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14. ABSTRACT

Colorectal adenomas are the precursors of colorectal cancer (CRC). The key question is what allows benign adenomas to grow and to eventually become cancerous. Understanding the molecular mechanisms underlying colorectal adenoma formation and growth would offer tremendous opportunities for CRC prevention and interception. The nuclear hormone receptor PPARδ is a transcription factor that is involved in fatty acid metabolism, obesity, wound healing, inflammation, and cancer. However, the role of PPARδ in colorectal adenoma biology and progression remains unclear. Particularly, the role of PPARδ in tumor immune evasion is unknown. Since we found that both PD-1 and PD-L1 promoters contain potential PPARδ binding sites and our preliminary *in vitro* data show that activation of PPARδ induces PD-L1 expression in colorectal tumor cells and PD-1 expression in $CD8^+$ T cells and macrophages, we hypothesize that PPARδ induction of PD-L1 in colon tumor epithelial cells induces their resistance to $CD8^+$ T cell cytotoxicity (Aim 1) and activation of PPARδ induces tumor immune evasion by suppressing colorectal $CD8^+$ T cell cytotoxicity and inhibiting macrophage phagocytosis via induction of PD-1 (Aim 2). Since certain fatty acids and fatty acid derivatives are natural ligands of PPARδ, we postulate that PPARδ mediates the effect of dietary fats on PD-L1 in tumor cells and PD-1 in $CD8^+$ T cells and macrophages. This proposal will provide a rationale for developing PPARδ antagonists as agents to target immune checkpoint pathways and to enhance the efficacy of checkpoint inhibitors in treatment of CRC patients.

15. SUBJECT TERMS Colorectal Cancer (CRC), tumor immune evasion, peroxisome proliferator-activated receptor δ (PPARδ), immune checkpoint receptors, PPRE: PPAR response element, PD-1: programmed cell death protein 1, PD-L1: programmed death-ligand 1, $CD8^+$ T cell cytotoxicity, macrophage phagocytosis, high-fat diets.

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1. Introduction

Colorectal cancer (CRC) is the third most common malignancy and the second leading cause of cancer-related deaths in the USA. CRC is also the second most common cancer among USA veterans. However, colonoscopy screening is an effective way to detect and prevent CRC by removing polyps before malignant conversion, over fifty percent of patients still present to their physician with advanced disease (stage III-IV). Unfortunately, existing therapies have limited efficacy, especially for stage 4 disease. Understanding the molecular mechanisms underlying CRC formation and growth would offer tremendous opportunities for CRC prevention and interception. Certain lifestyles, such as high intake of the Western pattern diet (WPD), are also a risk factor for cancer, including CRC. WPD often contains high saturated fat, red, and processed meat, with an excess of total calories. However, how dietary fat intake contributes to CRC remains elusive. The nuclear hormone receptor peroxisome proliferator-activated receptor δ (PPARδ) is a transcription factor involved in fatty acid metabolism, obesity, wound healing, inflammation, and cancer. Despite decades of research, the role of PPARδ in CRC remains unclear. Our group and others have investigated the role of PPARδ in normal and tumor intestinal epithelial cells. However, the role of PPARδ in tumor immune evasion is unknown. In addition, it is not fully understood how immune checkpoint molecules such as PD-1 (an immune checkpoint receptor) and its ligand PD-L1 are regulated in the tumor microenvironment. Since we found that both PD-1 and PD-L1 promoters contain potential PPARδ binding sites and our preliminary *in vitro* data show that activation of PPARδ induces PD-L1 expression via binding to the PD-L1 promoter in colorectal tumor cells and PD-1 expression in $CD8^+$ T cells and macrophages via binding to the PD-1 promoter, we hypothesize that PPARδ induction of PD-L1 in colon tumor epithelial cells induces their resistance to $CD8^+$ T cell cytotoxicity (Aim 1) and activation of PPARδ induces tumor immune evasion by suppressing colorectal $CD8^+$ T cell cytotoxicity and inhibiting macrophage phagocytosis via induction of PD-1 (Aim 2). In addition, our preliminary results reveal that a high-fat diet promotes intestinal adenoma burden and induction of PD-L1 expression in tumor epithelial cells.

Since certain fatty acids and fatty acid derivatives are natural ligands of PPARδ, we postulate that PPARδ mediates the effect of dietary fats on PD-L1 in tumor cells and PD-1 in $CD8^+$ T cells and macrophages. **Aim 1** and **Aim 2** are designed to address these questions. The results from this proposal will not only uncover a previously unrecognized role of PPARδ in affecting tumor immune evasion but also reveal a potential mechanism underlying the contribution of dietary fat to CRC. For long-term studies, our findings will provide a rationale for developing PPARδ antagonists as agents to target immune checkpoint pathways and to enhance the efficacy of checkpoint inhibitors in the treatment of CRC patients, including military personnel, veterans, and their families. Our two aims are outlined below.

Aim 1: To determine whether the PPARδ-PD-L1 pathway mediates the effect of dietary fats on the induction of tumor cell resistance to $CD8^+$ T cell cytotoxicity

Aim 2: To examine whether the PPARδ-PD-1 pathway mediates the effect of dietary fats on suppressing $CD8^+$ T cell cytotoxicity and macrophage phagocytosis against tumor cells

2. Keywords

Colorectal Cancer (CRC), tumor immune evasion, peroxisome proliferator-activated receptor δ (PPARδ), immune checkpoint receptors, PPRE: PPAR response element, PD-1: programmed cell death protein 1, PD-L1: programmed death-ligand 1, $CD8^+$ T cell cytotoxicity, macrophage phagocytosis, high-fat diets.

3. Accomplishments

- **What were the major goals of the project?**

Specific Aim 1	Timeline	Site
Aim 1. To determine whether the PPARδ-PD-L1 pathway mediates the effect of dietary fats on induction of tumor cell resistance to $CD8^+$ T cell cytotoxicity		
Major Task 1A: To assess whether activation of PPARδ induces tumor cell resistance to $CD8^+$ T cell cytotoxicity via PD-L1 *in vitro*	Months 1-12	DuBois
Subtask 1: Generation of PD-L1 promoter and mutant PD-L1 promoters and evaluation of PD-L1 promoter activity after PPARδ activation in CRC cells	Months 1-3	DuBois

Subtask 2: Evaluation of tumor epithelial cell resistance to $CD8^+$ T cell cytotoxicity *in vitro* in CRC cells with or without PPARδ or PD-L1 knockdown after PPARδ activation	Months 4-12	DuBois
Milestone Achieved: We expect that 1) treatment of tumor cells with GW501516 will increase their resistance to $CD8^+$ T cell cytotoxicity, 2) knockout or knockdown of PPARδ will block the effect of GW501516 on tumor cell resistance to $CD8^+$ T cell cytotoxicity, and 3) inhibition of PD-L1 by treatment with a neutralizing antibody or knockdown will inhibit the effect of PPARδ activation on tumor cell resistance to $CD8^+$ T cell cytotoxicity. The expected results will demonstrate that activation of PPARδ induces tumor cell resistance to $CD8^+$ T cell cytotoxicity via induction of PD-L1 *in vitro*.		
Major Task 1B: To assess whether the PPARδ-PD-L1 pathway mediates the effect of dietary fats on induction of tumor cell resistance to $CD8^+$ T cell cytotoxicity	Months 1-24	DuBois
Subtask 1: Generation of 84 *Ppard$^{fl/fl}$/Apc$^{fl/fl}$/CDX2-Cre* mice and 84 their control littermates (*Ppard$^{+/+}$/Apc$^{fl/fl}$/CDX2-Cre*) as well as 84 *PD-L1$^{fl/fl}$/Apc$^{fl/fl}$/CDX2-cre* mice and 84 their control littermates (*PD-L1$^{+/+}$/Apc$^{fl/fl}$/CDX2-cre*)	Months 1-12	DuBois
Subtask 2: To determine whether PPARδ mediates the effect of its agonist (GW501516) or a high-fat diet on induction of tumor burden (measurement of tumor number and size), PD-L1 expression in colorectal adenoma epithelial cells (isolation of cells from tumors and analyses of $PD\text{-}1^+$ cells by Flow Cytometry), and tumor cell resistance to $CD8^+$ T cell cytotoxicity in *Apc$^{fl/fl}$/CDX2-Cre* mice (isolation of cells tumor epithelial cells and measurement of their resistance to $CD8^+$ T cell cytotoxicity	Months 13-18	DuBois
Subtask 3: To determine whether PD-L1 mediates the effect of PPARδ or a high-fat diet on tumor burden and tumor cell resistance to $CD8^+$ T cell cytotoxicity	Months 18-24	DuBois
Milestone Achieved: We expect that 1) GW501516 or the high-fat diet will induce PD-L1 expression in tumor epithelial cells and enhance tumor cell resistance to $CD8^+$ T cell cytotoxicity accompanied with an increase of colorectal tumor burden and 2) deletion of either PPARδ or PD-L1 will attenuate the effect of GW501516 or the high-fat diet on colorectal tumor burden, PD-L1 expression in tumor epithelial cells, and tumor cell resistance to $CD8^+$ T cell cytotoxicity. The expected results will demonstrate that activation of PPARδ accelerates colorectal tumor growth, in part, by inducing tumor cell resistance to $CD8^+$ T cell cytotoxicity via induction of PD-L1 and PPARδ mediates the effect of a high-fat diet on tumor cell resistance to $CD8^+$ T cell cytotoxicity.		
Specific Aim 2	**Timeline**	**Site**
Aim 2: To examine whether the PPARδ-PD-1 pathway mediates the effect of dietary fats on suppressing $CD8^+$ T cell cytotoxicity and macrophage phagocytosis against tumor cells		
Major Task 2A: To determine whether activation of PPARδ suppresses $CD8^+$ T cell cytotoxicity and macrophage phagocytosis via induction of PD-1 *in vitro*	Months 1-12	DuBois
Subtask 1: Generation of PD-1 promoter and mutant PD-1 promoters and evaluation of PD-L1 promoter activity after PPARδ activation in $CD8^+$ T cells and macrophages	Months 1-3	DuBois
Subtask 2: Evaluation of the effect of PPARδ on $CD8^+$ T cell cytotoxicity against tumor cells *in vitro*	Months 4-8	DuBois
Subtask 3: Evaluation of the effect of PPARδ on macrophage phagocytosis against tumor cells *in vitro*	Months 4-8	DuBois
Subtask 4: To examine whether PD-1 mediates the effect of PPARδ on $CD8^+$ T cell cytotoxicity and macrophage phagocytosis against tumor cells *in vitro*	Months 9-12	DuBois
Milestone Achieved: We expect that 1) treatment of $CD8^+$ T cells and macrophages with GW501516 will suppresses $CD8^+$ T cell cytotoxicity against tumor cells and impairs macrophage phagocytosis against tumor cells, 2) knockout or knockdown of PPARδ will block the effect of GW501516 on $CD8^+$ T cell cytotoxicity and macrophage phagocytosis, and 3) inhibition of PD-1 by its neutralizing antibody or knockout will inhibit the effect of PPARδ on $CD8^+$ T cell cytotoxicity and macrophage phagocytosis. The expected results will demonstrate that activation of PPARδ suppress $CD8^+$ T cell cytotoxicity and macrophage phagocytosis		

against tumor cells by induction of PD-1.		
Major Task 2B: To determine whether PPARδ mediates the effect of GW501516 or a high-fat diet on induction of PD-1 in colonic CD8$^+$ T cells and macrophages and promotion of tumor immune evasion	Months 1-24	
Subtask 1: Generation of 84 *Ppard$^{fl/fl}$/Apc$^{fl/fl}$/CDX2-Cre* mice and 84 their control littermates (*Ppard$^{+/+}$/Apc$^{fl/fl}$/CDX2-Cre*)	Months 1-12	DuBois
Subtask 2: To examine whether GW501516 and a high-fat diet induces PD-1 expression in colorectal CD8$^+$ T cells and macrophages (isolation of immune cells from tumors and matched normal tissues and analyses of PD-1$^+$ cells in total CD8$^+$ T cells and macrophages by Flow Cytometry) and reduces colorectal CD8$^+$ T cell abundance and cytotoxicity (isolation of CD8$^+$ T cells from colon tumors and matched normal tissues and measurement of their cytotoxicity against tumor cells) and macrophage phagocytosis (isolation of macrophages from colon tumors and matched normal tissues and measurement of their phagocytosis against tumor cells) accompanied with promotion of colonic adenoma burden and if PPARδ mediates the effects of GW501516 and a high-fat diet in *Apc$^{fl/fl}$/CDX2-Cre* mice	Months 18-24	DuBois
Milestone Achieved: We expect that GW501516 or a high-fat diet will induce PD-1 expression in colorectal CD8$^+$ T cells and macrophages, reduces colorectal CD8$^+$ T cell abundance and cytotoxicity (isolation of CD8$^+$ T cells from colon tumors and matched normal tissues and measurement of their cytotoxicity against tumor cells), suppresses colorectal macrophage phagocytosis in both tumors and matched normal tissues, and promotes colorectal adenoma burden. We also expect that deletion of PPARδ will attenuate the effects of GW501516 and a high-fat diet. The expected results will demonstrate that activation of PPARδ or a high-fat diet suppresses colorectal CD8$^+$ T cell cytotoxicity and macrophage phagocytosis via reduction of PD-1 and PPARδ mediates the effects of a high-fat diet.		
Major Task 2C: To determine whether PD-1 in CD8$^+$ T cells is required for PPARδ- or dietary fats-induced tumor immune evasion	Months 13-30	DuBois
Subtask 1: Generation of 84 *PD-1$^{fl/fl}$/Cd8a-Cre/Apc$^{Min/+}$* mice and 84 their control littermates (*PD-1$^{+/+}$/Cd8a-Cre/Apc$^{Min/+}$*)	Months 13-24	DuBois
Subtask 2: To assess whether deletion of PD-1 in CD8$^+$ T cells attenuates the effect of PPARδ or a high-fat diet on CD8$^+$ T cytotoxicity and adenoma burden in *Apc$^{fl/fl}$/CDX2-Cre* mice	Months 25-30	DuBois
Milestone Achieved: We expect that deletion of PD-1 in CD8$^+$ T cells will attenuate the effect of PPARδ activation or the high-fat diet on tumor burden and tumor-infiltrating and normal tissue-infiltrating CD8$^+$ T cell cytotoxicity and abundance. The expected results will demonstrate that activation of PPARδ accelerates colorectal adenoma formation by suppressing CD8$^+$ T cell cytotoxicity against transformed epithelial cells in normal tissues and promotes colorectal adenoma growth by suppressing tumor-associated CD8$^+$ T cell cytotoxicity against tumor cells via induction of PD-1. These results will also demonstrate that the PPARδ-PD-1 pathway mediates the effect of a high-fat diet on tumor burden and CD8$^+$ T cell cytotoxicity.		
Major Task 2D: To determine whether PD-1 in macrophages is required for PPARδ- or dietary fats-induced tumor immune evasion	Months 13-36	DuBois
Subtask 1: Generation of 84 *PD-1$^{fl/fl}$/LysM-CreER^{T2}/Apc$^{Min/+}$* mice and 84 their control littermates (*PD-1$^{+/+}$/LysM-CreER^{T2}/Apc$^{Min/+}$*)	Months 13-24	DuBois
Subtask 2: To determine whether deletion of PD-1 in macrophages attenuates the effect of PPARδ or a high-fat diet on macrophage phagocytosis and adenoma burden	Months 30-36	DuBois
Milestone Achieved: We expect that deletion of PD-1 in macrophages will attenuate the effect of PPARδ activation or the high-fat diet on tumor burden and tumor-infiltrating and normal tissue-infiltrating macrophage phagocytosis. The expected results will demonstrate that activation of PPARδ promotes colorectal adenoma formation by suppressing macrophage phagocytosis against transformed epithelial cells in normal tissues and accelerates colorectal adenoma growth by suppressing TAM phagocytosis against tumor cells via induction of PD-1. These results will also demonstrate that the PPARδ-PD-1 pathway mediates the effect of a high-fat diet on tumor burden and macrophage phagocytosis.		

- **What was accomplished under these goals?**

Major Task 1A: To assess whether activation of PPARδ induces tumor cell resistance to CD8$^+$ T cell cytotoxicity via PD-L1 *in vitro*

Subtask 1: Generation of PD-L1 promoter and mutant PD-L1 promoters and evaluation of PD-L1 promoter activity after PPARδ activation in CRC cells

To examine whether activation of PPARδ induces PD-L1 promoter activity and whether mutations in either of the PPARδ binding sites in the PD-L1 promoter lead to a loss of responsiveness to PPARδ activation, we generated a PD-L1 promoter luciferase vector by inserting a human PD-L1 promoter (-520 to +20) into pGL3 luciferase reporter vector. In addition, mutant PD-L1 promoter luciferase vectors were generated by the deletion of the PPRE1 or PPRE2 element. Then we performed PD-L1 promoter luciferase report assays. As shown in Fig. 1, treatment of a PPARδ agonist (GW501516) resulted in the induction of PD-L1 transcription compared to vehicle in LS-174T cells. In contrast, the deletion of PPRE1 in the PD-L1 promoter luciferase vector, but not the deletion of PPRE2, attenuated the effect of GW501516 on the induction of PD-L1 transcription. These results demonstrated that activation of PPARδ induces PD-L1 transcription via directly binding to the PPRE1 of the PD-L1 promoter resulting in the initiation of its expression. Thus, we have completed this subtask.

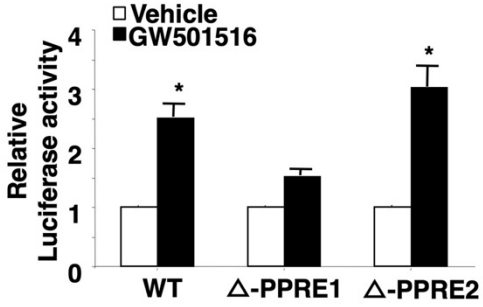

Fig. 1. Activation of PPARδ stimulates PD-L1 promoter activity by binding to PPRE1 in LS-174T cells. The LS-174T cells were transiently transfected with WT or mutant PD-L1 luciferase reporter vector plus pRL-SV40 plasmid followed by treatment with GW501516 (1μM) for 24 h. The dual-luciferase assays were performed using Duel-luciferase report assay kit. Data are represented as the mean ± SE of relative luciferase activity from three independent experiments. * indicates p<0.05.

Subtask 2: Evaluation of tumor epithelial cell resistance to CD8$^+$ T cell cytotoxicity *in vitro* in CRC cells with or without PPARδ or PD-L1 knockdown after PPARδ activation

To further examine whether PPARδ-induced PD-L1 expression results in tumor epithelial cell resistance to CD8$^+$ T cell cytotoxicity *in vitro*, we first evaluated whether PPARδ is required for colon tumor epithelial cell resistance to CD8$^+$ T cell cytotoxicity by performing assays for CD8$^+$ T cell cytotoxicity against CRC cells. HCT-116/WT, HCT-116/*Ppard*$^{-/-}$, LS-174T/vector, or LS-174T/shPPARδ cells were co-cultured with human activated CD8$^+$ T cells at a ratio (E:T=1:1) for 18 hr. ACCORDING TO THE MANUFACTURER'S INSTRUCTIONS, CD8+ T cells were isolated from human PBMC using a human CD8+ T Cell Isolation Kit (Miltenyi Biotec Inc.). CD8$^+$ T cells were then activated by adding anti-CD3/CD28 hAb-coated beads (Dynabeads Human T-Activator CD3/CD28, Gibco) according to the manufacturer's instructions. Tumor cell lysis by activated CD8$^+$ T cells was measured using CytoTox96 Non-Radioactive Cytotoxicity Assay kit (Promega) according to the manufacturer's instructions. Our results showed that deletion or knockdown of PPARδ led to induction of CD8$^+$ T cell cytotoxicity against CRC cells (Fig. 2A), demonstrating that deletion or knockdown of PPARδ in CRC cells resulted in a reduction of their resistance to CD8$^+$ T cell cytotoxicity. Next, we determined whether PD-L1 mediates the effect of PPARδ on CRC cell resistance to CD8$^+$ T cell cytotoxicity. Indeed, a PD-L1 antibody (Atezolizumab) attenuated the effect of PPARδ on the induction of CRC cell resistance to CD8$^+$ T cell cytotoxicity (Fig. 2B), demonstrating that PD-L1 mediates the effect of PPARδ on CRC cell resistance to CD8$^+$ T cell cytotoxicity. Collectively, these results demonstrate that

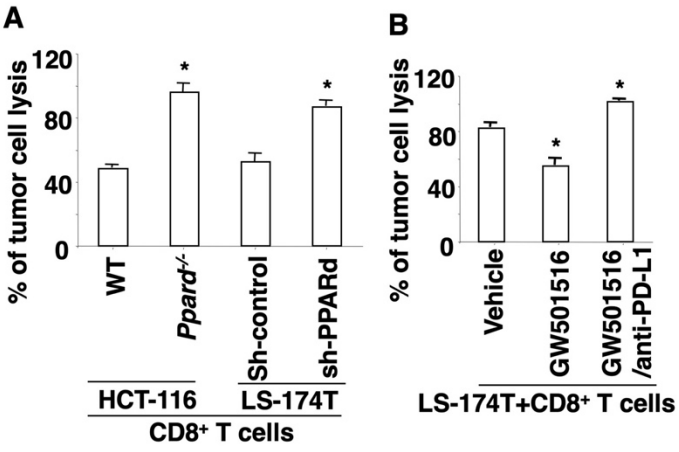

Fig. 2. Activation of PPARδ induces tumor cell resistance to CD8$^+$ T cell cytotoxicity via induction of PD-L1. (A) Indicated CRC cells were co-cultured with human activated CD8$^+$ T cells at a ratio (E:T=1:1) for 18 hr. (B) LS-174T cells were treated with vehicle, GW501516 (1μM), and/or anti-PD-L1 (10μg/ml) for 8 hr. After treatment, LS-174T cells were co-cultured with human activated CD8$^+$ T cells at a ratio (E:T=1:1) for 18 hr. After co-culture, percentage of tumor cell lysis were measured. Data are represented as the mean ± SE of percentage of tumor cell lysis from three independent experiments. * indicates p<0.05.

activation of PPARδ induces tumor cell resistance to $CD8^+$ T cell cytotoxicity via induction of PD-L1 *in vitro*. This subtask has been completed.

Major Task 1B: To assess whether the PPARδ-PD-L1 pathway mediates the effect of dietary fats on the induction of tumor cell resistance to $CD8^+$ T cell cytotoxicity

Subtask 1: Generation of 84 *Ppard$^{fl/fl}$/Apc$^{fl/fl}$/CDX2-Cre* mice and 84 their control littermates (*Ppard$^{+/+}$/Apc$^{fl/fl}$/CDX2-Cre*) as well as 84 *PD-L1$^{fl/fl}$/Apc$^{fl/fl}$/CDX2-cre* mice and 84 their control littermates (*PD-L1$^{+/+}$/Apc$^{fl/fl}$/CDX2-cre*)

We have generated *Ppard$^{fl/fl}$/Apc$^{fl/fl}$/CDX2-Cre* mice and their control littermates (*Ppard$^{+/+}$/Apc$^{fl/fl}$/CDX2-Cre*) mice by conventionally crossing *Apc$^{fl/fl}$/CDX2-Cre* on C57BL/6J genetic background with *Ppard$^{fl/fl}$* mice (*Ppard^{tm1Rev}/J*) mice on a C57BL/6J genetic background. In addition, *PD-L1$^{fl/fl}$/Apc$^{fl/fl}$/CDX2-are* and their control littermates (*PD-L1$^{+/+}$/Apc$^{fl/fl}$/CDX2-cre*) mice were also generated by conventionally crossing *Apc$^{fl/fl}$/CDX2-Cre* mice with *PD-L1$^{fl/fl}$* mice on C57BL/6J genetic background. Thus this subtask has been completed.

Subtask 2: To determine whether PPARδ mediates the effect of its agonist (GW501516) or a high-fat diet on induction of tumor burden, PD-L1 expression in colorectal adenoma epithelial cells (isolation of cells from tumors and analyses of $PD\text{-}1^+$ cells by Flow Cytometry), and tumor cell resistance to $CD8^+$ T cell cytotoxicity in *Apc$^{fl/fl}$/CDX2-Cre* mice (isolation of cells tumor epithelial cells and measurement of their resistance to $CD8^+$ T cell cytotoxicity)

Not Yet Initiated.

Subtask 3: To determine whether PD-L1 mediates the effect of PPARδ or a high-fat diet on tumor burden and tumor cell resistance to $CD8^+$ T cell cytotoxicity

Not Yet Initiated.

Major Task 2A: To determine whether activation of PPARδ suppresses $CD8^+$ T cell cytotoxicity and macrophage phagocytosis via induction of PD-1 *in vitro*

Subtask 1: Generation of PD-1 promoter and mutant PD-1 promoters and evaluation of PD-1 promoter activity after PPARδ activation in $CD8^+$ T cells and macrophages

To determine whether activation of PPARδ induces PD-1 promoter activity and if mutations in the PPARδ binding site of the PD-1 promoter lead to a loss of responsiveness to PPARδ activation, a PD-1 promoter luciferase vector was generated by inserting a human PD-1 promoter (-850 to +20) into pGL3 luciferase reporter vector. In addition, a mutant PD-1 promoter luciferase vector was generated by point mutations in only one PPRE element. Our results showed that treatment of TALL-104 cells (human leukemic $CD8^+$ T cells) with GW501516 increased PD-1 transcription in a dose-dependent manner (Fig. 3A). In contrast, mutant PD-1 promoter luciferase vector failed to respond to GW501516 induction of PD-1 transcription. Similar results in macrophages (IC-21) were obtained (data not shown). These results demonstrated that activation of PPARδ induces PD-1 transcription via directly binding to the PPRE of the PD-1 promoter resulting in the initiation of its expression. Thus, we have completed this subtask.

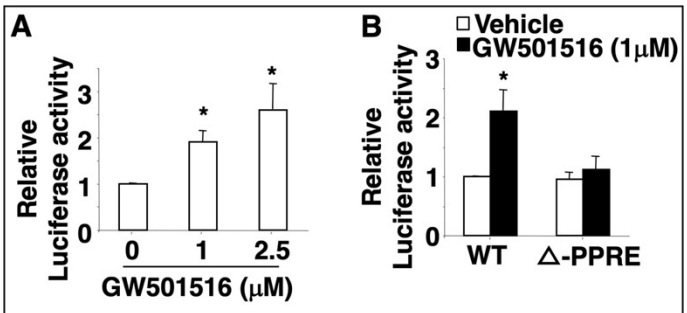

Fig. 3. Activation of PPARδ stimulates PD-1 promoter activity by binding to PPRE in TALL-104 cells. The TALL-104 cells were transiently transfected with WT or mutant PD-1 luciferase reporter vector plus pRL-SV40 plasmid followed by treatment with GW501516 for 24 h. The dual-luciferase assays were performed using Duel-luciferase report assay kit. Data are represented as the mean ± SE of relative luciferase activity from three independent experiments. * indicates $p<0.05$.

Subtask 2: Evaluation of the effect of PPARδ on $CD8^+$ T cell cytotoxicity against tumor cells *in vitro*

To further determine whether PPARδ is required for suppression of $CD8^+$ T cell cytotoxicity against CRC cells *in vitro*, HCT-116, LS-174T, HCA-7, or HT-29 cells were co-cultured with mouse-activated $CD8^+$ T cells at a ratio (E:T=1:1) for 18 hr. Mouse $CD8^+$ T cells were isolated from WT and PPAR□ knockout mice spleens using a mouse $CD8^+$ T Cell Isolation Kit (Miltenyi Biotec Inc.) according to the manufacturer's instructions. $CD8^+$ T cells were then activated by adding anti-CD3/CD28 mAb-coated beads (Dynabeads Mouse T-Activator CD3/CD28,

Gibco) according to the manufacturer's instructions. Tumor cell lysis by activated CD8$^+$ T cells was measured as described above. Our results showed that deletion of PPARδ in activated CD8$^+$ T cells resulted in the induction of their cytotoxicity against CRC cells (Fig. 4A), demonstrating that PPARδ is required for suppression of CD8$^+$ T cell cytotoxicity against CRC cells *in vitro*. To evaluate whether PPARδ mediated the effect of GW501516 on suppression of CD8$^+$ T cell cytotoxicity against CRC cells, mouse WT and PPARδ-deficient splenic activated CD8$^+$ T cells were treated with vehicle or GW501516 as described above. Following treatment, CD8$^+$ T cells were subjected to assays for measuring CD8$^+$ T cell cytotoxicity against tumor cells, as described above. The results showed that treatment of mouse splenic activated CD8$^+$ T cells with GW501516 led to suppression of CD8$^+$ T cell cytotoxicity against CRC cells. In contrast, treatment of mouse PPARδ-deficient splenic activated CD8$^+$ T cells with GW501516 failed to suppress CD8$^+$ T cell cytotoxicity against CRC cells (Fig. 4B), demonstrating that PPARδ is mediated the effect of GW501516 on suppression of CD8$^+$ T cell cytotoxicity against CRC cells. These results demonstrate that activation of PPARδ suppresses CD8$^+$ T cell cytotoxicity against CRC cells *in vitro*. This subtask has been completed.

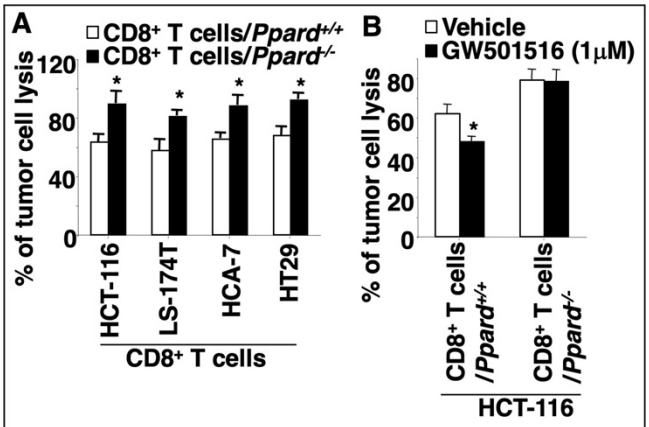

Fig. 4. Activation of PPARδ suppresses CD8$^+$ T cell cytotoxicity against CRC cells. (A) Indicated CRC cells were co-cultured with mouse activated CD8$^+$ T cells at a ratio (E:T=1:1) for 18 hr. (B) Indicated CD8$^+$ T cells were treated with vehicle or GW501516 for 8 hr. After treatment, CD8$^+$ T cells were cocultured with HCT-116 cells as described above. After co-culture, percentage of tumor cell lysis were measured. Data are represented as the mean ± SE of percentage of tumor cell lysis from three independent experiments. * indicates p<0.05.

Subtask 3: Evaluation of the effect of PPARδ on macrophage phagocytosis against tumor cells *in vitro*

Similar approaches, as described in the Subtask 2, were used to determine whether PPARδ is required for the suppression of macrophage phagocytosis against CRC cells and if PPARδ is mediated the effect of GW501516 on the suppression of macrophage phagocytosis against CRC cells. Mouse WT and PPARδ-deficient bone marrow-derived macrophages and IC-21/WT and IC21/shPPARδ were used to test their ability to phagocytose HCT-116 cells after GW501516 treatment. However, the results were not significant, although activation of PPARδ tended to suppress the ability of macrophages to phagocytose HCT-116 cells (data not shown). Now we are working on this subtask by using mouse colon adenocarcinoma cells to test phagocytosis of mouse WT and PPARδ-deficient bone marrow-derived macrophages as well as IC-21/WT and IC21/shPPARδ. We hope to complete this subtask in two months.

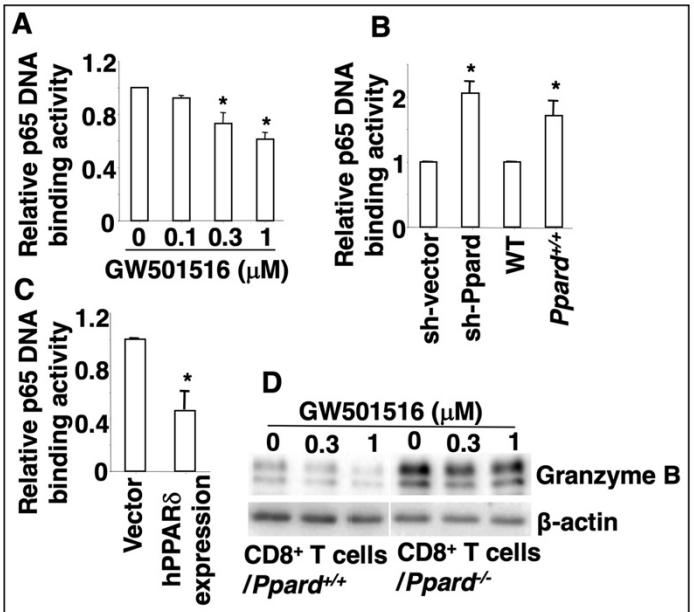

Fig. 5. Activation of PPARδ downregulates Granzyme B expression via inhibition of the DNA binding capacity of p65 NF-κB. (A) Human CD8$^+$ T cells were treated with GW501516 for 8 hr. The DNA binding activity of p65 NF-κB was measured. (B) The DNA binding activity of p65 NF-κB in human CD8$^+$ T cells/vector and CD8$^+$ T cells/sh-*Ppard* as well as mouse WT and PPARδ-deficient CD8$^+$ T cells was measured. (C) The DNA binding activity of p65 NF-κB in human CD8$^+$ T cells/vector and CD8$^+$ T cells/PPARd overexpression was measured. Data are represented as the mean ± SE of percentage of tumor cell lysis from three independent experiments. * indicates p<0.05. (D) Mouse WT and PPARδ-deficient CD8$^+$ T cells were treated with GW501516 for 8 hr. Granzyme B levels in these cells were measured by Western blot assays. The images are representative of three independent experiments.

Subtask 4: To examine whether PD-1 mediates the effect of PPARδ on CD8$^+$ T cell cytotoxicity and macrophage phagocytosis against tumor cells *in vitro*

We performed the experiments outlined in Aim 2A and found that PD-1 did not mediate the effect of PPARδ on CD8$^+$ T cell cytotoxicity. Then we investigated how activation of PPARδ suppresses CD8$^+$ T cell cytotoxicity. Previous studies revealed that NF-κB is required for the capacity of CD8$^+$ T cells to kill tumor

cells via induction of Granzyme B expression (PMID: 25648675, 16547254, and 12360212). It is also well established that one of the main functions of PPARδ in acute inflammation is to inhibit NF-κB activation by directly binding to p65 NF-κB to prevent the binding of p65 NF-κB to its target genes (PMID: 30927048). Based on these previously published results, we postulated that activation of PPARδ suppresses $CD8^+$ T cell cytotoxicity via an NF-κB-Granzyme B pathway. We first determined whether activation of PPARδ prevents the binding of p65 NF-κB to its consensus DNA binding site. Activated $CD8^+$ T cells from human PBMC were treated with GW501516 for 8 hr. After treatment, the DNA binding capacity of p65 NF-κB was measured using TransAM NF-κB (p65) kit (Active Motif). As shown in Fig. 5A, GW501516 treatment decreased the DNA binding capacity of p65 NF-κB in a dose-dependent manner.

Moreover, deletion or knockdown of PPARδ in mouse splenic or human-activated $CD8^+$ T cells increased the DNA binding capacity of p65 NF-κB (Fig. 5B). In contrast, overexpression of PPARδ in human-activated $CD8^+$ T cells decreased the DNA binding capacity of p65 NF-κB (Fig. 5C). These results demonstrate that activation of PPARδ prevents the binding of p65 NF-κB to its consensus DNA binding site. In addition, mouse PPARδ-deficient $CD8^+$ T cells had higher levels of Granzyme B than WT $CD8^+$ T cells (Fig. 5D). GW501516 treatment downregulated Granzyme B expression in mouse WT $CD8^+$ T cells, but not in mouse PPARδ-deficient $CD8^+$ T cells (Fig. 5D), demonstrated that PPARδ mediates the effect of GW501516 on Granzyme B expression. Collectively, these results indicate that activation of PPARδ suppresses $CD8^+$ T cell cytotoxicity by reduction of Granzyme B expression via inhibiting NF-κB activation. This subtask has been completed.

Major Task 2B: To determine whether PPARδ mediates the effect of GW501516 or a high-fat diet on the promotion of tumor immune evasion

Subtask 1: Generation of 84 *Ppard$^{fl/fl}$/Apc$^{fl/fl}$/CDX2-Cre* mice and 84 their control littermates (*Ppard$^{+/+}$/Apc$^{fl/fl}$/CDX2-Cre*)

This subtask has been completed (please see Subtask 1 of Major Task 1B).

Subtask 2: To examine whether GW501516 and a high-fat diet reduces Granzyme B expression in colorectal $CD8^+$ T cells and macrophages and reduces colorectal $CD8^+$ T cell abundance and cytotoxicity and macrophage phagocytosis accompanied with the promotion of colonic adenoma burden and if PPARδ mediates the effects of GW501516 and a high-fat diet in *Apc$^{fl/fl}$/CDX2-Cre* mice

Not Yet Initiated.

Major Task 2C: To determine whether PD-1 in $CD8^+$ T cells is required for PPARδ- or dietary fats-induced tumor immune evasion

Not Yet Initiated.

Major Task 2D: To determine whether PD-1 in macrophages is required for PPARδ- or dietary fats-induced tumor immune evasion

Not Yet Initiated.

- **What opportunities for training and professional development has the project provided?**

In addition to our significant progress on this proposal, Dr. Bo Cen and Jie Wei were provided with opportunities for professional development. Both have added the necessary knowledge base of colorectal cancer research to become more effective tumor immunological scientists during this granting period. At the bench, they have established the skills needed to complete the experiments proposed in this application independently. In addition, they have interacted with various mentors, colleagues, collaborators, and researchers from around the world through meetings, seminars, and presentations. At MUSC, we have weekly lab meetings to allow them to present their data and discuss progress. They attend several weekly seminar series within the Department of Biochemistry, Immunology, and the Hollings Cancer Center, as well as the annual Hollings Cancer Center retreat, which broadens their knowledge on various topics. All their works will be published after completion. The preparation of the manuscripts will provide them with further opportunities to improve their writing skills and to create effective ways to communicate their results.

- **How were the results disseminated to communities of interest?**

Our findings will be presented at Oncology Conferences such as AACR Annual Meeting. In addition, we will also present our results to seminars organized by the Department of Biochemistry, Immunology, and the Hollings Cancer Center. Finally, our findings will be published for the scientific community, cancer patients, and the general population, including military personnel, veterans, and their families.

- **What do you plan to do during the next reporting period to accomplish the goals?**

As outlined in the SOW, we will start to work on Major Task 1B and Subtask 2 of Major Task 2B. We expect that we can complete these tasks during the next reporting period. As mentioned earlier, we are evaluating whether PPAR□ is required to suppress macrophage phagocytosis against CRC cells.

4. Impact

- **What was the impact on the development of the principle discipline(s) of the project?**

Our results during this reporting period not only uncover a previously unrecognized role of PPARδ in affecting tumor immune evasion *in vitro* but also provide a rationale to evaluate our hypotheses *in vivo* as outlined in the SOW. For long-term studies, our findings will provide a rationale for developing PPARδ antagonists as agents to target immune checkpoint pathways and to enhance the efficacy of checkpoint inhibitors in the treatment of CRC patients, including military personnel, veterans, and their families.

- **What was the impact on other disciplines?**

PPARδ regulation of $CD8^+$ T cell activation may be applicable to other diseases, such as obesity and diabetes.

- **What was the impact on technology transfer?**

Nothing to report at this time.

- **What was the impact on society beyond science and technology?**

Any advances in colorectal cancer (CRC) prevention and treatment will affect such a large population in the United States and worldwide, which will have a significant social impact. However, colonoscopy screening is an effective way to detect and prevent CRC by removing polyps before malignant conversion, over fifty percent of patients still present to their physician with advanced disease (stage III-IV). Unfortunately, existing therapies have limited efficacy, especially for stage 4 disease.

5. Changes/Problems

- **Changes in approach and reasons for change**

As described in Subtask 4 of Major Task 2A, we initially postulated that PD-1 mediates the effect of PPARδ on $CD8^+$ T cell cytotoxicity against tumor cells. However, our results showed that PD-1 did not mediate the effect of PPARδ on $CD8^+$ T cell cytotoxicity *in vitro*. Previous studies have revealed that NF-κB is required for the capacity of $CD8^+$ T cells to kill tumor cells via induction of Granzyme B expression (PMID: 25648675, 16547254, and 12360212). It is also well established that one of the main functions of PPARδ in acute inflammation is to inhibit NF-κB activation by directly binding to p65 NF-κB to prevent the binding of p65 NF-κB to its target genes (PMID: 30927048). Based on these previously published results, we postulate that activation of PPARδ suppresses $CD8^+$ T cell cytotoxicity via an NF-κB-Granzyme B pathway. Our results demonstrate that activation of PPAR□ suppresses CD8+ T cell cytotoxicity by reducing Granzyme B expression via inhibiting NF-κB activation.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

As described in Subtask 3 of Major Task 2A, activation of PPARδ tended to suppress the ability of macrophages to phagocytose HCT-116 cells. However, the results were not significant. Now we are working on this subtask by using mouse colon adenocarcinoma cells to test phagocytosis of mouse WT and PPARδ-deficient

bone marrow-derived macrophages as well as IC-21/WT and IC21/shPPARδ. We hope to complete this subtask in two months.

- **Changes that had a significant impact on expenditures**

  Nothing to report at this time.

- **Significant changes in the use or care of human subjects, vertebrate animals, biohazards, and select agents**

  Nothing to report at this time.

6. Products

Nothing to Report

7. Participants & Other Collaborating Organizations

- **What individuals have worked on the project?**

Name:	Raymond N. DuBois, M.D., Ph.D.
Project Role:	Principal Investigator
Researcher Identifier ORCID ID	0000-0002-6385-7154
Nearest person month Worked:	0.24 months
Contribution to Project:	Advised and guided the research conducted by Dr. Bo Cen & Jie Wei.
Funding Support:	Funded through NIH and Institutional support

Name:	Bo Cen, Ph.D.
Project Role:	Research Assistant Professor
Researcher Identifier ORCID ID	0000-0002-6640-2120
Nearest person month Worked:	6 months
Contribution to Project:	Dr. Bo performed all experiments proposed in Aim 1A.
Funding Support:	50% from Institutional support

Name:	Jie Wei, M.S.
Project Role:	Research Associate
Researcher Identifier ORCID ID	0000-0001-7111-1945
Nearest person month Worked:	4.8 months
Contribution to Project:	Mr. Wei performed all experiments proposed in Aim 2A. In addition, she conducted all experiments proposed in the subtask 1 of major task 1B and the subtask 1 of major task 1B.
Funding Support:	60% from Institutional support

- **Has there been a change in the active other support of the PD/PI(s) or senior personnel since the last reporting period?**

  Nothing to Report

- **What other organization were involved as partners?**

  Nothing to Report

8. Special Reporting Requirements

Nothing to Report

9. Appendices

Nothing to Report