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GSTO1-1 is an upstream suppressor of M2 macrophage skewing and HIF-1 α -induced eosinophilic airway inflammation

GSTO1-1 regulates HIF-1 α and M2 Macrophages

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List of Abbreviations

AB-PAS- Alcian Blue PAS

AHR- Airways Hyperreactivity

BALF- Bronchoalveolar Lavage Fluid

BMDM- Bone Marrow-Derived Macrophages

CD- Cluster Differentiation

gDNA- Genomic DNA

GSH- Glutathione

GST- Glutathione-S-Transferase

HDM- House Dust Mite

IFN- Interferon

IL- Interleukin

LPS- Lipopolysaccharide

NF- Nuclear Factor

qPCR- Quantitative Polymerase Chain Reaction

ROS- Reactive Oxygen Species

SAL- Saline

Th- T-helper Cell

TLR- Toll-Like Receptor

WT- Wild Type

Abstract

Background: Glutathione S-Transferases Omega Class 1 (GSTO1-1) is a unique member of the GST family regulating cellular redox metabolism and innate immunity through the promotion of LPS/TLR4/NLRP3 signaling in macrophages. House Dust Mite (HDM) triggers asthma by promoting Type 2 responses and allergic inflammation via the TLR4 pathway. Although linked to asthma, the role of GSTO1-1 in facilitating Type 2 responses and/or HDM-driven allergic inflammation is unknown.

Objective: To determine the role of GSTO1-1 in regulating HDM-induced allergic inflammation in a preclinical model of asthma.

Methods: Wild-type and GSTO1-1 deficient mice were sensitized and aeroallergen challenged with HDM to induce allergic inflammation and subsequently hallmark pathophysiological features characterized.

Results: By contrast to HDM challenged WT mice, exposed GSTO1-1-deficient mice had increased numbers of eosinophils and macrophages and elevated levels of eotaxin-1 and -2 in their lungs. M1 macrophage associated factors, such as IL-1 β and IL-6, were decreased in GSTO1-1-deficient mice. Conversely, M2 macrophage factors such as Arg-1 and Ym1 were upregulated. HIF-1 α expression was found to be higher in the absence of GSTO1-1 and correlated with the upregulation of M2 macrophage markers. Furthermore, HIF-1 α was shown to bind and activate the eotaxin-2 promotor. Hypoxic conditions induced significant increases in the levels of eotaxin-1 and -2 in GSTO1-deficient BMDMs, providing a potential link between inflammation-induced hypoxia and the regulation of M2 responses in the lung. Collectively, our results suggest that GSTO1-1-deficiency promotes M2-type responses and increased levels of nuclear HIF-1 α , which regulates eotaxin(s) induced eosinophilia and increased disease severity.

Conclusion & Clinical Implication: We propose that GSTO1-1 is a novel negative regulator of TLR4 regulated M2 responses acting as an anti-inflammatory pathway. The discovery of a novel HIF-1 α -induced eotaxin pathway identifies an unknown connection between hypoxia and the regulation of the severity of allergic inflammation in asthma.

Introduction

Asthma is a chronic disorder of the airways associated with episodes of bronchial constriction, mucus production, and immune cell infiltration. The inflammation associated with disease can be driven by allergic or non-allergic mechanisms, where the activation of Toll-like receptors (TLRs) on epithelial cells and macrophages regulates the initial nature of the innate inflammatory response (1-7). TLR4 activation in response to bacterial components, such as lipopolysaccharide (LPS), will drive Nuclear Factor (NF)- κ B-driven inflammatory programming towards IL-1 β , IL-6, and TNF- α production in macrophages (8-10), promoting the activity of Type 1 (M1) resident populations and neutrophilic inflammation. Conversely, stimulation of the IL-4 receptor will result in STAT6 activation, which promotes the development of Type 2 (M2) resident macrophage populations. M2 macrophages are associated with tissue repair/remodeling and allergic inflammation via the production of eotaxin in response to IL-4/IL13 (11-14), where both eotaxin and IL-13 regulate eosinophilia and hallmark features of allergic inflammation (15-17). While higher M1 ratios in tissues are associated with pathogen clearance and neutrophilic inflammation, M2-skewing in asthma is generally associated with worsened eosinophilic inflammation and airway remodeling (3, 18-22).

Glutathione (GSH) is a critical intracellular thiol that protects cytoplasmic proteins against degradation by ROS. GSH is dependent on catalytic enzymes known as glutathione-S-transferases (GSTs) that facilitate GSH-redox homeostasis (23-25). Of the 13 known classes of GSTs, the Omega class of GSTs (GSTOs) have distinct catalytic functions unrelated to the GSH-conjugating activity catalyzed by most other GSTs (26, 27). The isoform GSTO1-1 has been identified as a

downstream component of TLR4 signaling, and shRNA inhibition of GSTO1-1 in LPS-exposed macrophages have reduced interleukin (IL)-1 β , IL-6 and NLRP3-inflammasome activation with concurrent attenuation of glycolysis and hypoxia-inducible factor-1 (HIF-1) nuclear translocation (28, 29). In addition, GSTO1-1 catalyzes the deglutathionylation of NEK7 and regulates the maturation and release of IL-1 β via the NLRP3 inflammasome (30). The use of GSTO1-1-deficient mice in inflammatory disease models have supported these *in vitro* findings, where LPS-induced inflammation in GSTO1-1-deficient mice resulted in lower levels of IL-1 β , IL-6, and TNF- α expression compared to wild type (WT) controls, in addition to reduced liver necrosis and spleen enlargement (31). Similar observations were observed in models of DSS-induced colitis and obesity-induced inflammation (31), both of which are critically regulated TLR4 and MyD88 regulated inflammatory cascades. In these models, GSTO1-1 was found to regulate bactericidal pathways in macrophages (such as phagocytosis) in addition to the production of ROS and inflammatory cytokines (through TLR4 signaling) (29, 31), in which its inhibition can contribute to decreased severity of disease. House dust mite triggers asthma exacerbations and is known to regulate allergic Type 2 inflammation through TLR4 signaling (32, 33). However, the role of GSTO1-1 in regulating Type 2 inflammation and allergic diseases remains mostly unexplored.

Post-hoc analysis of microarray data from asthma patients that have experienced exacerbations demonstrated that GSTO1-1, compared to other GSTs, is distinctly upregulated peripheral blood mononuclear cells and nasal lavage fluid compared to patients in remission (Fig S1 A&B) (34, 35). Given the expression of GSTO1-1 in active asthma and the involvement that GSTO1-1 has in TLR4 signaling, we utilized GSTO1-1-deficient mice in a house dust mite (HDM) model of allergic asthma to determine if GSTO1-1 may regulate hallmark features of disease (Type 2 allergic inflammation, airways hyperreactivity (AHR), development of fibrotic lesions and mucus hypersecretion). Our findings revealed that GSTO1-1 deficiency led to increased eosinophilic inflammation, mucus production, and fibrosis within the lung, with no increase in Type 2-associated cytokines except for eotaxin-1 and eotaxin-2 levels. We identified GSTO1-1 as a crucial regulator of M1/M2 macrophage skewing, whereby GSTO1-1-deficiency resulted in increased populations of M2 macrophages in the lung. We further demonstrate that both glycolysis and HIF-1 α are increased as a result of GSTO1-1 deficiency in response to HDM, and provide direct evidence that HIF-1 α is a transcriptional regulator of eotaxin-2 in GSTO1-1-deficient bone marrow-derived macrophages (BMDM). Together, these studies characterize how GSTO1-1

maintains M1/M2 balance in the allergic lung by negatively regulating M2 function. Thus, we have identified a novel negative regulatory pathway of Type 2 responses, whereby GSTO1-1 regulates M2 skewing and glycolytic rate, and levels of nuclear HIF-1 α that regulates eotaxin transcription and eosinophil accumulation in the airways.

Methods

Mice and House Dust Mite Model

C57BL/6 male mice (WT littermates or GSTO1-1-deficient) (31) were bred at the John Curtin School of Medical Research (ANU, Canberra) Bioresources Facility, and were transferred to Hunter Medical Research Institute (Newcastle) Bioresources facility. All experiments were conducted with approval from the Animal Care and Ethics Committee (The University of Newcastle).

Mice were sensitized with 50µg of HDM extract (Citeq Biologics, The Neverlands) in 50µl of sterile saline (SAL) intranasally under light isoflurane anesthesia, once a day for three days. As a control, SAL alone was administered. Eleven days post sensitization, mice were intranasally challenged with HDM (5µg/50µl) under light isoflurane anesthesia once a day for four days. Mice were then sacrificed 24 hours after the last HDM challenge.

Bronchoalveolar Lavage Fluid Analysis

Mice were trachealized, and a syringe was used to flush 1ml of Hanks Balanced Salt Solution to the right lung lobes of the mice. The collected cells were then red blood cell lysed, and a cell count was performed to determine the total cell number. Cells were cytospun onto a microscope slide, stained with May Grunwald and Giemsa solution, and a differential count was performed to determine the cell populations present in the lung airways (adjusted to the total cell count).

Airways Hyperresponsiveness (Flexivent)

Mice were anesthetized interperitoneally with ketamine (100mg/kg) and xylazine (10mg/ml, Troy Laboratories, NSW, Australia) (final volume of 200µl per mouse) and the tracheas of each mouse was cannulated. Airway resistance was determined using the Flexivent apparatus FX1 system (Scireq, Montreal, Canada) to measure pulmonary resistance at a rate of 450 breaths/minute (8ml/kg.) Increasing doses of methacholine (Sigma-Aldrich, NSW, Australia) were routed to anesthetized mice via nebulization, and dose-dependent resistance values were determined at each methacholine dose. The airways resistance for each methacholine dose was normalized as a percentage increase from baseline (PBS).

Macrophage isolation from whole mouse lung

Cells were isolated from exercised mouse lung tissue, as previously described (36). 15ml of Histopaque-1083 (Sigma-Aldrich, NSW, Australia) was carefully layered under the cell suspension, and the cells were centrifuged for 30 minutes (3,000g) without the break setting. The opaque cell suspension layer was collected and washed, and cells were plated at 6×10^6 cells/well with DMEM (10% FCS).

RNA extraction and qPCR

RNA was extracted from whole lung tissue or isolated macrophages using TRIzol® (Invitrogen, VIC, Australia) as per the manufacturer's instructions. RNA was separated from the TRIzol®

suspension using chloroform (250µl), and RNA was precipitated using isopropanol (300µl) (Sigma-Aldrich, NSW, Australia). RNA was then washed with ethanol and resuspended in nuclease-free water. RNA was then reverse-transcribed into cDNA using random primers and M-MLV (Invitrogen, VIC, Australia). Primers in the presence of cDNA and SYBR green dye underwent qPCR reaction (Viia7, Life Technologies) to determine CT values for genes of interest. Results are expressed as fold change normalized to the control gene HPRT. Sequence for all primers (murine) used in this study are GSTO1-1 (F: 5'-ATGTCGGGAGAATCCTCCA-3' R: 5'-CCCTTCAGAGCCCATAATCAC-3'), IL-1β (F: 5'-GGATGAGGACATGAGCACCTTCTT-3', R: 5'-TGTCGTTGCTTGGTTCTCCTTGTA-3'), IL-6 (F: 5'-AGAAAACAATCTGAAACTTCCAGAGAT-3', R: 5'-GAAGACCAGAGGAAATTTTCAATAGG-3'), IL-10 (F: 5'-CATTTGAATTCCCTGGGTGAGAAG-3', R: 5'-GCCTTGTAGACACCTTGGTCTTGG-3'), Arg-1 (F: 5'-GCTCCAAGCCAAAGTCCTTAGAGAT-3', R: 5'-AGGAGCTGTCATTAGGGACATCAAC-3'), Yml (F: 5'-GACAGGCCAATAGAAGGGAGTTTCA-3', R: 5'-GACGGTTCTGAGGAGTAGAGACCAT-3'), iNOS (F: 5'-CCTCGGAGGTTTACCTCACTGT-3', R: 5'-CAGAACTTCGGAAGGGAGCAAT-3'), TGF-β (F: 5'-CCCGAAGCGGACTACTATGCTAAA-3', R: 5'-GGTAACGCCAGGAATTGTTGCTAT-3'), CD38 (F: 5'-CTGCTGGAGAGGTTATTCCTCG-3', R: 5'-GGAAGAGTGAGCGGCGCATCAAGG-3), CD206 (F: 5'-CATGGATGTTGATCGCTACTGGAG-3', R: 5'-CCATAGAAAGGAATCCACGCAGTC-3')

Protein isolation and quantification

RIPA buffer (Sigma-Aldrich, NSW, Australia) was used to extract protein from whole lung tissue or bone marrow-derived macrophages (BMDM). Protein expression for eotaxin-1, eotaxin-2, IL-5, IL-13, and IL-25 was determined by ELISA (R&D systems, VIC, Australia), while the NLRP3 inflammasome expression was quantified using a pre-coated ELISA kit from MyBioSource (CA, USA). Protein expression was normalized to total protein determined by BCA (ThermoFisher).

Histological Analysis of Mouse Lung

Lung sections for histology were taken from the left lobe of euthanized mice and were fixed in 10% formalin solution. Lung sections were embedded in paraffin, and lung sections were cut at a

thickness of 5µm before being fixed on a microscope slide. Slides were either allocated for HIF-1α staining or underwent staining for collagen (Masson-Trichrome) or mucus (AB-Pas). Light microscope was then used to determine the degree of goblet-cell hyperplasia in each lung section (per high powered field), and image analysis (ImageProPlus software) was employed to assess the degree of fibrosis of the lung airways.

HIF-1α staining and scoring of tissue sections

Paraffin-embedded lung tissue underwent deparaffinization using Xylene and Ethanol, and underwent antigen retrieval using citric-acid buffer (Sigma-Aldrich, NSW, Australia). Lung tissue was then blocked with 10% sheep serum and were then exposed to rabbit anti-mouse HIF-1α antibody (Novus Biological, CO, USA) at 4°C overnight. Lung tissue sections were then supplemented with an anti-rabbit HRP-conjugated antibody (BioLegend, WA, Australia, WA, Australia) for immunohistochemistry analysis and were then counterstained with DAB (Sigma-Aldrich, NSW, Australia). HALO software (Indica Labs, NM, USA) was then used to determine the H-score (IHC intensity grade/area) for both epithelial and parenchymal regions of HIF-1α stained lung tissue.

Immunofluorescence staining for HIF-1α and CD206

Isolated macrophages from mouse whole lung were cytospun on a microscope slide and were fixed in 10% formalin for 20 minutes before antigen retrieval and blocking as described above. Macrophages were supplemented with Triton-X (Sigma-Aldrich, NSW, Australia) for one hour after blocking, and cells were supplemented with an anti-mouse antibody targeting HIF-1α (Novus Biological, CO, USA) or CD206 (BioLegend, WA, Australia) at 4°C overnight. Isolated lung macrophages were then stained with Alexaflour™555 or 647 (BioLegend, WA, Australia) conjugated antibodies, and cells were then counterstained using DAPI (Sigma-Aldrich, NSW, Australia). Immunofluorescent images were then captured using the Zeiss Imager M2 microscope platform (Zeiss, Jena, Germany), and ImageJ software was used to determine the average cellular intensity of HIF-1α staining in isolated lung macrophages or to determine the number of dual HIF-1α/CD206 positive cells per High Powered Field.

Flow Cytometry of mouse lung cells and macrophages

Cells were isolated from mouse lungs using the GentleMACS™ tissue lyser, as previously described (37). Lung cells and isolated macrophages from mouse whole lung were incubated on ice for 10 minutes with Fc Block CD16/CD32 (Biocompare, CA, USA) before being stained with surface-marker antibodies for 30 minutes (CD45, Siglec F, CD125, F4/80, CD11b, CD206) (BioLegend, WA, Australia). For HIF-1 α staining, cells were treated with PFA (Sigma-Aldrich, NSW, Australia) before being resuspended in saponin buffer (Sigma-Aldrich, NSW, Australia), and HIF-1 α rabbit, anti-mouse primary antibody (Novus Biological, CO, USA) and its respective anti-rabbit secondary antibody (BioLegend, WA, Australia) were added to each sample for 30 minutes each. All cells were fixed using 1% buffer fixative, and cell populations were determined on the BD LSPFortessa™ X-20 flow cytometer. Analysis of cell populations was conducted using FlowJo™.

Extracellular Acidification Rate measurement of isolated lung macrophages

Extracellular Acidification Rate (ECAR) was determined using the Seahorse™ Glycolysis Stress Test Kit and the Seahorse™ FX96 plate reader (Agilent, VIC, Australia) in accordance with the manufacturer's instructions. Isolated macrophages were plated at a concentration of 3×10^5 cells per well on a 96 well Seahorse cell culture plate and were left to incubate overnight to adhere in Seahorse™ media and glutamine. Cellular ECAR was then determined on the FX96 plate reader coupled with the kits drug cartridge, which was loaded to deliver glucose, oligomycin, and 2-Deoxy-D-glucose (Agilent, VIC, Australia) to the cells at set time points. Readings were normalized to each sample cell count post-analysis.

Differentiation and hypoxia exposure of bone marrow-derived macrophages (BMDM)

Femurs were exercised from WT, and GSTO1-1-deficient mice and bone marrow were collected using Hanks Balanced Salt Solution (Sigma-Aldrich, NSW, Australia). Bone marrow cells were cultured for one week in the presence of L929 conditioned media to facilitate BMDM enrichment. BMDM were collected and plated in six-well tissue culture plates at a concentration of 1×10^6 cells per well. Both WT and GSTO1-1-deficient BMDM were subjected to hypoxic conditions in a hypoxia chamber (PLAS Laboratories, MI, USA) at 1% O₂, 5% CO₂ for either two, four, or six hours. Alternatively, cells were treated with 1 μ M of 17-Dimethylaminoethylamino-17-demethoxygeldanamycin (DMAG17) (Sigma-Aldrich, NSW, Australia) or vehicle control

(DMSO) and underwent hypoxia exposure for six hours. Cells were collected in RIPA buffer, and eotaxin expression levels were determined using ELISA.

Differentiation of naïve macrophages into macrophage phenotypes

Naïve BMDM were subjected to either no stimulus (M0), LPS (Invitrogen, VIC, Australia) with IFN- γ (M1), IL-4 (M2), or IL-10 (M3) (PreproTech, VIC, Australia) to differentiate the naïve macrophages into their allocated phenotype. Cells were lysed in TRIzol™ (Invitrogen, VIC, Australia) and collected for RNA extraction and qPCR analysis, or underwent hypoxia exposure for six hours where cells were collected in RIPA buffer.

Chromatin immunoprecipitation pull-down of HIF-1 α binding site and qPCR

Differentiated BMDM (6×10^6 cells/well) underwent hypoxia exposure (Plas Laboratories, Hypoxia chamber, MI, USA) at 1% O₂, 5% CO₂, for six hours before being detached and collected at a final volume of 500 μ l. Chromatin immune-precipitation was performed on cells per the MAGnify™ Chromatin Immunoprecipitation protocol (Invitrogen, VIC, Australia). Cells underwent chromatin cross-linkage using 13.5 μ l of 37% formaldehyde and were incubated for 10 minutes at room temperature before the reaction was inhibited using glycine (57 μ l at 1.25M). Crosslinked cells were washed with PBS and underwent lysis using Lysis buffer with protease inhibitors (50 μ l per million cells, protease cocktail inhibitors used at a concentration of 1/200). Crosslinked cells then underwent sonication using the UP50H Compact Lab Homogenizer (Hielscher Ultrasonics, Teltow, Germany), and the chromatin from each sample was resuspended and separated from the cell debris. Sheared chromatin was diluted in dilution buffer, and protease inhibitors (50 μ l per million cells) before 2 μ g of HIF-1 α antibody (that were previously bound to DynabeadsTTCA®) or control antibody (Novus Biological, CO, USA) was added to each diluted sample. The chromatin cells were washed, and cross-linkage was reversed using Proteinase K (1 μ l per reaction). Purification Magnetic Beads were then added to the samples, which were then washed and eluted. Primers for eotaxin-2 (F: 5'-TTCACGCAATAAGCCACAGA-3', R: 5'-TTTCTTGTCAGTGGAGTCA-3') were used to determine expression of eotaxin-2 in HIF-1 α -bound chromatin samples relative to input DNA.

Transfection of RAW 264.7 macrophages with cloned EOTAXIN-2 promoter region for Luciferase Assay

RAW 264.7 macrophages (ATCC™, Granville, NSW) were plated out on a 96 well white culture plate in DMEM supplemented with L-glutamine at 1×10^5 cells per well. Eight hours post-plating, cells were transfected with clones for eotaxin-2 (either WT or with mutated hypoxia-responsive element (HRE) binding sites) using pLightswitch Promotor vectors and FuGene HD for 16 hours (Active Motif, Carlsbad, CA). Untreated cells and vector-only transfected cells were used as a negative control. Cells were then subjected to six hours of hypoxia (1% O₂, 5% CO₂) before cell media was removed, and 100µl of Luciferase Complete Assay Buffer (Active Motif) was added to each well. Cells were left to incubate at room temperature for 30 minutes, and each well was read for two seconds on the SpectraMax M5 on the Luminator L setting.

Statistical analysis

Significance between experimental groups was determined using either One-way ANOVA (Dunnett's multiple comparison test), 2way ANOVA for experiments involving dose-response/time courses, and unpaired student t-tests when only two experimental groups were present. All statistical analysis was conducted in GraphPad Prism software, and significance was declared if the p-value determined between two groups was <0.05 .

Results:

GSTO1-1-deficiency leads to increased airway eosinophilia and remodeling following HDM

HDM induced allergic airways inflammation, AHR, mucus hypersecretion, and collagen deposition in both GSTO1-1-deficient and wild-type (WT) littermate control mice, which were sensitized and aerochallenged with this allergen. Analysis of bronchoalveolar lavage fluid (BALF) revealed significant increases in the number of airway macrophages, lymphocytes, and in particular eosinophils (3-fold increase) in the absence of GSTO1-1 (Fig 1A). While no significant differences in AHR were observed between HDM challenged WT and GSTO1-1-deficient mice in response to methacholine challenge (Fig 1B). The increased number of airway eosinophils in HDM challenged GSTO1-1-deficient mice was confirmed using flow cytometry (Fig 1C and D). By contrast, there were no significant differences in neutrophil numbers in HDM challenged WT and GSTO1-1-deficient mice, linking negative regulation of inflammation to Type 2 eosinophilia and not Type 1 regulated neutrophilia (Fig 1E). The increased number of eosinophils was associated with the inflammatory infiltrating eosinophil-phenotype rather than expansion resident eosinophils (Fig S2 A-C). GSTO1-1 mRNA in mouse whole lung confirmed that WT HDM exposed mice had a dramatically increased GSTO1-1 expression when compared to WT saline control (Fig 1F) directly linking HDM induced activation of TLR4 with increased expression. We have previously demonstrated that TLR4 activation is critical for the induction of downstream Type 2 responses to HDM (7). Features of airway wall remodeling (goblet cell hyperplasia and collagen deposition) were also found to be markedly increased in HDM exposed GSTO1-1-deficient mice by contrast to controls (Fig 1G-I).

GSTO1-1-deficiency increases eotaxins, but not Type 2 cytokine expression, in the allergic lungs following HDM exposure

Key Type 2 regulatory factors were measured in the lung homogenates from all groups of WT and GSTO1-1-deficient mice by ELISA (Fig. 2). Out of these factors, only eotaxin-1 (Fig 2A) and

eotaxin-2 (Fig 2B) were shown to be significantly increased in the absence of GSTO1-1. No changes in the protein levels of IL-5 (Fig 2C), IL-13 (Fig 2D), or the epithelial Type 2 factors IL-25 (Fig 2E) and IL-33 (Fig 2F) were observed between HDM exposed groups. This suggests that the impact of GSTO1-1 on airway eosinophilia may be mediated specifically through suppression of eotaxin production that reduces eosinophil recruitment and the severity of disease.

GSTO1-1 suppresses production M2 macrophage factors and enhances activation M1 factors

GSTO1-1 has previously been demonstrated to be crucial for IL-1 β and IL-6 expression in macrophages exposed to LPS *in vitro* (28, 29). Given this, we investigated in whole lung RNA if GSTO1-1-deficiency resulted in similar changes. M1/M2 skewing IL-1 β (Fig 3A), IL-6 (Fig 3B), and NLRP3 (Fig 3C) expression were found to be significantly decreased in GSTO1-1-deficient mice following HDM exposure. By contrast, expression of factors associated with M2 macrophages, such as IL-10 (Fig 3D), Arg-1 (Fig 3E), and Ym1 (Fig 3F), were significantly upregulated as a result of GSTO1-1-deficiency, suggesting an M2 skewing effect in the absence of GSTO1-1.

GSTO1-1 acts as a negative feedback loop in M2 macrophages

To confirm our observation of M2 macrophage phenotype skewing in the lung in the absence of GSTO1-1, we purified macrophages from the lungs of both WT and GSTO1-1-deficient mice that had been exposed to HDM. HDM exposed macrophages from WT mice were found to have a higher expression of the M1 surface marker CD38 when compared to macrophages from GSTO1-1-deficient mice (Fig 4A). However, iNOS, IL-1 β , and IL-6 remained unchanged (Fig 4B-D). The expression of the classical M2 factors CD206, Arg-1, Ym1, TGF- β , and IL-10 were all significantly upregulated in HDM-exposed GSTO1-1-deficient lung macrophages compared to WT counterparts (Fig 4E-I). These data were confirmed using flow cytometry to show that cell membrane protein expression of CD206 was increased in GSTO1-1-deficient lung macrophages compared to WT controls (Fig 4J-K), and in lung cells stimulated *ex vivo* with HDM (Fig 4L). We next investigated the induction signal for GSTO1-1 using M0, M1, M2, and M3 differentiated macrophages from mouse bone marrow. This data demonstrated that only under M2-inducing conditions was the expression of GSTO1-1 up-regulated (Fig 4M). Combined, these data suggest

that GSTO1-1 expression is upregulated specifically under M2 conditions and acts as a negative feedback signal on M2 skewing.

GSTO1-1-deficiency leads to altered macrophage metabolic activity and enhanced HIF-1 α expression in the lung

Our observation of increased Arg-1 expression in GSTO1-1 deficient macrophages suggests that GSTO1-1 could influence the Krebs/Citric Acid cycle through the Urea cycle, which is known to regulate cell metabolism and HIF-1 α activity (38, 39). GSTO1-1 has previously been shown to upregulate both glycolysis and HIF1- α upon LPS activation (29). Thus, we investigated the expression of HIF1- α in HDM exposed GSTO1-1-deficient mice, and the effect GSTO1-1 had on HDM-exposed macrophage glycolysis. We assessed the characteristics glycolysis in macrophages extracted from HDM exposed mice using the Seahorse™ glycolysis stress assay. GSTO1-1-deficient HDM exposed macrophages had a higher extracellular acidification rate (ECAR) of glycolysis in response to glucose and oligomycin (mitochondrial inhibition) exposure when compared to macrophages from WT HDM-exposed mice (Fig 5A). While at baseline glycolysis did not differ (Fig 5B), oxidative phosphorylation (OXPHOS) was significantly higher in the GSTO1-1-deficient macrophages compared to WT controls (Fig 5C). HIF-1 α in macrophages is known to regulate glycolysis. Unlike previous observations in LPS-treated macrophages, immunohistochemistry for HIF-1 α *in vivo* revealed that expression was increased in the peribronchial regions in the absence of GSTO1-1, while there was no observable changes in the airway epithelium (Fig 5D-F). To confirm this result, we performed Immunofluorescent HIF-1 α staining on lung macrophages isolated from HDM-exposed GSTO1-1-deficient or WT control mice. While HDM increased the expression of macrophage HIF-1 α , this was further increased in the absence of GSTO1-1 (Fig 5G & H).

Co-expression of HIF-1 α in macrophages with the M2 marker CD206 was increased in GSTO1-1-deficient mice

We next determined what phenotype of macrophage was primarily responsible for HIF-1 α expression in both WT and GSTO1-1-deficient HDM-exposed *ex vivo* lung macrophages. Immunofluorescence staining of BALF cells for both HIF-1 α and the M2 macrophage marker CD206 revealed the co-expression of these proteins within the same cell (HIF-1 α ⁺/CD206⁺ macrophages) and an increase in these dual positive cells in the absence of GSTO1-1 (Fig 6A &

B). This indicated that M2 macrophages are primarily expressing HIF-1 α as a result of HDM exposure. This finding was further supported by increased co-expression of CD206 and HIF-1 α in the absence of GSTO1-1 in whole lung slices from HDM-treated mice (Fig 6C & 6D), and flow cytometry analysis of isolated lung macrophages (Fig 6E & 6F). This data clearly demonstrates that the interaction between GSTO1-1, glycolysis, and HIF-1 α is immune context-dependent, and in the case of the HDM allergic lung environment, unlike Type 1 immune response or LPS-treated macrophages, GSTO1-1 suppresses HIF-1 α .

BMDM have increased eotaxin-1 and 2 protein expression under hypoxic conditions through a GSTO1-1/HIF-1 α -dependent mechanism

We next investigated how the increased level of HIF-1 α expression and airway eosinophilia both observed in the absence of GSTO1-1 may be linked together. Two key drivers of eosinophilia that were elevated in our HDM models were eotaxin-1 and 2. Examination of both eotaxin-1 and eotaxin-2 promoter regions using MatInspector (Genomatix, Munich, Germany) revealed the presence of HIF-Responsive Elements (HRE) (CACGT, CACGC, and CACGG). A notable enrichment of HREs was located between 1,892 and 2,942 base pairs upstream of the transcription start site of eotaxin-2 (Fig S3 C), suggesting the potential for direct binding and regulation of transcriptional activity driven by HIF-1 α . Due to heightened activity of HIF-1 α in GSTO1-1 deficient macrophages, we investigated if hypoxia alone would be sufficient to induce eotaxin expression in GSTO1-1-deficient BMDM when compared to WT BMDMs. Incubating BMDMs in hypoxic conditions (1% O₂, 5% CO₂) did not induce eotaxin protein expression in the WT control group. However, GSTO1-1-deficient BMDM had increased eotaxin-1 protein at the four and six-hour time points (Fig 7A) and eotaxin-2 protein at six hours of hypoxia (Fig 7B). Administration of DMAG17, a selective inhibitor of the HIF-1 α chaperone protein (Hsp90), ablated eotaxin-1 (Fig 7C), and eotaxin-2 (Fig 7D) protein expression post-hypoxia (six hours) when compared to the vehicle control. This data demonstrates HIF-1 α can drive eotaxin expression. To demonstrate direct promoter binding by HIF-1 α , we examined eotaxin-2 expression in hypoxic RAW264.7 macrophages that were transfected with WT or HRE-mutated eotaxin-2 promoter-region clones driving a luciferase reporter. Under hypoxic conditions, the HRE-mutated eotaxin-2 promoter clone demonstrated significantly reduced luciferase activity compared to the WT HRE control (Fig 7E). Furthermore, ChIP pull-down of DNA from either WT or GSTO1-1-deficient BMDM

revealed that HIF-1 α binding was enriched in the promoter region of eotaxin-2, and this binding was observed only in the absence of GSTO1-1 (Fig 7F).

Discussion

GSTO1-1 has previously been identified as a pro-inflammatory factor involved in driving LPS/TLR4 responses in macrophages, and its inhibition in RAW macrophages resulted in reduced inflammatory cytokine release and ROS production in response to LPS (29, 31). Such studies further linked a GSTO1-1 pro-inflammatory role with the stabilization of HIF-1 α , in addition to NLRP3 cleavage of IL-1 β and NF- κ B induced transcription of TNF- α and IL-6. A role for GSTO1-1 in the regulation of inflammation has further been extended *in vivo*, where GSTO1-1 deficient mice were found to be protected against LPS-induced septic shock and inflammation induced by high-fat diet-induced insulin resistance (31), implicating GSTO1-1 as an important mediator of these inflammatory diseases. GSTO1-1 has been linked with exaggerated inflammation of the lungs due to asthma exacerbations (Fig S1 A&B), and we have previously demonstrated that HDM induced allergic airways disease in a preclinical model of asthma is critically regulated by TLR4 (7), suggesting that GSTO1-1 may play a regulatory role. For this reason, our present study explored the role of GSTO1-1 deficiency in a mouse model of HDM induced asthma. HDM is an aeroallergen that contains PAMPs such as LPS and β -glucans and structural elements that mimic ligands, which activate TLR4 to mediate lung inflammation (40).

Despite GSTO1-1's pro-inflammatory role in the context of LPS/TLR4-driven inflammation (or type 1 inflammation), our results demonstrated that HDM-induced inflammation (type 2 inflammation) was negatively regulated by GSTO1-1, where its inhibition resulted in more significant infiltration of eosinophils and macrophages into the lung. The majority of these eosinophils were determined to be infiltrating inflammatory cells, with smaller populations of resident eosinophils found to be increased in the lung (Fig S2 A-C). This infiltration occurred concurrently with increased levels of eotaxin-1 and -2, mucus production, and collagen deposition. We observed that the recruitment of these immune cells occurred in the GSTO1-1-deficient group without any noticeable increase in AHR or Th2-associated inflammatory mediators, except for the eotaxin chemokines. Quantifying IL-1 β , IL-6, and NLRP3 in the whole lung revealed that these M1 macrophage factors were downregulated as a result of GSTO1-1 deficiency, as was observed

in previous studies (28, 29). By contrast, the expression M2 macrophage factors were significantly upregulated (e.g., Arg-1, Ym-1, and IL-10). Given the lack of change in Th2 cytokines and the modification of M1/M2 factors in the whole lungs of these mice, we investigated the macrophage populations that accumulate due to HDM exposure in WT and GSTO1-1-deficient mice.

By extracting macrophages from the lungs of HDM exposed WT and GSTO1-1-deficient mice, we observed a decline in the M1 marker CD38 gene expression while the M2 marker, CD206, was significantly upregulated in the GSTO1-1 deficient group, as was Arg-1, Ym-1, IL-10, and TGF- β . Despite inhibition resulting in an upregulation of factors relating to M2 macrophages, GSTO1-1 gene expression of differentiated M0, M1, M2, and M3 BMDM revealed that M2 macrophages were the only population that expressed GSTO1-1 when compared to naïve macrophages. As evident in the GSTO1-1 deficient mice, both in whole lung and isolated macrophages, the lack of GSTO1-1 expression results in attenuated M1 cytokines and a significant increase in M2 factors, implicating GSTO1-1 as a mediator of macrophage phenotype.

A previous investigation of GSTO1-1 identified a pro-inflammatory role for HIF-1 α in the context of LPS induced inflammation, where inhibition of GSTO1-1 resulted in reduced HIF-1 α stabilization and succinate accumulation (29). In asthma, HIF-1 α has been shown to have a functional role in promoting inflammation and airways remodeling, as well as macrophage phenotype polarization (41, 42). Notably, we observed significantly increased expression of HIF-1 α in the peribronchial regions of the lung, while IF staining of isolated macrophages revealed a marked increase of HIF-1 α expression in GSTO1-1-deficient macrophages. IF-staining of isolated macrophages and whole lung showed that HIF-1 α co-localizes with CD206 expression in isolated GSTO1-1-deficient macrophages, suggesting that M2 macrophages are experiencing heightened hypoxia due to GSTO1-1 deficiency. Glucose metabolism of isolated lung macrophages revealed that extracellular acidification rates, a marker for glycolysis which is regulated by HIF-1 α , was notably heightened in GSTO1-1 deficient cells compared to WT in response to glucose and oligomycin administration. While HIF-1 α activation and glycolysis are processes that are associated primarily with M1 macrophage function, this data suggests that the pathways GSTO1-1 regulates in M2 macrophages suppress glycolysis and HIF-1 α activity in response to HDM. A lack of GSTO1-1 expression could compromise the regulation of glucose-metabolism pathways in M2 macrophages, increasing the conversion of glucose to pyruvate that could result in cell exhaustion and a subsequent upregulation of HIF-1 α transcription.

We determined the consequence of HIF-1 α upregulation in GSTO1-1 deficient macrophages by exploring the inflammatory response of naïve GSTO1-1-deficient BMDM under hypoxic conditions. M2 macrophages have also been shown to secrete eotaxin-2 in response to Th2 cytokines (12), and scanning of the eotaxin-1 and eotaxin-2 promoter regions demonstrated the presence of HRE regions, which indicate potential binding sites for the HIF-1 α transcription factor (Fig S3 A-C). Exposing BMDM to hypoxic conditions demonstrated that both eotaxin-1 and eotaxin-2 could be induced in GSTO1-1-deficient BMDM but not WT BMDM, and inhibition of the chaperone protein (HSP90) using DMAG17 attenuated eotaxin protein expression in GSTO1-1-deficient BMDMs. The marked increase of eotaxins in the whole lung without elevated IL-13 protein levels suggests that the increased eosinophilia observed in our model could be due to eotaxin derived from increased M2 macrophages undergoing hypoxia. As a proof of principle, we choose to investigate HIF-1 α regulation of eotaxin-2 transcription. The employment of both ChIP-pulldown of HIF-1 α bound chromatin and luciferase reporters demonstrated that eotaxin-2 transcription could be induced by HIF-1 α provided that there are available HREs for HIF-1 α binding. Although HIF-1 α has previously been associated with eotaxin-2 production in lung macrophages (42), this is the first time HIF-1 α has been demonstrated, to our knowledge, to bind to the promoter region of an eotaxin directly. Thus, subsequent dysregulation of HIF-1 α as a result of GSTO1-1 deficiency could result in substantial eotaxin-induced eosinophil accumulation in the lungs in response to HDM (Figure 8).

Collectively, these results suggest an anti-inflammatory role for GSTO1-1 in HDM-driven allergic airways disease through the regulation of M1 to M2 skewing, the suppression of HIF-1 α function, and glycolysis in M2 macrophages, and in attenuating HIF-1 α -induced production of eotaxin-2. Further investigation of GSTO1-1 in a clinical setting due to its observed upregulation in exacerbation patient PBMCs, where GSTO1 polymorphisms could identify subgroups of asthmatic that may be prone to worsened eosinophilic inflammation during exacerbation.

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Author Contribution

L.S performed and designed mouse experiments, analyzed data, generated figures, drafted, and edited the manuscript. B.G, S.S, and F.E performed experiments and analyzed data. P.B, G.K, and S.K contributed resources, interpreted data, conceptualized mouse and *in vitro* experiments, and edited the manuscript. M.Y and P.S.F conceptualized, coordinated, designed, and supervised mouse experiments, interpreted data, drafted and edited the manuscript. All authors contributed to data discussion and revised the manuscript.

Conflict of interests

The authors declare no conflict of interest

Data availability statement

The data that support the findings found in Supplementary Figure 1 are available in the Gene Expression Omnibus at <https://www.ncbi.nlm.nih.gov/geo/>, reference number GSE16032 (Fig S1 A), GSE30326 (Fig S1 B), GSE22528 (Fig S1 C), and GSE41649 (Fig S1 D)

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Figure Legends

Figure 1: GSTO1-1 knockout mice present with increased eosinophilia, macrophage infiltration, and remodeling in response to HDM exposure. (A), Differential counts normalized to total cell number in BALF (Fig 1A) collected from one lung lobe (n=6-8). (B), Airways resistance (relative to baseline expression) in response to increasing doses of methacholine (Fig 1B) determined by flexivent analysis, significance determined using two-way ANOVA (n=5-6). (C-E), Flow cytometry analysis (Fig 1C) of cells gated as eosinophils (CD45+ve, CD125Int, Siglec F+ve, Fig 1D) and neutrophils (CD45+ve, CD125+ve, Siglec F-ve, Fig 1E) expressed as a percentage of CD45+ve cells (n=4). (F), Gene expression of GSTO1-1 (Fig 1F) in mouse whole lung expressed as fold change from WT SAL normalized to HPRT (n=7-9). (G&H), Histological analysis of mouse whole lung tissue for AB-PAS stained goblet cells (Fig 1G) and Masson's Trichrome stained collagen (Fig 1H) under light microscopy and image analysis software **with representative images (Fig 1I)** (n=5). All data expressed as SEM+/- . Unless stated, significance was determined by one-way ANOVA ($P<0.05$ =* or #, $P<0.01$ ** or ##, $P<0.001$ *** or ###, $P<0.0001$ **** or ####).

Figure 2: Eotaxin-1 and eotaxin-2, but not Th2 cytokines, are upregulated in GSTO1-1 deficient mice in response to HDM. (A-F), Protein expression of eotaxin-1 (Fig 2A), eotaxin-2 (Fig 2B), IL-5 (Fig 2C), IL-13 (Fig 2D), IL-25 (Fig 2E) and IL-33 (Fig 2F) determined by ELISA, normalized to total protein determined by BCA (n=5-9). All data expressed as SEM+/- . Significance was determined by one-way ANOVA ($P<0.05$ =* or #, $P<0.01$ ** or ##, $P<0.0001$ **** or #####).

Figure 3: Expression of M1 factors are lower in the lungs of GSTO1-1-/- mice, while M2 factors are increased. (A-B), mRNA expression of whole lung IL-1 β (Fig 3A), and IL-6 (Fig 3B) expressed as fold change from WT SAL normalized to HPRT (n=6-8). (C), Protein expression of NLPR3 inflammasome (Fig 3C) determined by ELISA, normalized to total protein determined by BCA (n=6-8). (D-F) mRNA expression of whole lung IL-10 (Fig 3D), Arg-1 (Fig 3E), and Ym1 (Fig 3F) expressed as fold change from WT SAL normalized to HPRT (n=4-9). All data expressed as SEM+/- . Significance was determined by one-way ANOVA ($P<0.05$ =* or #, $P<0.01$ ** or ##, $P<0.001$ *** or ###, $P<0.0001$ **** or #####).

Figure 4: GSTO1-1-/- mice have increased M2 macrophage populations compared to WT mice after HDM exposure. (A-I), qPCR data of mRNA extracted from lung isolated macrophages in HDM exposed mice (normalized

to HPRT) for CD38, iNOS, IL-1 β , IL-6, CD206, Arg-1, Ym1, TGF- β and IL-10 (Fig 4A-I) shown as relative expression (n=3-4). (J-L), M2 macrophage populations from isolated macrophages (Fig 4J-K) and lung cells stimulated ex vivo with HDM (Fig 4L) gated as F4/80+ve, CD11b+ve, CD206+ve, and expressed as a percentage of total macrophages (n=3-4). (M), GSTO1-1 mRNA expression in macrophages differentiated into M0, M1, M2, and M3 phenotypes, significance determined using one-way ANOVA (n=6) (Fig 4M). All data expressed as SEM $\pm$ /. Significance was determined using unpaired student t-test unless otherwise stated. ($P<0.05$ =*, $P<0.01$ =**, $P<0.0001$ =****).

Figure 5: HIF-1 α expression is upregulated in the lungs and immune cells of GSTO1-1/- mice in response to HDM-induced allergic airways disease. (A) ECAR measurements (mpH/min) of isolated lung macrophages normalized to cell count as a basal reading or in response to glucose, oligomycin, and 2-DG exposure. Statistics determined using 2-way ANOVA (Fig 5A). (B-C) Basal readings of isolated macrophages measuring ECAR (Fig 5B) and OCR (Fig 5C), significance determined by unpaired student t-test. (D-F), Immunohistochemistry staining (Fig 5D) of mouse whole lung for HIF-1 α and H-Score analysis for HIF-1 α expression in the lung epithelium (Fig 5E) and peribronchial (Fig 5F) regions (n=5). (G-H), Immunofluorescence staining of HIF-1 α (Fig 5G) in macrophage enriched cells isolated from whole lung, with cellular fluorescent intensity (Fig 5H) determined through image analysis (n=2-4). All data expressed as SEM $\pm$ /. Significance was determined by one-way ANOVA unless otherwise stated ($P<0.05$ =* or #, $P<0.01$ =** or ##, $P<0.0001$ =**** or #####).

Figure 6: HIF-1 α is expressed in M2 macrophages, which is more prevalent in GSTO1-1/- HDM exposed mice. (A-D), Immunohistochemistry staining of HIF-1 α and CD206 in macrophage enriched cells isolated from mouse whole lung tissue (Fig 6A-B) and mouse whole lung tissue (Fig 6C-D) determined as number of dual-positive cells within a set high-powered field (n=4-5). (E-F), Flow cytometry of M2 macrophages expressing HIF-1 α (Fig 6E) gated as F4/80+ve, CD11b+ve, CD206+ve, HIF-1 α +ve cells expressed as a percentage of total macrophages (Fig 6F), (n=4). All data expressed as SEM $\pm$ /. Significance was determined by one-way ANOVA or unpaired student t-test ($P<0.05$ =* or #, $P<0.001$ =*** or ###).

Figure 7: HIF-1 α drives eotaxin-1 and eotaxin-2 production in BMDM deficient in GSTO1-1 exposed to hypoxic conditions. (A-B), Protein expression of eotaxin-1 (Fig 7A) and eotaxin-2 (Fig 7B) normalized to total protein in BMDM incubated with a hypoxia chamber (O_2 levels >1%) for a period of 2, 4 or 6 hours (n=3). (C-D) Protein expression of eotaxin-1 (Fig 7C) and eotaxin-2 (Fig 7D) normalized to total protein in BMDM incubated with a hypoxia chamber (O_2 levels >1%), which have been treated with DMAG17 or vehicle control (n=7-9). (E), Luciferase activity of RAW macrophages transfected with either an empty vector (Vector), mutated eotaxin-2 promoter sequence (Mut), or wild type eotaxin-2 promoter sequence (WT) expressed as a fold change from untreated cells (Fig 7E) (n=8). (F), HRE present on the eotaxin-2 promoter region (Fig 7F) and qPCR data of eotaxin-2 in gDNA bound to HIF-1 α isolated from ChIP (relative to Input DNA) (n=3). All data expressed as SEM $\pm$ /. Significance was determined by one-way ANOVA ($P<0.05$ =* or #, $P<0.01$ =** or ##, $P<0.0001$ =**** or #####).

Figure 8: Schematic of GSTO1-1 proposed role in regulating M2 macrophage phenotype and HIF-1 α -induced eotaxin-2 production. Normal expression of GSTO1-1 in macrophages facilitates TLR4/MyD88 activation of NF- κ B

in response to LPS derived from HDM while regulating IL-4/13 receptor activation of STAT6 due to Th2 cytokines elevated by HDM. Similarly, GSTO1-1 regulates glycolysis and HIF-1 α induction of M1 associated cytokines. Additionally, HIF-1 α could negatively regulate GSTO1-1 expression as observed previously (43). Deficiency of GSTO1-1 results in reduced TLR4 activation of NF- κ B and upregulated Arg-1 and Ym1 expression due to upregulate STAT6. Lack of GSTO1-1 resulted in dysregulated glycolysis and upregulated HIF-1 α expression in skewed M2 macrophages, resulting in HIF-1 α activation of the eotaxin-2 promotor and heightened eosinophilia in the HDM-exposed lung.

Supplementary Figure 1: PBMCs from exacerbated asthmatic patients have increased GSTO1 expression in PBMCs and Nasal Lavage when compared to convalescent and post-exacerbation patients. (A-B), Post-hoc analysis of published array data taken from the GEO-data repository GSE16032 (Fig S1 A) and GSE30326 (Fig S1 B) of GSTO1, GSTO2, GSTA2, GSTM1, GSTP1 and GSTT1 expression in PBMC or Nasal Lavage as fold change relative to the non-exacerbation group (convalescence or post exacerbation). PBMCs: n=5, Nasal Lavage: n=16. (C-D) Post-hoc analysis of human lung macrophages (Fig S1 C) and airway epithelium (Fig S1 D) for GSTO1 from the GEO-data repositories GSE22528 and GSE41649 respectively (n=4-5). All data expressed as SEM $\pm$ SE. Significance was determined using unpaired student t-test. ($P<0.05$ =*, $P<0.001$ =***).

Supplementary Figure 2: Flow cytometry analysis of lung eosinophils phenotype, lymphocytes and CD11c cells. (A-C) Eosinophils (CD45+ve, CD125Int, Siglec F+ve) from mouse whole lung were determined as inflammatory or resident based on the expression of CD101 (inflammatory), or CD62L (Resident) expressed as a percentage of CD45+ve cells (Fig S2 A-C) (n=4). (D) Percentage of CD11b-ve, CD11c+ve macrophages from ex-vivo HDM stimulated cells that express CD206 (Fig S2 D) (n=2-3). (E-F) Percentage of Th2 (Fig S2 E) and Th17 (Fig 2S F) cells in whole lung (n=4). All data expressed as SEM $\pm$ SE. Significance was determined using unpaired student t-test. ($P<0.05$ =*, $P<0.01$ =**).

Supplementary Figure 3: Promoter Regions of the Murine Eotaxin-1 and Eotaxin-2 genes (C57BL6) with highlighted HIF-1 α Responsive Elements. (A-C), HIF-1 α consensus sequences (Fig S3 A) identified in the eotaxin-1 (Fig S3 B) and eotaxin-2 (Fig S3 C) promoter regions determined using MatInspector (Genomatix).

Figure 1

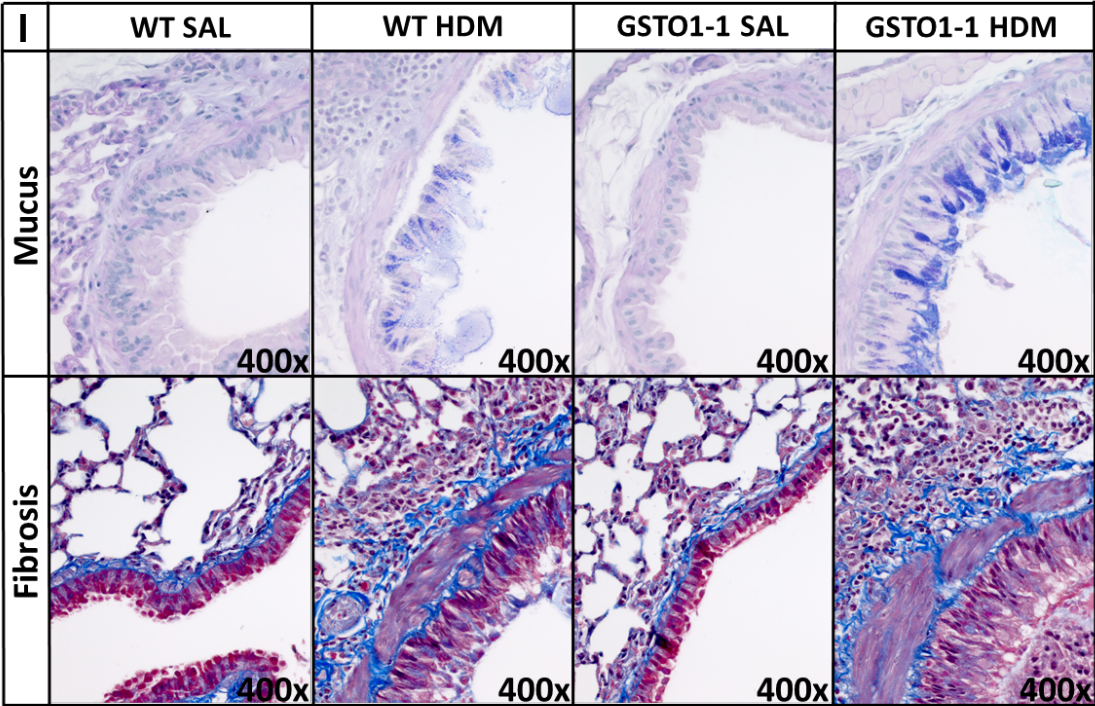
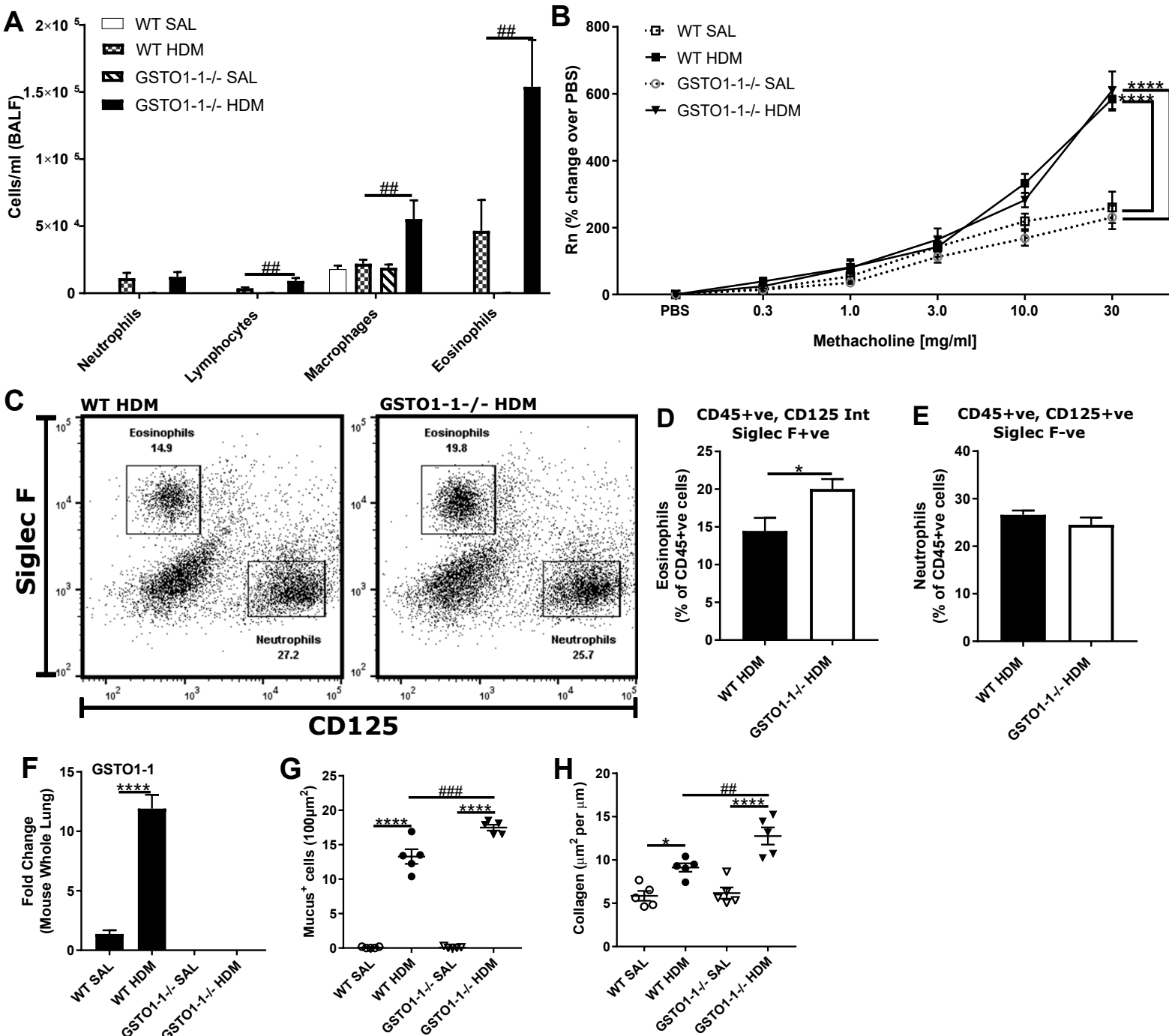


Figure 2

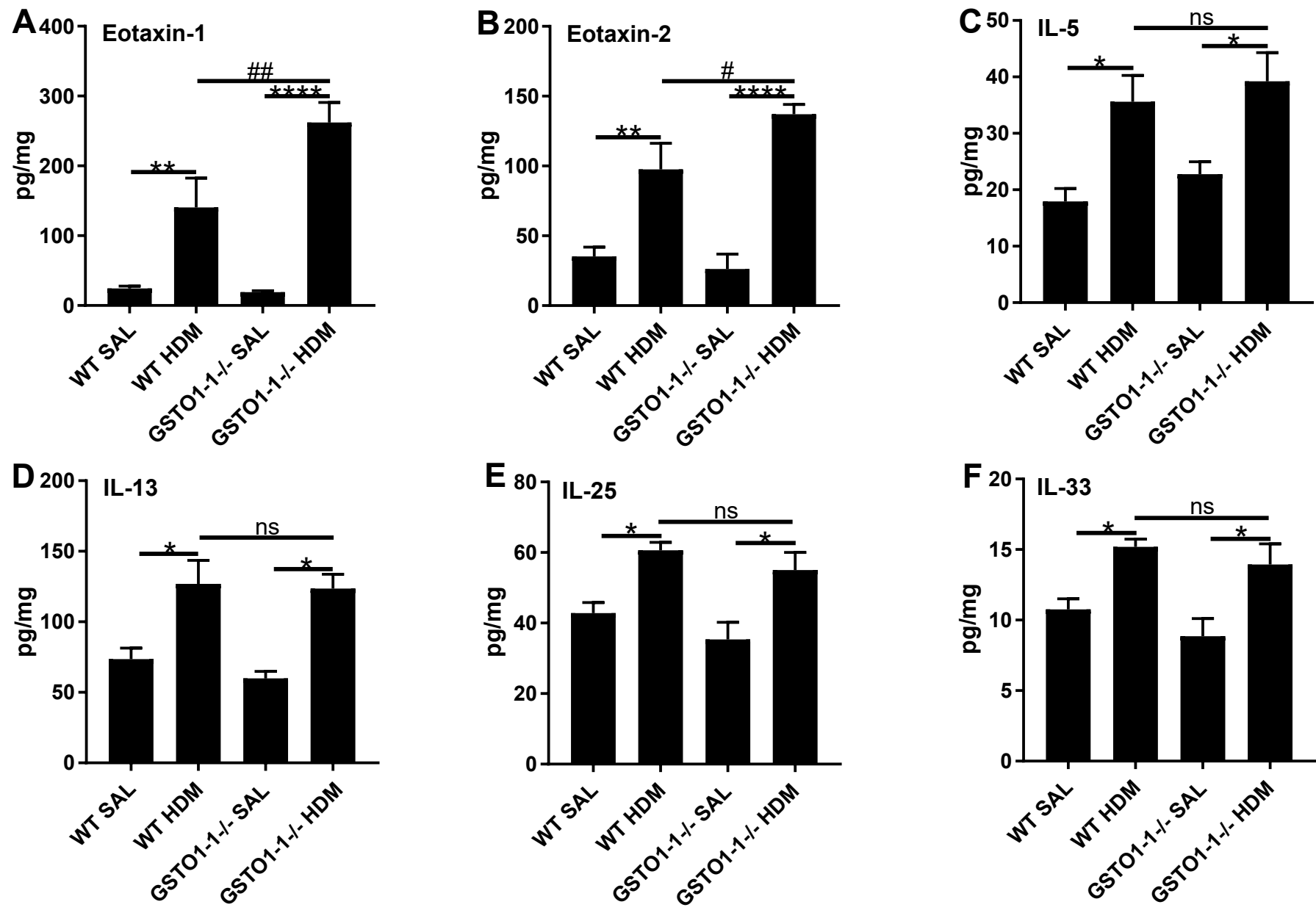


Figure 3

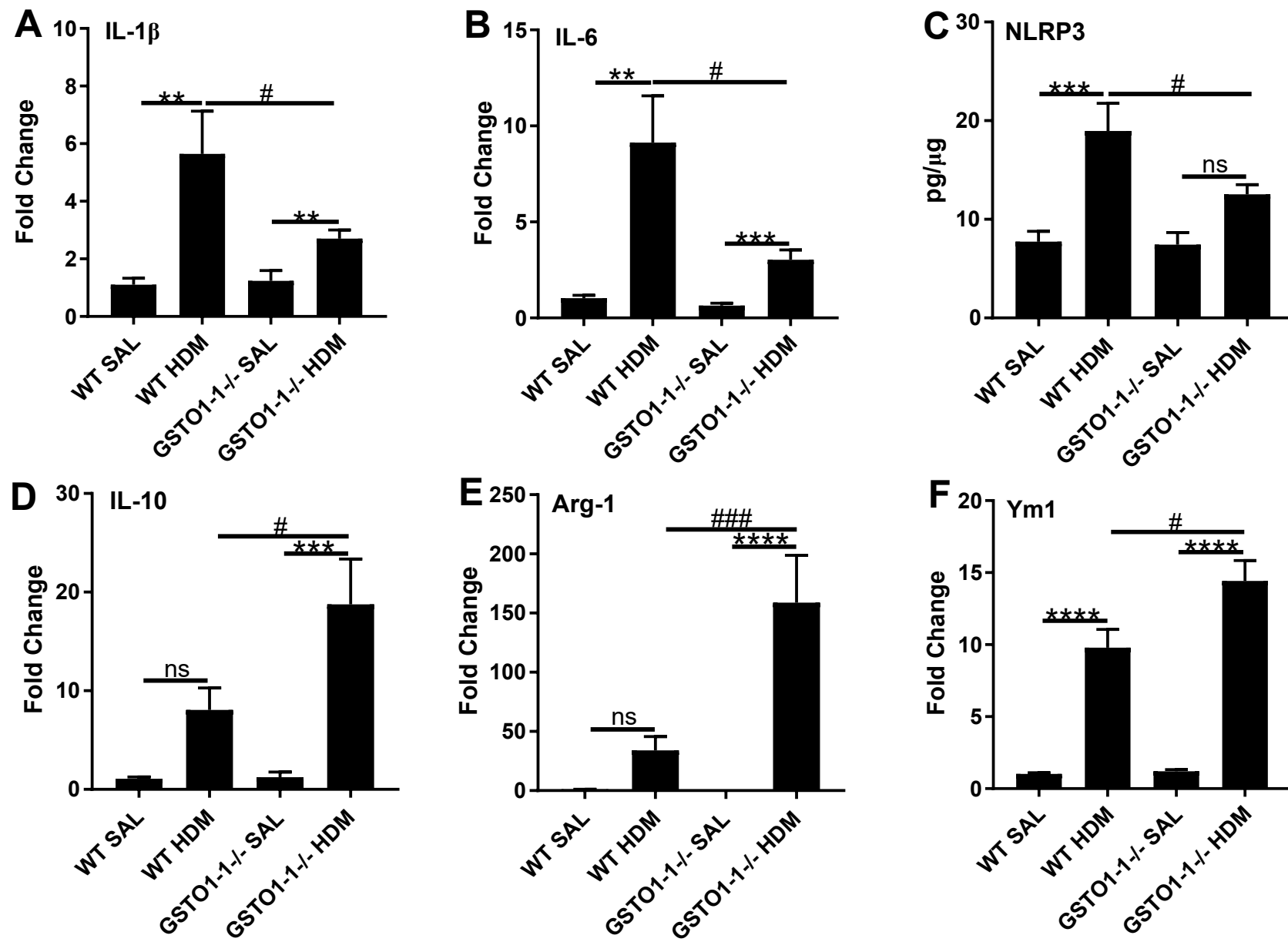


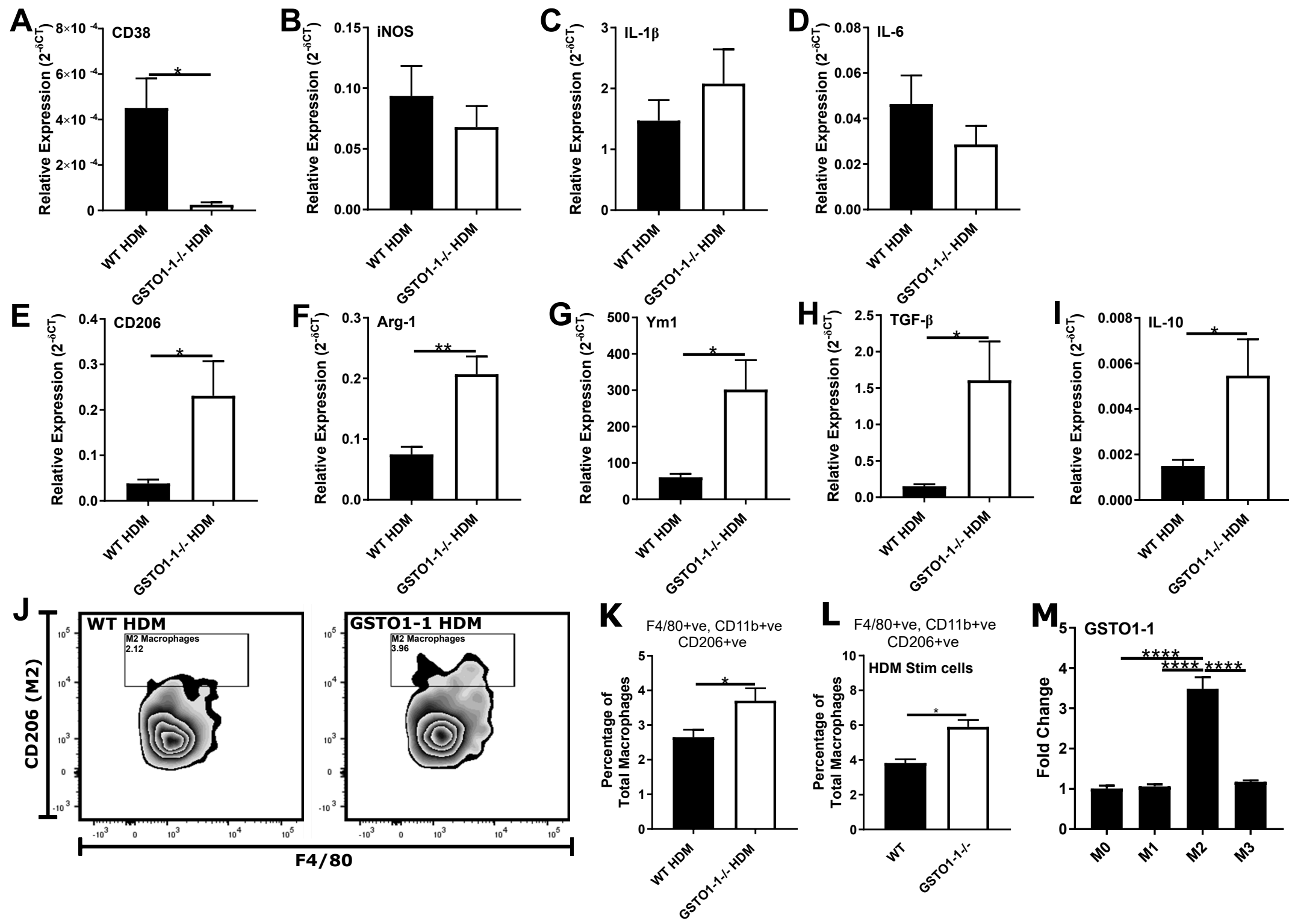
Figure 4

Figure 5

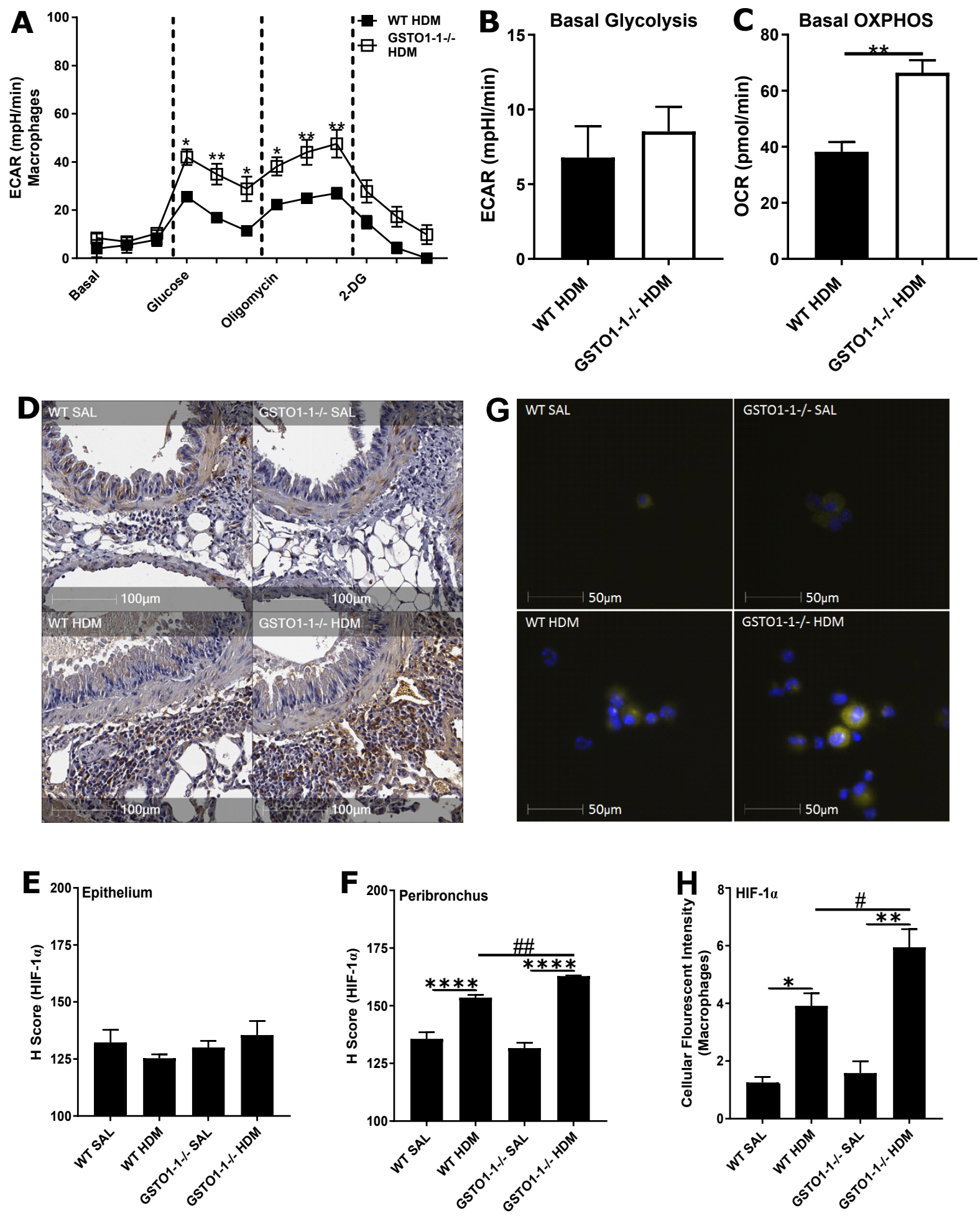


Figure 6

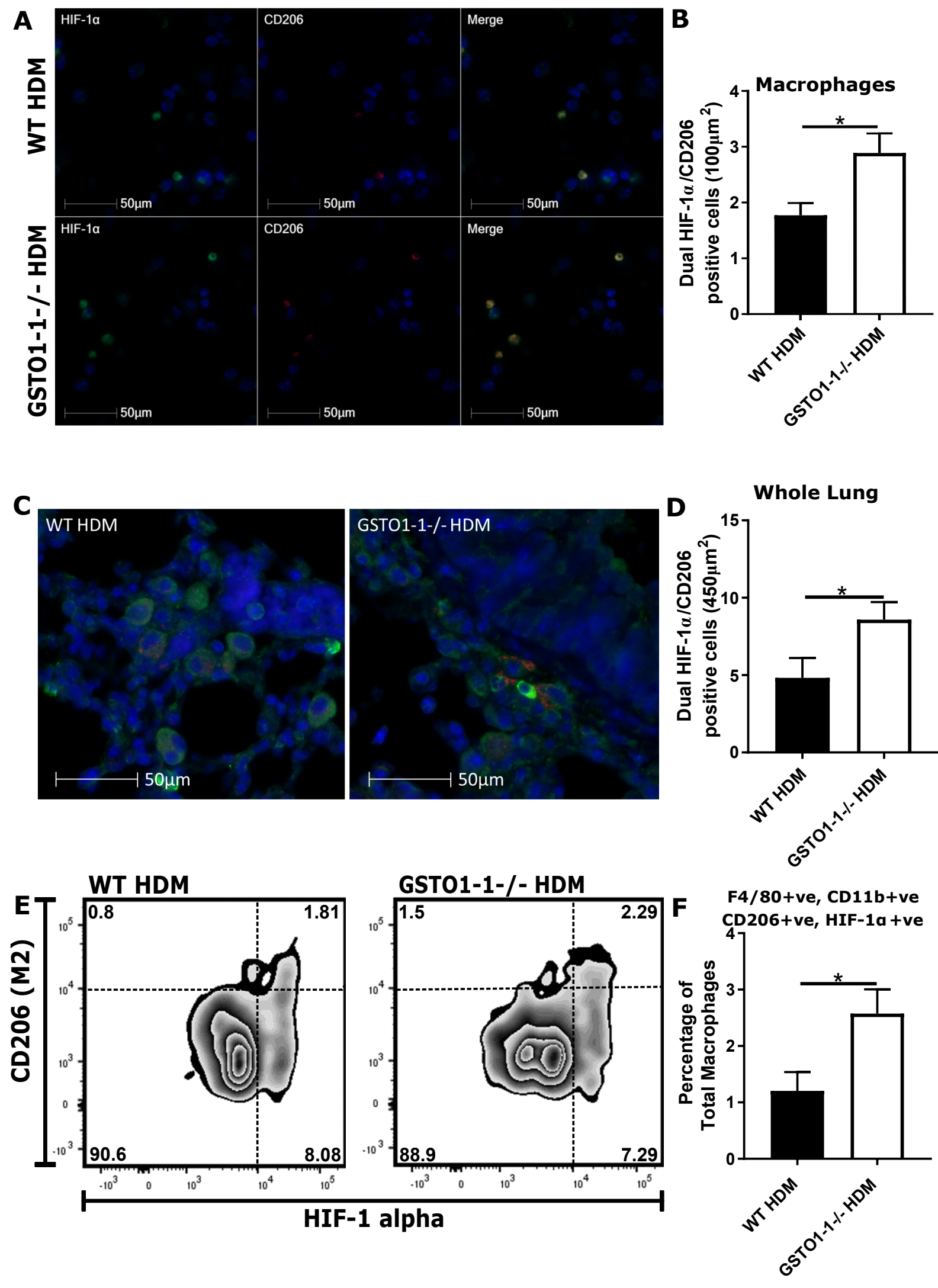


Figure 7

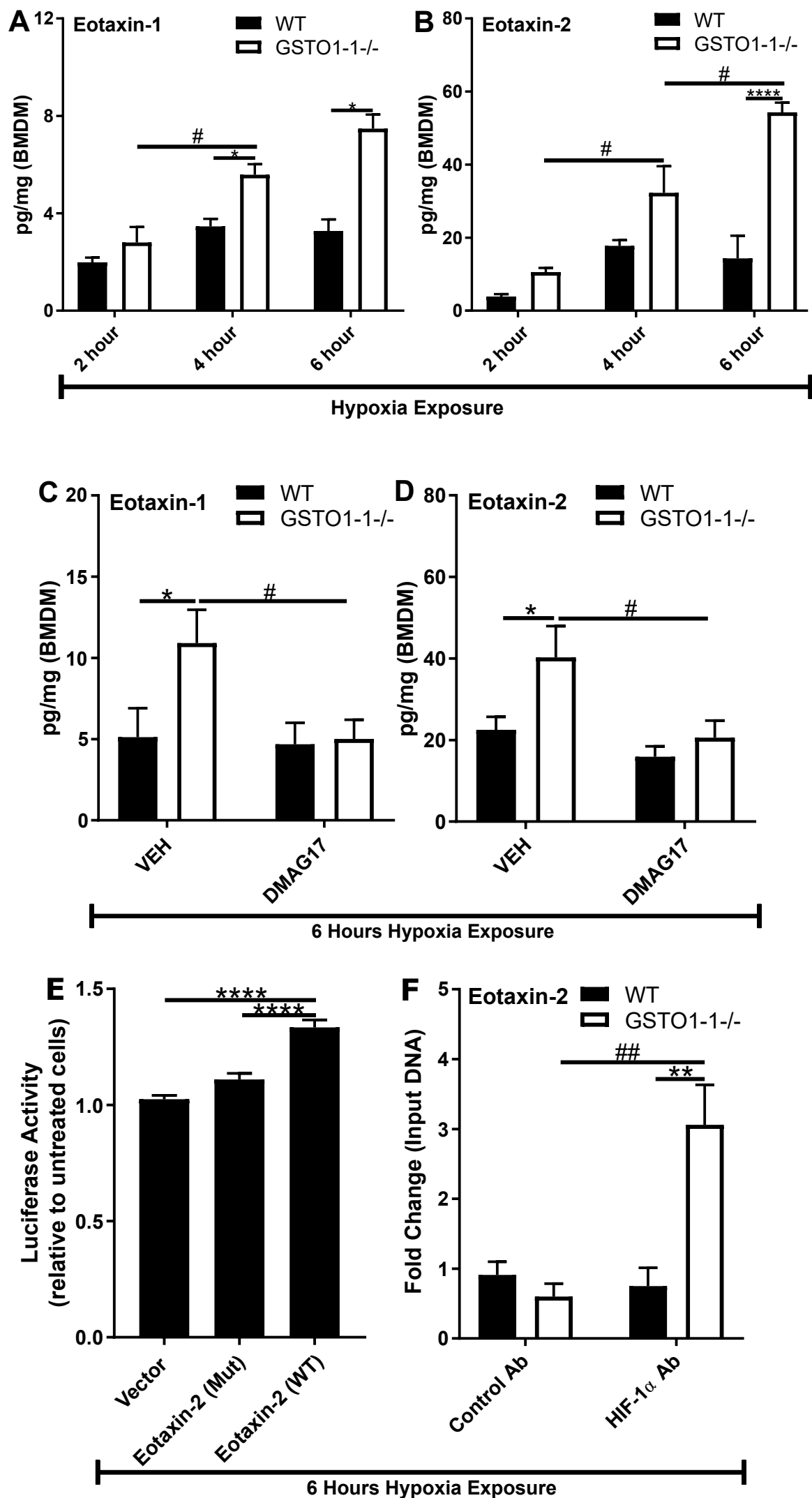
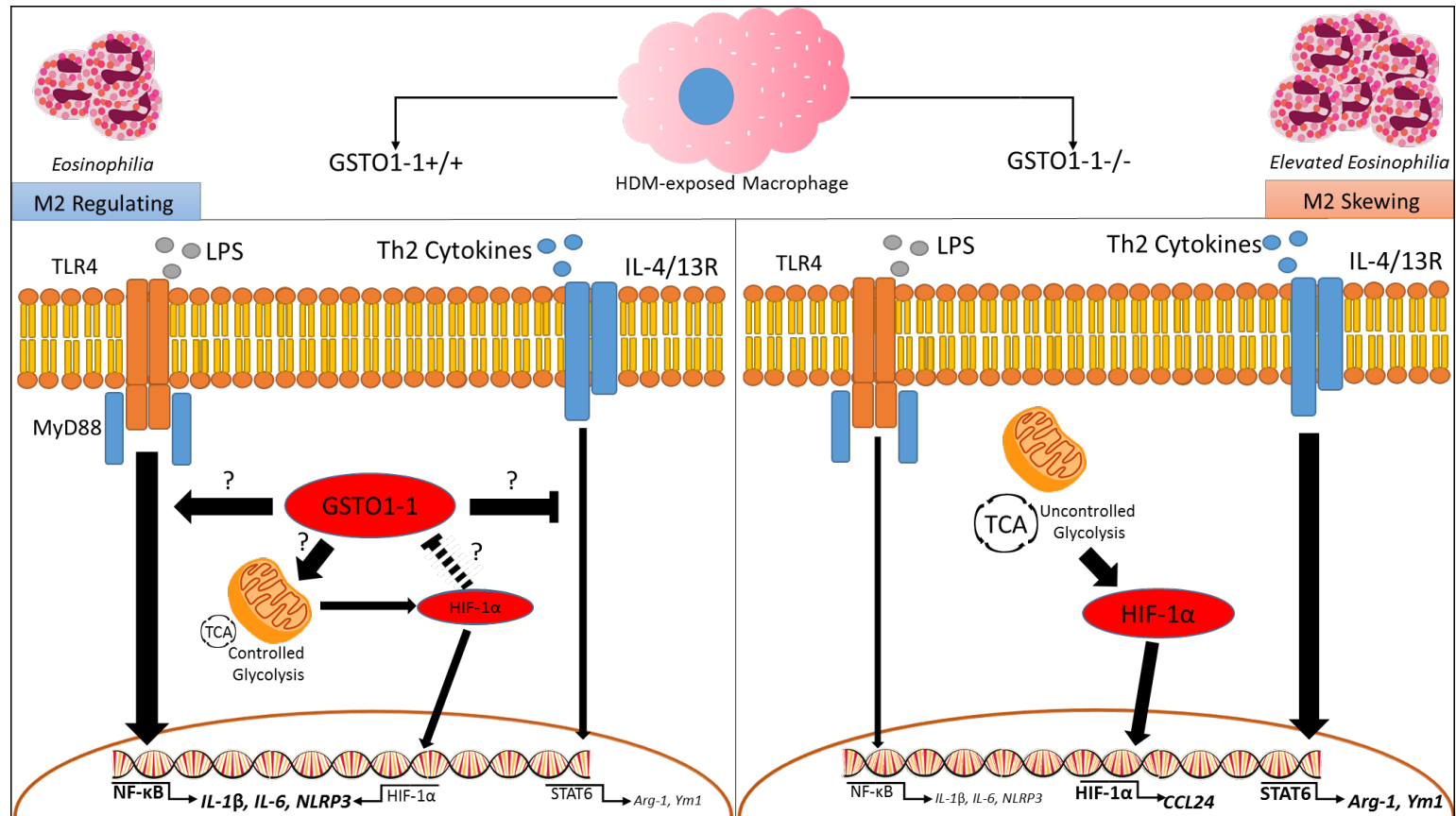
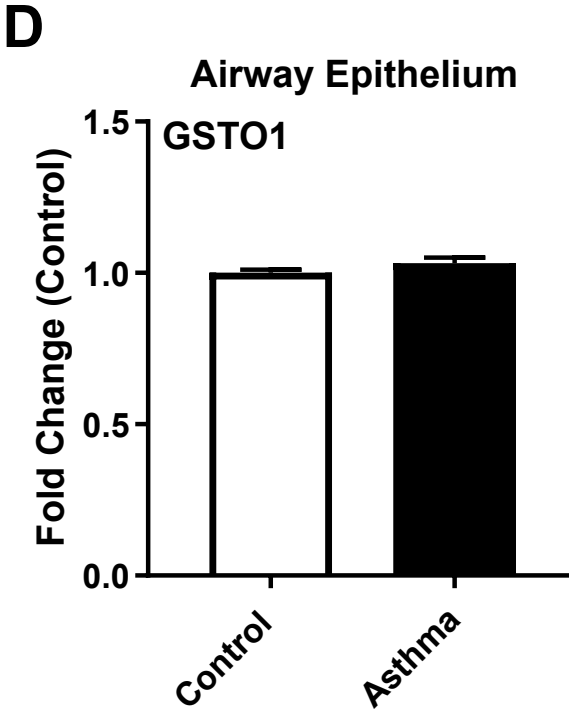
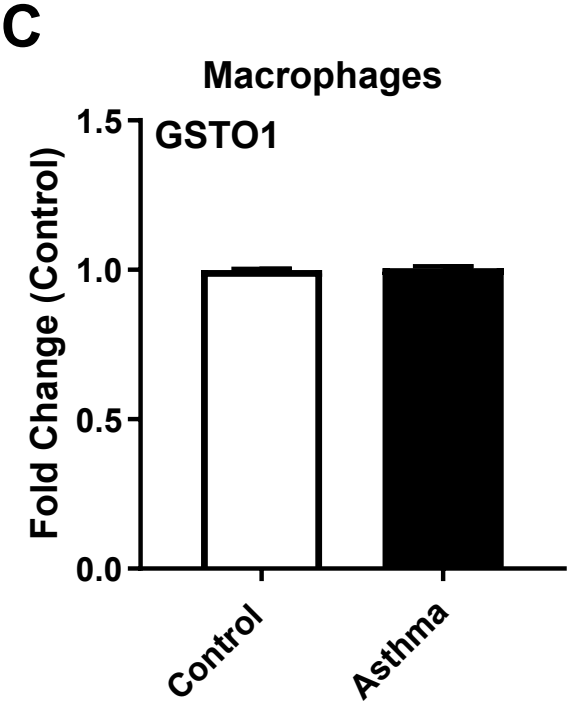
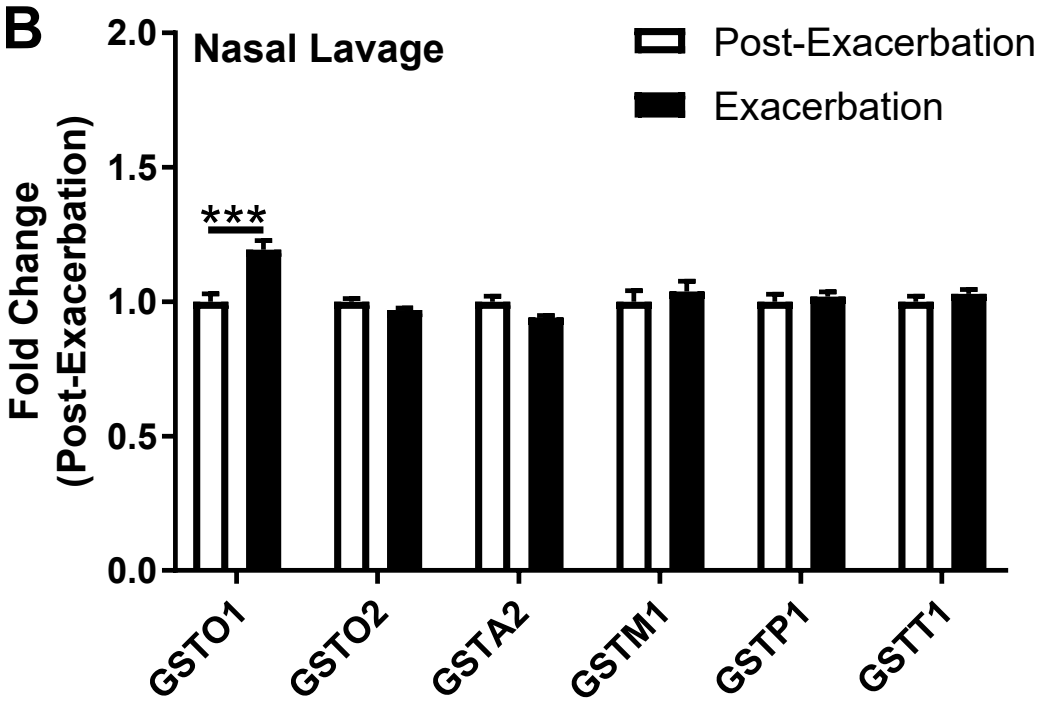
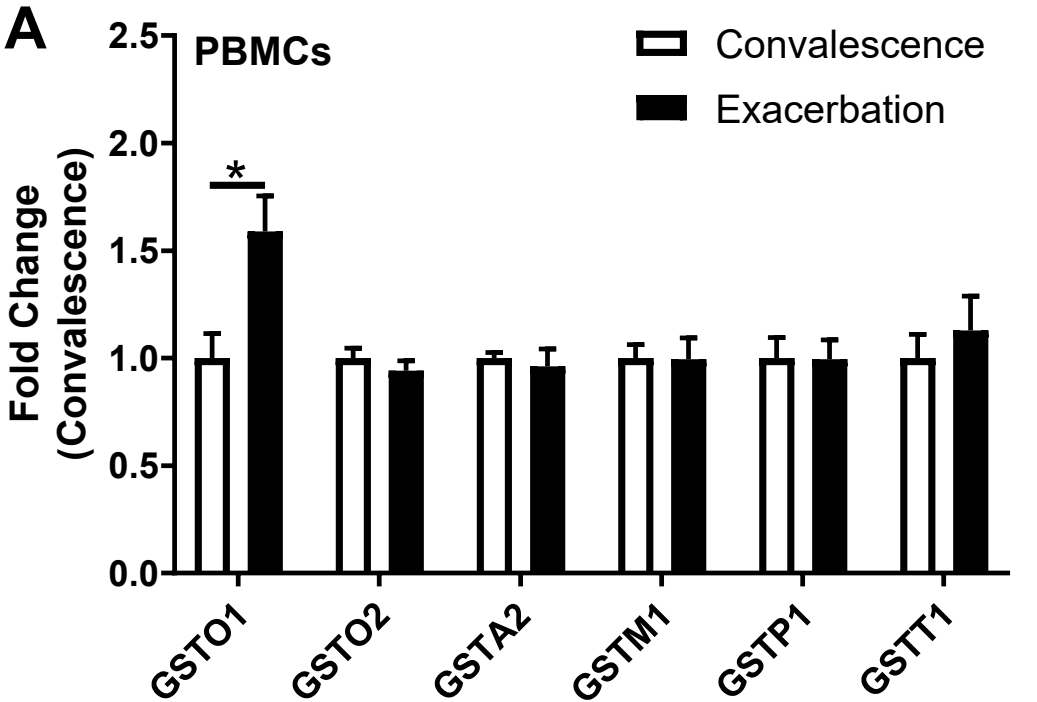


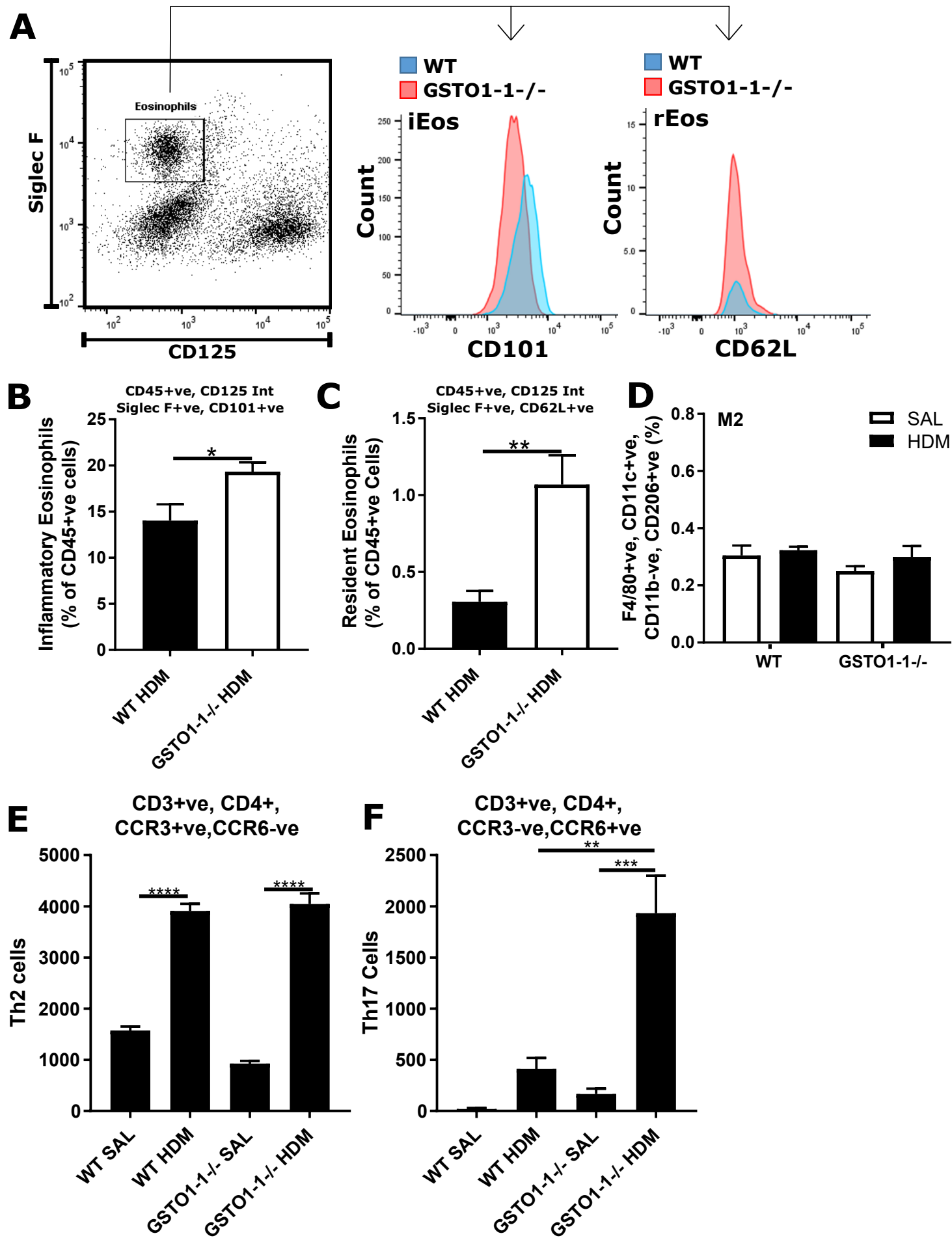
Figure 8



Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3

A HIF consensus sequences

5′-3′: 1. **ACGTG** 2. **GCGTG** 3. **CCGTG**

3′-5′: 1. **CACGT** 2. **CACGC** 3. **CACGG**

Transcription start site (TSS): **TATA**

B Murine CCL11 – Eotaxin 1 (C57BL6) chr11:82,056,823-82,058,042 1,220 bp.

TGCTGCACCAGGGGTTGAGAACAGCAAGCATGAGCAGAACAGCGAGAGGCTCCATACAAAAGCACAAAATAGACATGGC**ACGG**AAGCACTACAAAGGCAGCAATGGGTCTGAGTGAGATCGCTCCAGAGTCTCATGCTGTCATATTCCTGGGTTTCCTTCAGAACGTCAGCAAGTGAGTTAGCCATAGGGCAGGGGAATGTGGGTAGATTCCGTGTTTTGATTGGCAGGCTTAGGTTACCTAAGGCTTTACTTGCTGCATACTGTCCCTCCCCACAATGCACCGGAATTTCATCATATGTACATCCAAT**CACGC**ATGGGATGACAGAGAGAGTTTCCCTAAACGTTCAATTCTGGGAACATAGCTTGCCCATCCAAGCCAGATGCACTCCTCCTCTAATTCCAGAATGCATACTGAGATGTCCCTGAATAACTTCTAACCAGTGAAAGTTGGGTGGGGTGCTAAGGAA TTACCGGAGATTATTAGGGCAGAGAAACCAGTTTCTGCAATTCCTTGGAACAAGGATGTATATGTAGCTTGGTACAGGTG GTGGTCTGTATTGCCAACCACTGGTGTGATGTGTGATGTGTGATCTGTCTGTTTGCTGTCTGTCTGTGTGAAGC TCAAGCCAAGTGTGATCTTAGTTTTCTAAGCTGTCCCTACACCTGATGCTTCCACAATGCTAAGTTCCTGGACACTAA GATTGTATGACCATTGAATTACATAAAAAGGGCAAGCATGACTATCATGCCCCTGTTTTCTGAGAAGGGCTCTTCTGGGA CTCCAAAACACCAGGTGAACAGGAACCAGGACTGGCTTCCACATGTCATCTCCTGGCTTCTTTGTTCCACTGCACAATG ATCAGTCGCAATTTTCATAATCGCACAAATCTCTTCAGTTTTCACTCCCATCCCTTAACAGATGCCAAGTCCCTCCACGGT TTTGTGTTTTCTTCTATTTGCTGTCTTTCCAGGATCTGAGAGTGTTCCACTGC**TCAAAGCCCTCCTTCATGACCTTGTG CAGGCAGTTTTCTCTGGAAGCTTCCACCACCTCTGCTCCCTATAAAGCTAGTCGGGAGAGCCTACAGAGCCCAGAGGCT GAGATCCAAGCAGTAACTTCCATCTGTCTCCCTCCACCATGCAGAGCTCCACAGCGCTTCTATTCTGTCTGT**CACGGT CACTTCCTTCACCTCCAGGTGCTGGCTCACCCAG****

C Murine CCL24 Eotaxin 2 (C57BL6) chr5:135,568,937-135,574,049 5,113 bp.

CTTGTGTATTTTATAAGGTCCCAGGGTCCTCCTCTCCCCCTCTATTGGTCCTGTTTAGATACCTACCCTGTGTCTTCTT ACCATCTCTCATCCTAATGTCTTCTGGGAACAAGCCATCATCTCAGAGAGCCTGCTTTTGCTCCTTTTAGTCCCTTTA GATGGATAGATGGATGGGCAGGTAGAGTGAGGGATGGATGGATGGATGGATAGATGGATGGATGTAGAGATCAATGGAA GAATAGGTAGAAAGAAAGATGGACAGGTAGATGTACGTATGTATGAACAGGTAGACCAATGGATGGATAGATAGATCAA TGGATGAATGGGTGAAAGATGGATAGACAGGTAGATGAATGAATGGATAGATAGATGGATAGGTAGATGTATGTGTAA ATGTATGTATGTATGTATGTATGTATGTATGTATGTATGTATGTATGGACAGGTTGATCAATGGATGGATGATAGGCAGATCAAAGG ATGTATGGATAGATAGATGGATGGATAGGCAGATCAGTAGATGGATGGATGGATGGATGGATGGATGGATGGATGGATGG ATGGATGGATGGATGGACAGGTAGATGGATGGATAGATGGATGAATGGTTGGATGGATAGACAGACAGGTAATCAATA GATGAATGAGTGGACAGAAGGCAGGGAGATAAAGGAGACTTACTCACTATGCAGCTACAAAACACTGAGGCTCAGAGAA CCAAGTAACTTACCAGGATTT**CACGC**AATAAGCCACAGAGCAAAGTCATCTAAAACATTTTCCCTTGGCTGTGGCCCA GGCAGGATGACAGCCCTCCCCAGCTTTTCTCTTGCCAGAACCCCAATAATTGCTGACTCCACTGGACAAGAAATGGGC AACAACTTCCCCATTTTTTGACCACAGAATGCTCAACGATTTTCGAGGAAGT**CCGTG**GGGGAGAGGGCGGGAGGGGACA CCAGAGCCTCTATCTCCTGAGCTGTCCATATAAAGACCAGTGGGAGGGAGAT**CGGGGTGTCCTCATATCTCCAGGATCT CTTTTCTGCACCCCACTTTGAACTCTGAGCTGTGCCTGACCTCCAGAACATGGCGGGCTCTGCTACGATCGTTGCCGG CCTCCTTCTCCTGGTAGCCTGC**CGGTG**TTGCATCTTCCCCATAG**GTGAGACTTCTGGGCCAGTGTGGGGCTGCTGGTGA CAAAAGCTTCTAAGGCCATGGCTGGTTTGGCACAGTGAAGGGTGAGGGAACCAAGAAGTCTGGAGCTGGGGCTGAGGGA GGCATTAAGTGGCTGGGGTATGACAGGCTCTGTGCTGTGCAGTTGAGCCTACGGCAGGCTGGATACTGACCTGATTTCTT CTTCTCCTTTTCAG**ATTCTGTGACCATCCCTCATCTTGTCTGCACGTCTTTATTTCCAAGAAAATTCCAAGAAAACCGAG TGGTTAGCTACCAGTTGGCCAACGGCAGCATCTGTCCCAAGGCAGGGGTCAT**GTGAGTATCTAACTCCAGGGTCCCAGAA CTCCAGGGTTCTCCTCCTCCCCAGGAGGTCCAGCCCTGGGCATCTCTGAAGAAGGCCTAGACTTCATGAACCTTCAGCT TCCTCACCCAGGAACTAGGGGGAGAGCAGGGCGAGGGAGGTAGGTCAAGTTCGTAGTATGCTTGCTTAGGATACATGAAG CCCTGCGTGGTTCCATCCCTAATATCACATAAACCAACAGGACATGGTATAGGCCAGTAATTCACAACTCAGGAGGCTA AGAAGCTGACCAAGTACCAATGTGGGCAAGGTATTGTG**CACGG**AGGGGAAATACATGATATGCCATCAGTCTAACTAG TCTTCTCAACAATTTTCATAAGACAATGCCAGAACCCTGCCTAGAATCCCCAGGGAGGGGCTGGGACGTGGCTCATC

AATAGTCTACTTTTCTTAACCTTGTAGAAAACCTCTGGGTTTCATTCCCAGCATGGCAAAACCAAAAACAATCTCACAGAGA ACATAGGACTTTTTCCATTTTATGAAGGTAGTTGAGGCTCAGTAAGACCTCAGGCTCAAGGCTGCATGACCACCCAGG TCTCTAGATTTTCACATAGCTTCAGAGCCACCTTTTACCTGGGGTAACAAGCATGACCTCGTGTCTTAAGCTATGCTGT ACAGGGGGAATGACATGTTAATATTCCCCAGCTGACATCAGAGCGTCCCTGAGAGCTGGCACCAAGGGGGCATCCAG GGCTCACCTCTCAGCTATAGCAGGCCCAGGAGCCATGTTTTCCATGCCTAGTGTGATCTGATGATGTTTTACCTTTAAAAA ATGGGTATGTGTGTAGATGAGCTGGGCATGAGTGTC**CACGT**CATGTGTGTGCATGTCTGTGGAGACCAGAGGCTAATTT TGGGTGTCCCAAGTATACTGAGCACCTCCTTCAAAATAGAAGTCTCTCTCTCTTTTTTAAATTAAGTTTTGTGTGTGT GTGTGTGTGTGTGTGTGTGCATGCACACATGCATGCACTCTGCAGAACATGTATAGGGGTTCTCTTATTCTACCTGTG GGTCCCAGAAAATAGAACTCAGGCTTGGCGACAAGCACTTTCCCTGTGAACCGTCTCTGGCTTCCCTACCAGCCTGGAG CTCACCAAGTAGTTTTAGGCTAGCTGGCTGTCAAGCTGTGGGAATCCCCCTAGCTCTGCCTCCTCAGTGTGAGATTAC AAGCTCATGCCACCATGCTTGGAGTCCTGACAATTGA**ACGTG**TATCCTCACAGTTGTAAGGCATACATTTTCCCCAGCT AAGCTGACCTCTCAGATCTGTTTCTCCTGTATAGATCTGACACAGACCATACTGTGTATCACCATATCCAGCACCATACT TCAAAGGCAGAGCCTCCTTTGTCTCACAGGGACGCCACAGAATGAGCGTATCCGCACACTCCACCATCCTGTCCCCCAG TGGTAAAACACAGAATTCCGAGAGAAGTCTCATGTCAAGGGCTTCCTAGCGCCAAGGGCAGGCGGGCAGCTGAGACTCG CTTGCGCTCACTTCCCTATAAAGGGAAGTTAGTCTCACACACCTGGCAGGACGTCAACTAAACCCCTGCCCTTCTTTCC TACAG**CTTTCATCACCAAGAAGGGCCATAAGATCTGTACTGACCCCAAGCTGCTGTGGGTTTCAGAGGCACATACAAAAAC TGGATGCCAAGAAAAACCAGCCTTCTAAAGGGGCCAAGGCAGTGAGAACCAAGTTTGTGTCTCAGAGACGCCGTGGCAA TAGCACCGAGGTTTAGCCGCCCTCTCCTCTGTCCCTGAACTTGGACATAGGTATTGACGCTTTACCAGGCTGCTTTGGGG TTTTGACAGACCAGCTGGGATGAGGTCTAAGTTTCAATTTCCCCATTCTCAGCATTTTCTTTTAGGGGATGTGTTTATTCTGT GAGAACCTGAGTGAGCTGCAAGCATGCCAGAGTTCTGGTGTCTCCTGTGTCTGAGTGAAAAGATTCCAGAAGAATATCAG TTGTCTGTGTTATCTCTTGTGTGGCTCAAAGGCTTCATCAGAGCCCAGGTGCATGAGCCCCGAGTCTCTGCAGGCGGA CCTTGTCTTGCAAGCTTCCATAGGGTTCTTGTCTTAGGGTGGGGCTGAGGGCTGTCTGTCTGTCCATCTCTGGGTG AAGATCTGTCTAACAGCAGTAACCTCCCAAACATGCTGTATGGGTATACCAGGCTGGTCCCATCCCCAGAACTTGGAAACAGA TAAAAAAAGTTGGCAGAAACGGAGGATTTCAACAGCAGCATTTTTTAAACCCATCAGTCTTTGTAAAGCCGGAGGTGTAA CTCAGATGGTGGTGTGCTTGCTGGCATGCATGGGGCATTGGGTTTCAACCACAGCACCGCAAAAACCTAGGCATGATGG TGCATGCCTAAGAAGATCAGAAATTTCAAGCTTAGCCAGACATCCCAGAAGGCAGAGGCAAGAGGATCTCTGTGAGTTCA ATGCCACCCTGGTCTACATAGCGAGCTCCAGGACAGCCAGGGCTACATAGAGAGACCATTTCTCAAACACCCTTCCCTA AAAATAAGTTCAAGGCTACATAGCCACACAGAAAATTTGAGGCCAGCCTGGCCCAAGGTTGCCCTCCTCTTGTGTGCCAG GATGT**AGAACCTCTCAGTACCGCTCCAGCACCATGTCTGCCTGGATGGCAACAAGCTTCCCTCCATGATGATAATGGAC TGAATCTCTGAAGCTATAAGCCAGCCCCAACTAATGTTTTTCCCTTTAAGAGTTGTCTGTGGCCATGGTGTCTCTTCATAG CACTAGAAATCCTAACTCAGACAGGTTCTGAGACTAAATTTACATAAAAAATTCAGCATAC**ACGTG**AAAAGCCAGGTA TGTGTGGGAGTGGGTATAGCCTGTTCTGGGGCAGAGGAAGAGAGAGCCTGGGAGATGACAAACCAGCCAGCTTTGCTAG AAGAAAGGATAGCTTTGGGTACACTGAGAGAACCCTATCTCATGGGAAAAGGGCACATAAGAGAGAAGAACACATCAG CCCTTCTCTGACCTCTGCATATTCATGTCCGTATGTGTGCCACCCACAGACT**CACGTG**CACACACACACACACACACA CACACACACAAAAGGTAGAGAACAATTGAGAAAGACACCAGATGTTATCTGGCTTCCATGCTCACACATGAGTACATA TATATAATAAAACAAATAATGTAAAAATAAAGATAAAAAGAAAG**ACGTG**ATATATAGCTGAAGGCAGAGTTCATTCTTCCA GGAACGTACCCAGGCCCCACCCTCTGAGCTAGCCCTCATTTCTGACACATGCCATTGAT**CCGTG**TCAGGGGAAGAGTGACT TTTCCCTAGTTAATAAGAAAAACAAGCAGG**GCGTG**TGGCATATACCTTTAATCCCAGCACTTGGGAGGCAGAGGTAGG CAGATTTCTGAGTTCGAGGCTAGCCTGGTCTACAGAGTGAGTTCAGGACAGCCAGGGCTATACAGAGACACCCTGTCT CGAAAAACCAATTAATAAAAAATAAAAGAAAAAAGAAAGAAAAACAAAGTCTGAGAAAACGAAGTGAAGAGCGGT TCTAAAAAGGAGAAGTGCAAAATGGCCAGAAAACACATGAAAAAAAATGTTCACTAT