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13. SUPPLEMENTARY NOTES

14. ABSTRACT: The overall objective of this proposal is to evaluate the therapeutic efficacy of human, bone marrow-derived mesenchymal stem cells (hMSCs) in a mouse model of inflammatory bowel disease (IBD). As mentioned in my previous report, we encountered an unexpected problem with our mouse model of IBD following our relocation to TTUHSC. We have spent the last 2 years developing and characterizing a new model that has a much incidence of disease than what we observed following our relocation to TTUHSC. Unfortunately, this situation delayed the start of the studies outlined in Tasks 1 and 2. Nevertheless, we now have the studies outlined in Task 1 currently underway. We present new and exciting data demonstrating that the inflammation observed in our re-derived mouse model of IBD is characterized by the infiltration of much larger numbers of myeloid cells into the inflamed colon compared to our original model. In addition, we have made great progress in developing a more immunologically-relevant *in vitro* system to assess immuno-suppressive activity and mechanisms of suppression of hMSCs (Task 3). This antigen-specific, *in vitro* model more closely mimics the cellular and immunological interactions that occur in our *in vivo* mouse model of IBD. Surprisingly we find that hMSCs are much less effective at suppressing antigen-specific proliferation of mouse T cells compared to the immunosuppressive effects of mouse regulatory T cells or mouse MSCs. These data, together with our previous data demonstrating that hMSCs attenuate IBD in mice suggest that suppression of disease may not be due to suppression of T cell proliferation *in vivo*. We care currently exploring whether hMSCs suppress inflammatory cytokine generation by mouse T cells.

15. SUBJECT TERMS
inflammatory bowel disease; mesenchymal stem cells; Tregs; IL-10, TGFβ; colitis; intestinal inflammation; immunosuppression

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1. INTRODUCTION

The inflammatory bowel diseases (IBD; Crohn's disease; ulcerative colitis) are chronic inflammatory disorders of the small bowel and/or colon that affects approximately 1.5 million people in the US with a calculated *annual cost* for both medical expenses and work loss of almost $4 billion dollars. A recent study analyzing the Department of Veterans Affairs database from 1975-2006 reports that although rates of hospitalization for ulcerative colitis (UC) and Crohn's disease (CD) have begun to stabilize over the past few years, there has been a disproportionate increase in rates of hospitalizations for nonwhite vs. white US military veterans for both UC and CD. Currently, there are only a handful of medical treatments available to treat these debilitating inflammatory disorders with only a few new therapies projected to be available in the near future. Thus, there is a clear need for the development of additional therapeutic agents to treat patients with IBD. A great deal of excitement has been generated from recent studies demonstrating that adoptive transfer of syngeneic, allogeneic or xenogeneic (*human*) MSCs suppress the inflammation and tissue injury observed in animal models of autoimmune encephalomyelitis, allograft rejection, arthritis and graft vs. host disease. Because MSCs can be grown and expanded *in vitro* and exert their immuno-regulatory activity across major histocompatibility complex barriers *in vivo*, we are in the unique position to evaluate the therapeutic efficacy of *human* MSCs in our mouse model IBD. The ***overall objective*** of this proposal is to evaluate the therapeutic efficacy of *ex vivo*-generated, bone marrow-derived human MSCs in a well-characterized mouse model of *chronic* colonic inflammation. *Hypothesis:* We propose that *ex vivo*-generated MSCs suppress chronic gut inflammation by homing to the mesenteric lymph nodes (MLNs) and/or colonic lamina propria where MSC-derived TGFβ suppresses/limits the generation of colitogenic T-cells and/or induces the formation of IL-10-producing Tregs. In order to test this hypothesis we propose the following three specific aims: a) Evaluate the ability of human MSCs to suppress the *induction* of chronic gut inflammation; b) Determine the therapeutic efficacy of human MSCs in reversing *preexisting* colitis; and c) Define the immuno-regulatory mechanisms utilized by MSCs to attenuate chronic colitis.

2. KEYWORDS

inflammatory bowel disease; mesenchymal stem cells; Tregs; IL-10, TGFβ; colitis; intestinal inflammation; immuno-suppression;

3. ACCOMPLISHMENTS

Major Goals of the Project

Task 1. Evaluate the ability of human MSCs to suppress the induction of chronic gut inflammation (months 1-12).

Task 2. Determine the therapeutic efficacy of human MSCs in attenuating preexisting colitis (months 12-24).

Task 3. Define the immuno-regulatory mechanisms utilized by MSCs to attenuate chronic colitis (months 24-36).

Accomplishments for the Current Reporting Period

Tasks 1 and 2. Evaluate the ability of human MSCs to suppress the induction of chronic gut inflammation

In order to assess the therapeutic efficacy of human MSCs in a mouse model of chronic gut inflammation we will use our well-characterized T cell transfer model of chronic colitis. We induce chronic disease by adoptive transfer of *naïve* ($CD4^+CD45RB^{high}$) T-cells obtained from healthy wild type donors into recombinase-activating gene-1 deficient ($RAG\text{-}1^{-/-}$) mice. We have more than 15 years of experience with this mouse model in which we have routinely demonstrated that ~85% of the reconstituted mice develop moderate-to-severe colonic inflammation by 6-8 weeks post T cell transfer. Shortly after my relocation from LSU Health Sciences Center (LSUHSC) to my current position at Texas Tech Health Sciences Center (TTUHSC), we were surprised to find that only 30-40% of the $RAG\text{-}1^{-/-}$ mice housed in the TTUHSC animal facility developed chronic colitis at 8 weeks following T cell transfer compared to our historical incidence (at LSUHSC) of ~85%. We have spent the past *2 years* attempting to determine the reason(s) for this significant reduction in disease incidence. After ruling out animal vendor, housing conditions (ventilator, micro-isolator cages) and T cell preparations/administration, we determined, as reported in the previous Progress report, that the reduced incidence appeared to be due to large and significant differences in the microbial composition of mice housed in the animal facilities at the 2 different institutions. Thus, we have spent most of last year attempting to enhance the incidence of disease for our *in vivo* studies outlined in Tasks 1 and 2. We found that if we colonize (via gavage) $RAG\text{-}1^{-/-}$ mice housed at TTUHSC with feces obtained from colitic mice generated at LSUHSC for 1 wk prior to T cell transfer, we can induce severe colitis in >90% of these mice *following T cell transfer* (referred to as LSUHSCc→TTUSHC mice)(please see previous Progress report). Studies performed during the current funding cycle revealed that serial transfer of feces obtained from colitic LSUHSCc→TTUSHC mice into $RAG\text{-}1^{-/-}$ mice housed at TTUHSC (called TTUHSCc→TTUHSC mice) produced moderate but *not* severe disease following T cell transfer that was localized more to the proximal rather than distal colon. We did observe significantly more disease in $RAG\text{-}1^{-/-}$ mice colonized with feces obtained from colitic TTUHSCc→TTUSHC mice than in mice colonized with feces obtained from healthy $RAG\text{-}1^{-/-}$ recipients housed at TTUHSC (Figure 1). Another major finding we made during the current funding cycle was that the colonic inflammation in TTUHSCc→TTUHSC mice contained significantly more myeloid cells when compared to mice that received healthy feces from animals housed at TTUHSC (Figure 2). We are currently exploring the reasons for

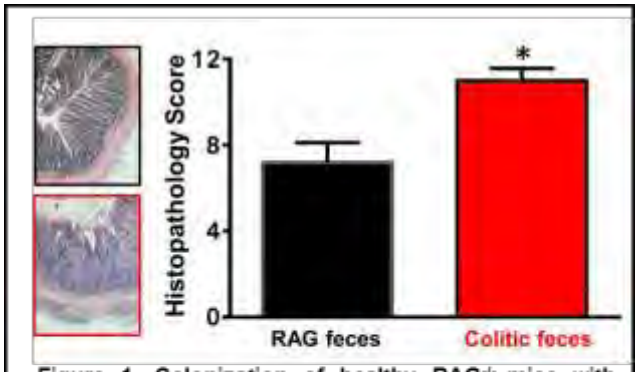

Figure 1. Colonization of healthy $RAG^{-/-}$ mice with colitic feces enhances the severity of chronic colitis following T cell transfer. Healthy $RAG^{-/-}$ mice were colonized (via gastric gavage) with feces obtained from healthy $RAG^{-/-}$ or colitic donors (2 mg feces/g bw). 7 days later, colitis was induced by transfer of naïve T-cells. Blinded histopathological analysis was determined at 8 wks post T cell transfer. Data represent the mean±SEM for N=6 mice/group. $p<0.05$ vs. RAG feces.

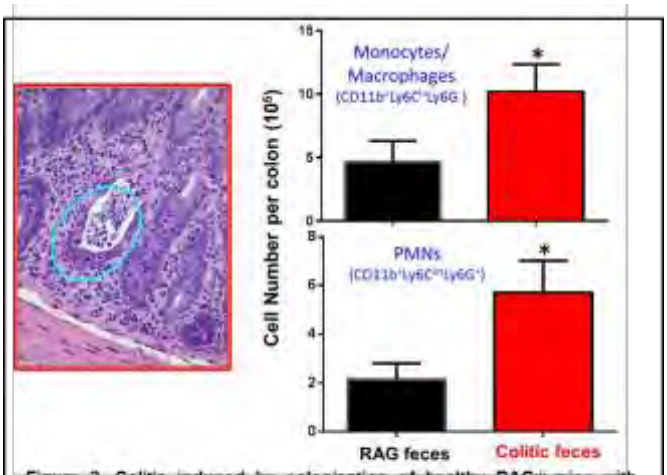

Figure 2. Colitis induced by colonization of healthy RAG$^{-/-}$ mice with colitic feces is characterized by increased presence myeloid cells within the colonic lamina propria. Fecal transplant and induction of colitis was performed as described in Figure 1. Blinded hisotopathology analysis and myeloid cell quantification was performed at 8 wks post T cell transfer. Data represent the mean ± SEM for N=6 mice/group. p<0.05 vs. RAG feces.

this interesting observation. While the initiation of studies outlined in Tasks 1 and 2 was delayed much longer than we anticipated due to the unexpected loss of disease phenotype, we felt that it was critical to invest a significant amount of time to either re-establish our mouse model of IBD or modify the original protocol to produce a more consistent model of chronic colitis before proceeding with our *in vivo* studies. In fact, the studies proposed in Tasks 1 and 2 are currently ongoing with the first results expected in mid-September 2015. We will forward these new data when they become available. Preliminary data in our original application demonstrated proof of concept that weekly injections (i.p.) of 5 million hMSCs for six weeks following T cell transfer significantly suppressed the development of chronic colitis in our mouse model of IBD. However, new clinical information has become available over the past year suggesting that the "dose" of MSCs we used in our preliminary studies (166 million/kg for a 30 gram mouse) is excessive and will never be used in the clinics to treat human IBD. Current clinical studies are using either "low dose" (2 million/kg) or "high dose" (8 million/kg) hMSCs to treat autoimmune and chronic inflammatory diseases (http://clinicaltrials.gov/show/NCT00294112). Furthermore, *intraperitoneal* administration of human MSCs will never be used in patients with IBD. In view of these clinical realities, our current, ongoing studies utilizes *intravenous* administration of clinically-relevant numbers of hMSCs to assess their therapeutic efficacy in our mouse model of IBD.

Task 3. Define the immuno-regulatory mechanisms utilized by MSCs to attenuate chronic colitis

To investigate the cellular and immunological interactions that occur between *human* MSCs and *mouse* immune cells in our mouse model of IBD, we have developed a novel xenogeneic *in vitro* system to quantify these interactions in a more controlled and immunologically-relevant environment. This is a very important, yet under-appreciated aspect of MSC therapeutic research in which *human* MSCs are administered to mice with acute or chronic inflammation. The vast majority of published studies that utilize hMSCs to treat mice or rats with chronic inflammation have used syngeneic *in vitro* assays to model the *in vivo* studies. That is, investigators have assessed the ability of *human* MSCs to suppress activation of *human* lymphocytes (e.g. T and B cells) *in vitro*. We believe this may be a critical shortcoming in interpreting results obtained from these studies because the more immunologically-relevant *in vitro* system would assess the immuno-modulatory effects of hMSCs on *mouse* immune cell function. As reported in our previous Progress Report, we demonstrated that hMSCs are capable of suppressing proliferation of mouse T cells activated with CD3 monoclonal antibody (mAb). While CD3 mAb-mediated proliferation of T cells *in vitro* has been used for many years to mimic lymphocyte activation *in vivo*, investigators agree that the mechanisms responsible for CD3 mAb-induced T cell activation/proliferation may be very different than those that occur

during *antigen-specific* activation *in vitro and in vivo*. Over the past year, we have assessed a number of different *in vitro* systems to more closely recapitulate the cellular and immunological interactions that would occur *in vivo* between mouse immune cells and hMSCs in our mouse model of IBD. These studies have led to the development of a novel, antigen-specific and immunologically-relevant assay system that quantifies immmuno-modulatory activity of *human* MSCs towards *mouse* immune cells. For this new assay, we activate mouse splenocytes obtained from OT2 transgenic *(tg)* mice with ovalbumin peptide (OVA) and quantify T cell proliferation *in vitro.* The T cell receptors (TCR) on $CD4^+$ T cells in OT2 tg mice recognize only OVA presented by the major histocompatibility complex II (MHC II) expressed on *mouse* antigen presenting cells (APCs; dendritic cells, macrophages). Thus T cell activation/proliferation in this novel xenogeneic assay is *antigen-specific*. The utility of this new system can be seen in Figure 3 in which we observe a potent, dose-dependent suppression of T cell proliferation by regulatory T cells (Tregs) flow purified from the spleens of OT2 mice. This antigen-specific assay system will have great utility and be very important for us as we will be able to quantify the immunosuppressive activity of virtually any mouse or human MSC clone. Surprisingly, when we activated mouse OT2 splenocytes with OVA in the presence of increasing numbers of *un-manipulated* or *irradiated* hMSCs, we observe little or no suppression of T cell proliferation (Figures 4 and 5).

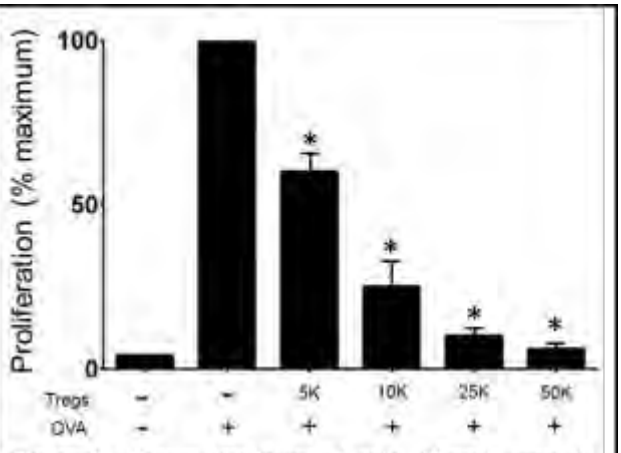

Figure 3. Mouse regulatory T cells (Tregs) potently suppress antigen-specific T cell proliferation. Mouse splenocytes ($5x10^4$/well; 50K) obtained from OT-2 transgenic mice were added to each well in the presence or absence of varying numbers of freshly isolated mouse OT2 regulatory T cells (Tregs). Antigen-specific activation of $CD4^+$ T-cells was induced by addition of ovalbumin peptide (OVA; 10µg/ml). Following a 72 hr incubation period, T cell proliferation was quantified by pulsing each well with ^{3}H-thymidine for the last 16 hrs of incubation. Data represent the mean ± SEM for triplicate determinations. P<0.05 vs. [–Tregs+OVA]

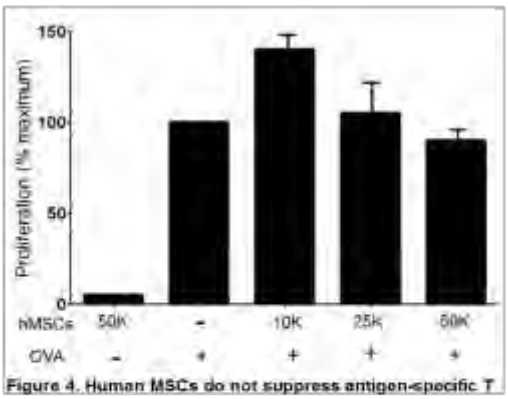

Figure 4. Human MSCs do not suppress antigen-specific T cell proliferation. Mouse splenocytes (50K) obtained from OT-2 transgenic mice were added to each well in the presence or absence of freshly isolated, un-manipulated human MSCs (hMSCs). Antigen-specific activation of $CD4^+$ T-cells was induced by addition of ovalbumin peptide (OVA; 10µg/ml). Following a 72 hr incubation period, T cell proliferation was quantified by pulsing each well with ^{3}H-thymidine for the last 16 hrs of incubation. Data represent the mean ± SEM for triplicate determinations.

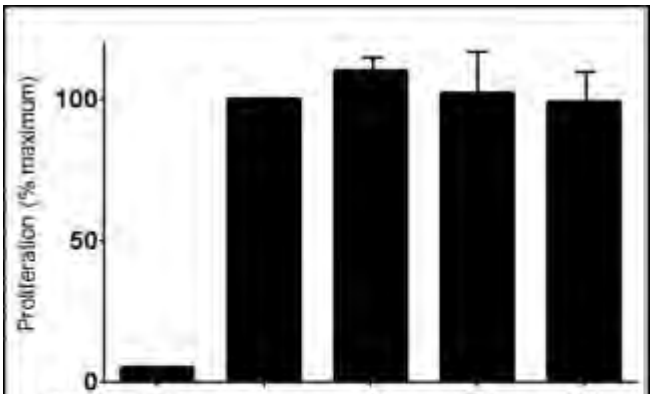

Figure 5. *Irradiated* human MSCs do not suppress antigen-specific T-cell proliferation. OT-2 splenocytes were incubated in the presence or absence of irradiated human MSCs (hMSCs) as described in previous figures. T cell proliferation was quantified using ^{3}H-thymidine incorporation as described. Data represent the mean ± SEM for triplicate determinations.

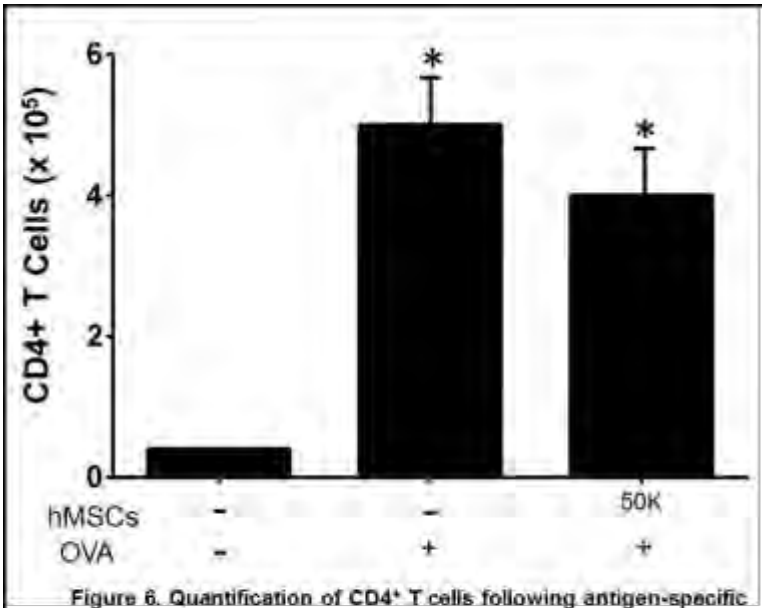

Figure 6. Quantification of CD4⁺ T cells following antigen-specific activation. OT-2 splenocytes were incubated in the presence or absence of irradiated human MSCs (hMSCs) as described in previous figures. Following the 72 hr. incubation, cells were stained with fluorescently-labeled CD3 and CD4 monoclonal antibodies and T cell number was quantified by flow cytometry. Data represent the mean ± SEM for triplicate determinations. P<0.05 vs. [-hMSCs-OVA].

We confirmed that the lack of suppressive activity was not due an artifact with using ^{3}H-thymidine incorporation to quantify cell proliferation as flow cytometric analyses revealed no significant reductions in $CD4^{+}$ T cell proliferation (Figure 6).

The lack of suppression of T cell proliferation appeared to be specific for hMSCs as we demonstrated that mouse MSCs (also derived from bone marrow) were very effective in suppressing OVA-induced T cell proliferation (Figure 7). These data contrast with those presented in our previous Progress Report where we show dose-dependent suppression of CD3 mAb-mediated T cell proliferation by hMSCs. Taken together with our previous data demonstrating suppression of CD3 mAb-induced proliferation mouse T cells by hMSCs suggest that the signaling pathways involved in T cell proliferation induced by nonspecific crosslinking of the TCR (via CD3 mAb) may be very different than those induced in an *antigen-specific* manner. Indeed, these differences may be critical for defining the mechanisms by which hMSCs suppress IBD *in vivo*.

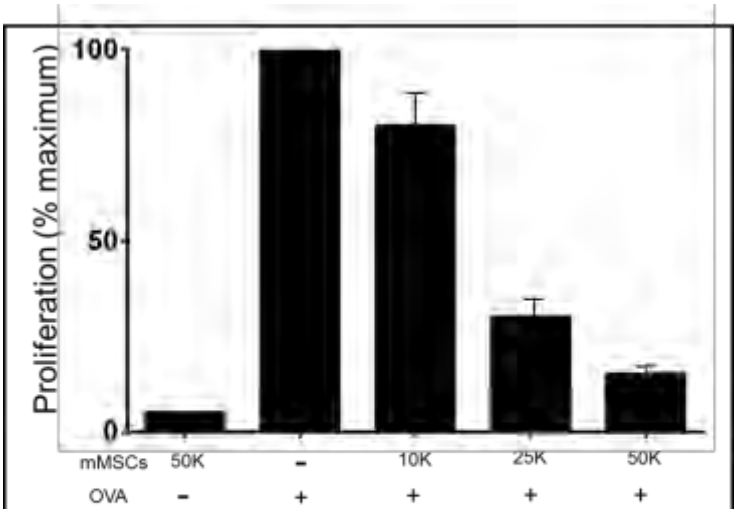

Figure 7. *Mouse* MSCs suppress antigen-specific T cell proliferation. OT-2 splenocytes were activated with OVA in the presence or absence of varying numbers of ***mouse*** MSCs as described in previous figures. T cell proliferation was quantified using ^{3}H-thymidine incorporation. Data represent the mean±SEM for triplicate determinations.

Opportunities for Training and Professional Development

Although the primary purpose of this application was not to provide training and professional development, my graduate student and postdoctoral fellow have received extensive training in immunology, use of mouse models of IBD and flow cytometry.

Dissemination of Results

Because of the time required to develop and characterize our new mouse model of IBD at our new institution, we have not, until recently, been able to fully implement the *in vivo* studies outlined in Tasks 1 and 2. However, we have presented our new model data in a departmental seminar and at the national MHSRS meeting that was held August 17-20, 2015 in Ft. Lauderdale, FL.

Plans for the Next Funding Period

We plan to devote our full attention to Tasks 1 and 2 for the next funding period. We plan to determine the therapeutic efficacy of clinically-relevant numbers of hMSCs in suppressing the development of chronic gut inflammation. In addition, we plan to assess the ability of hMSCs to reverse or attenuate preexisting disease in mouse model of IBD. Finally, we will initiate studies to assess the role of MLNs in hMSC-mediated suppression of disease.

4. Impact

Impact on discipline

We believe that data generated from our proposed studies will impact greatly those investigators using mouse models of IBD to evaluate therapeutic efficacy of different biologics and cell-based therapies. Our studies will represent the first studies to assess the ability of hMSCs to suppress chronic gut inflammation in a mouse model of IBD. All previous and current studies have used acute, self-limiting models of chemically induced IBD and hMSCs or have used the T cell transfer model of chronic colitis with mouse MSCs. The immunoregulatory effects reported in studies using MSCs have varied greatly with studies demonstrating protective effects while others have shown no effect or even exacerbation of colitis in different mouse models of IBD. In addition, data generated from studies that will be performed during current funding cycle will greatly impact how investigators perform and interpret their *in vitro* data describing the immunosuppressive properties of hMSCs in mouse models of autoimmune and chronic inflammatory diseases. The vast majority of published studies using human MSCs in mouse models of disease have not examined directly the immuno-regulatory properties of *human* MSCs towards *mouse* immune cells *in vitro*. We believe that it is critical to model the interactions between MSCs and immune cells in the more immunologically-relevant system using *human* MSCs and *mouse* immune cells. Data generated in the current funding cycle together with those present in our previous Progress Report describing the how activation of hMSCs by human or mouse inflammatory cytokines induce the dramatic up-regulation of certain immunosuppressive mediators that have not been interrogated in animal models of chronic inflammation will also have a major impact in the field of MSC therapy. Our studies may reveal new therapeutic targets for drug development.

Impact on other disciplines
Nothing to report

Impact on technology transfer
Nothing to report

Impact on society
Nothing to report

5. Changes/Problems

Changes in Approach
No major changes. Based upon new data from clinical studies using human MSCs in the treatment of different inflammatory diseases, we will concentrate our efforts on evaluating the immunosuppressive properties of *clinically-relevant* doses of human MSCs.

Problems and Delays
Because of the time required to develop and characterized our new mouse model of IBD, we were not able to fully implement the *in vivo* studies outlined in Tasks 1 and 2. We have however recently initiated the first of these studies with our new and improved model of IBD.

Changes that had a significant impact on expenditures
No major impact on expenditures

Changes in use of vertebrate animals
We have reestablished our mouse model of IBD.

6. Products

Publications
Koboziev I, Jones-Hall Y, Valentine JF, Reinoso Webb C, Furr KL, Grisham MB; Use of Humanized Mice to Study the Pathogenesis of Autoimmune and Inflammatory Diseases. Inflamm Bowel Dis. 2015 Jul;21(7):1652-73.

Fang K, Grisham MB, Kevil CG. Application of Comparative Transcriptional Genomics to Identify Molecular Targets for Pediatric IBD; Front Immunol. 2015 Apr 8;6:165.

Presentations
Data generated from these studies have been presented as seminars at Texas Tech University Health Sciences Center, Georgia Reagents University, Vanderbilt University and East Carolina University. I have also presented some of this work as part of a symposium at the American Gastroenterological Association at Digestive Diseases Week in Washington, DC (May 2015).

7. Participants and Other Collaborators

Individuals working on project

Name: Matthew Grisham, PhD
Project Role: PI
Nearest person months: 4 months
Contribution: Dr. Grisham is involved in designing, implementing and interpreting the experiments
Funding support:

Name: Iurii Koboziev, PhD
Project Role: Postdoctoral fellow
Nearest person months: 6 months
Contribution: Dr. Koboziev performs all cell preparations, molecular and *in vitro* assays and assists with the flow cytometry studies and animal model
Funding Support:

Name: Kathryn Furr, MS
Project Role: Laboratory Scientist
Nearest person months: 5 months
Contribution: Ms. Furr is my Lab Manager and Flow Cytometry Core Manager. She performs all flow cytometry experiments; She is generates the mouse model of IBD and assists with some of the MSC cell culture experiments.
Funding Support: TTUHSC/State Account

8. Special Reporting Requirements

Nothing to report

9. Appendices