the impact on the development of the principal disciplines of the project? Our exciting new findings as detailed in the current report provide important new insight into the roles of the endogenously generated RGDKGE collagen fragment in regulating immune checkpoint molecule and ovarian tumor growth. In particular, our studies suggest for the first time that integrin alpha-V beta-3 integrin may play a previously unknown role in regulating the expression of the immune checkpoint molecules LAG-3 and PD-L1. Importantly, we provide evidence that the protein kinase FAK, but not Src may play an important role in regulating PD-L1 and LAG-3 expression in T-cells. These findings suggest that selective targeting of integrin-ECM signaling may provide a strategy to regulate immune suppression during tumor growth. Finally, our new studies provide the first evidence that selective targeting of a unique RGDKGE collagen fragment may alter the accumulation of immune cells and stromal cells within distinct tissue compartments in mice. Collectively, these novel observations may lead to the development of new treatment strategies for ovarian cancer.

B). What was the impact on other disciplines? Our novel observations provide new evidence that integrin mediated cellular interaction with a unique collagen fragment may play a biologically important role in regulating immune suppression in vivo. Given that other cell types other than ovarian tumor cells also express integrin alpha-V beta-3, our studies may have implications in regulating the expressing of immune checkpoint molecules during other biological processes such as inflammation and additional processes characterized by differential stromal cell accumulation. Collectively, these novel observations may lead to a better molecular understanding of how mechano-transduction signaling pathways may regulate immune suppression in vivo.

C). What was the impact on technology transfer? Nothing to report.

D). What was the impact on society beyond science and technology? Nothing to report

5). Changes or Problems? Nothing to report**.**

6). Products:

A) **Manuscripts:** Nothing to Report
B) **Websites or Other Internet Sites**: Nothing to Report
C) **Technologies or Techniques**: Nothing to Report
D) **Inventions, patent applications, and/or licenses**: Nothing to Report
E) **Other Products**: Nothing to Report

7. Participants and Other Collaborating Organizations

Name	**Peter Brooks**
Project Role	**Principal Investigator**
Nearest Person Month Worked	1.8 calendar months
Contribution to Project	Provides overall scientific direction, analysis, writes manuscripts, supervises Staff Scientist
Funding Support	

Name	**Xianghu Han**
Project Role	**Staff Scientist**
Nearest Person Month Worked	6 calendar months
Contribution to Project	Performs cell biological, molecular, and animal experiments
Funding Support	

Has there been a change in the active other support of the PD/PI or senior/key personnel since the last reporting period? Yes

Brooks, Peter

ACTIVE:

(NEW)
Maine Cancer Foundation (Mohan, PI) 10/1/2017 – 09/31/2019 0.36 calendar months

NOHA: A Potentioal Blood-Based Biomarker for Estrogen Receptor Negative (salary only)
Breast Cancer
Goal: developing NOHA as a less-invasive, blood-based indicator for sensitive ER^- breast tumor prognosis in racially distinctive populations for early prognosis, screening and neoadjuvant tumor management.
Role: Collaborator

(NEW)

Cryptomedix
Maine Technology Institute/Cryptomedix LLC (Brooks, PI) 10/1/16-9/30/18 1.2 calendar months
Characterization of CMX-101 for the treatment of malignant tumors $172,735
The goal is to confirm the anti-tumor activity of the humanized antibody CMX-101 and to characterize potential mechanisms by which CMX-101 inhibits tumor growth in vitro and in vivo.

What other organizations were involved as partners? Nothing to report.

None Special Reporting Requirements: N/A

APPENDIX: N/A