

STRA6 is Differentially Expressed by Human PBMC Subsets

By

Crisha B. Barrett

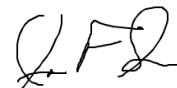
Thesis submitted to the Faculty of the
University of Michigan – Flint
in partial fulfillment of the requirements for
the degree of

Master of Science In Biology

Approved by:



Dr. Kanakadurga Singer, M.A., M.D.
Committee Member



Dr. Joseph Sucic
Committee Member



Dr. David Duriancik
Thesis Advisor

July 2019

Abstract

Stimulated by retinoic acid 6 (STRA6) was the first protein to be identified in a novel category of proteins, cytokine signaling transporters, due to its ability to function as both a cell surface receptor and a membrane protein that binds to retinol binding protein facilitating cellular uptake of retinol. Retinol is metabolized to all-*trans* retinoic acid which functions as a steroid hormone transcription factor ligand to regulate genes generally involved in cellular differentiation. The role of vitamin A, as all-*trans* retinoic acid, in immunity is well characterized, but the role of STRA6 in mediating cellular uptake of vitamin A in immune cells is not understood. The objective of this research was to characterize the expression of STRA6 on human peripheral blood mononuclear cell (PBMC) subsets. We hypothesized STRA6 would be expressed by all PBMC subsets, but at different intensities. We also hypothesized STRA6 would be co-localized with the lipid raft. A convenience sample of volunteers were recruited and PBMC were isolated by density gradient centrifugation. Multicolor flow cytometry was used to identify PBMC subsets and expression of STRA6. Confocal microscopy was used to determine cellular location and lipid raft association of STRA6 on cell lines and isolated PBMC. All T cell, natural killer cell, monocyte, and dendritic cell subsets analyzed expressed STRA6. Median fluorescence intensity of STRA6 was higher on pDC dendritic cells compared to mDC2 and mDC1 dendritic cells, cytotoxic T lymphocytes compared to T helper lymphocytes, intermediate compared to nonclassical and classical monocytes, and cytokine natural killer cells compared to both cytotoxic and CD56-/CD16 dim natural killer cells. Age was the best predictive factor for STRA6 expression. Confocal microscopy showed STRA6 was not associated with GM1 cholera toxin subunit B

staining and therefore not associated with lipid raft structures. These results provide preliminary data in which to target vitamin A signaling, through cellular uptake of retinol, in immune cells to improve immune homeostasis in diverse populations. With further research and development, STRA6 signaling in immune cells may be a therapeutic target for immune mediated diseases.

Acknowledgements

I would like to give my sincere appreciation to the members of my committee, Dr. Joseph Sucic, Dr. Kanakadurga Singer, M.A., M.D., and Dr. David Duriancik. Without their participation, support, and expertise this would not have been possible. Their guidance has assisted me throughout this process has been instrumental and the skills they have taught me will aid me in my future endeavors.

A very special thank you to my undergraduate lab mates Hana Fantin, Brent Smiles, and Christina Straham. These individuals spent many long hours in the lab with me and I could not have accomplished this work without them. Dominika Mazzola, Megan Wheeler, Rachelle Rowbotham, Alki Koutrouvidas, and Anthony Maxwell for going through this process alongside me. Their friendships have made the last year and a half so much easier and I will be forever grateful for them.

I want to conclude by thanking my biggest supporters, my family. Without their continuous love and support I would not have been able to accomplish this work. A very special note of appreciate to my sisters, Crystie and Kadie, who always know how to lift my spirits during times of stress.

Table of Contents

List of Tables	1
List of Figures	2
1. Literature Review	3
1.1 Stimulated by Retinoic Acid 6	3
1.2 Vitamin A Metabolism	4
1.3 Vitamin A and Immunity	5
1.4 Vitamin A and Immune Cell Metabolism	7
1.5 PBMC Phenotype and Function	7
1.6 Hypothesis	9
2. Methods	9
2.1 Participants	9
2.2 Blood Sample Collection and Processing	10
2.3 ARPE-19 Cell Processing	10
2.4 Flow Cytometry of PBMC Subsets	11
2.5 ARPE-19 Cells and PBMC Confocal Microscopy	14
2.6 Statistical Analyses	15
3. Results	15
3.1 Demographic and Anthropometric Analyses	15
3.2 PBMC STRA6 Expression	18
3.3 Predictive Factors for STRA6 Expression	25
4. Discussion	27
5. Conclusion	29
6. Future Direction	29
References	30

List of Tables

1. Antibody cocktails for flow cytometry.....	12
2. PBMC subset gating strategies.....	13
3. Demographic and anthropometric data.....	17

List of Figures

1. Expression of STRA6 on NKT, NK, and B lymphocytes.....	20
2. Expression of STRA6 on T helper and T cytotoxic lymphocytes.....	21
3. Expression of STRA6 on cytotoxic, cytokine, and CD56 ^{dim} /CD16 ⁻ NK cells.....	22
4. Expression of STRA6 on classical, nonclassical, and intermediate monocytes.....	23
5. Expression of STRA6 on mDC1, mDC2, and pDC dendritic cells.....	24
6. Age significantly predicts median fluorescent intensity on classical monocytes.....	26

1. Literature Review

1.1 Stimulated by Retinoic Acid 6

Stimulated by retinoic acid (STRA6), initially identified in 1995 using an embryonic carcinoma P19 cell line, was known to be a retinoic acid (RA) induced gene with unknown function (Bouillet et. al., 1997). In 2007, the function of STRA6 was fully characterized through the use of confocal microscopy and assays performed on bovine retinal pigmented epithelium cells and WiDr human colon adenocarcinoma cells. It was identified as the receptor for retinol binding protein (RBP) based on the following criteria; the ability to bind RBP to the plasma membrane, facilitate cellular uptake of retinol, and for its localization to the cellular locations expected of RBP receptor (Kawaguchi et. al., 2007). STRA6 is the first and only protein to be classified in a novel class of proteins called cytokine signaling transporters. It is classified as such for its dual ability to act as a membrane transporter facilitating intracellular uptake of retinol and a cell surface receptor activating the janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling cascade (Berry et. al., 2012).

STRA6 is a membrane bound protein with 19 distinct domains; 5 extracellular domains, 9 transmembrane domains, and 5 intracellular domains (Sun & Kawaguchi, 2007). The 9 transmembrane domains allow STRA6 to facilitate catalytic bidirectional transport of retinol from RBP and across the cellular membrane, which is distinct from any other known transport mechanisms. RBP exists in two forms, apo-RBP without retinol bound, and holo-RBP, which has retinol bound. When holo-RBP binds, STRA6 is phosphorylated, leading to the recruitment and activation of JAK2. Phosphorylated STRA6 has the ability to open holo-RBP, allowing retinol to disassociate from RBP and

be transported to the intracellular side of the cell via a defined pathway involving the transmembrane domains of STRA6 (Berry et. al., 2012 & Kawaguchi et. al., 2015). Intracellularly, retinol influences expression of over 500 genes associated with inflammation, cellular differentiation, proliferation, apoptosis, and metabolism (Amengual et. al., 2014). These effects are mediated by retinoic acid receptor (RAR)-retinoid X receptors (RXR) heterodimers or by RXR homodimers functioning as transcription factors that bind to retinoic acid response elements (RARE) (Conway, Henning, & Lerner, 2013). This pathway controls vitamin A concentrations by ensuring that vitamin A is only taken up by target cells when they have the ability to store it, preventing vitamin A from reaching toxic levels.

Phosphorylation of STRA6 by JAK2 also triggers the recruitment and activation of STAT3 or STAT5 and their corresponding signaling cascades. Upon activation, STAT3 dimerizes and translocates to the nucleus to function as a transcription factor driving oncogenesis (Karunanithi et. al., 2017). STAT5 activation induces the expression of its target genes including suppressor of cytokine signaling 3 (SOCS3), a known inhibitor of insulin signaling, leading to insulin resistance and reduced responses to inflammatory cytokines (Berry et. al., 2012).

1.2 Vitamin A Metabolism

Vitamin A is obtained from the diet by consumption of vitamin A precursors, retinyl esters or carotenoids. Precursors are converted to retinoids in the lumen of the proximal portion of the gut by enterocytes, incorporated into chylomicrons, and then enter circulation (Rosa Bono et. al., 2016). When chylomicrons reach the liver, retinyl esters leave circulation and are stored. In the liver retinyl esters are hydrolyzed into

retinol allowing them to bind to RBP and re-enter circulation maintaining a relatively narrow serum concentration of retinol. Intracellularly, retinol goes through two consecutive reactions to generate the main metabolite of vitamin A, retinoic acid (RA). Retinol is reversibly oxidized to retinaldehyde by retinol (or other non-specific alcohol) dehydrogenases and is then irreversibly oxidized to retinoic acid by one of three retinal dehydrogenase isoforms RALDH1, RALDH2, or RALDH3 (Conaway, Henning, & Lerner, 2013 & Rosa Bono et. al., 2016). RA exists as all-trans-RA or 9-cis-RA that bind to one of two nuclear hormone receptors, RXR or RAR. All-trans RA or 9-cis-RA binding and activation of RAR or 9-cis-RA binding and activation of RXR are responsible for mediating the majority of the immunological functions associated with vitamin A.

1.3 Vitamin A and Immunity

Vitamin A was initially described as an anti-infective agent in the early 20th century and is arguably the most multifunctional vitamin in the human body due to its role in human survival at every point in development (Ross, 2012 & Kawaguchi et. al., 2015). Vitamin A has pleiotropic effects in immunity and is associated with aspects of the innate and adaptive immune responses; maintaining skin integrity, mucosal barriers, lymph node development, and immune response and regulation, and leukocyte trafficking (Duriancik, Lackey, & Hoag, 2012; Ross, 2012; Rosa Bono et. al., 2016). Despite this knowledge, vitamin A deficiency remains one of the most common micronutrient deficiency and primary cause of immunosuppression worldwide (Rosa Bono et. al., 2016). In addition to impaired barriers, individuals suffering from vitamin A deficiency have impaired immune cell functions. Natural killer (NK) cell and neutrophil function is reduced in vitamin A deficient populations. Generally, vitamin A deficient

individuals have reduced T helper type 2 (T_H2) immunity and unaffected or increased T helper type 1 (T_H1) immunity. The effects on adaptive immunity are due to both direct effects of vitamin A deficiency on transcription regulation of B and T lymphocytes, as well as indirect effects of B and T lymphocyte stimulation through impaired activation of antigen presenting cells such as dendritic cells (DC).

Vitamin A deficiency causes immunosuppression by impacting immune cell responses and concentrations. Neutrophil concentration is increased in the spleen, peripheral blood, and bone marrow, but they are decreased in their ability to migrate to infection sites and phagocytose bacteria (Duriancik, et. al., 2012). Macrophages ability to migrate to infection sites and phagocytose bacteria is also compromised during deficiency. A decrease in overall NK cell cytotoxicity due to decreased concentration and decreased monocyte concentration in the spleen and bone marrow is observed during vitamin A deficiency (Duriancik, et. al., 2012). Deficiency also impacts immune cell responses by mediating the responses of $CD4^+$ T lymphocytes.

Differentiation of naïve $CD4^+$ T lymphocytes is dependent on their cell to cell contact with antigen presenting cells, DC, macrophages, and B lymphocytes, whose cytokine production are mediated by retinoic acid (Duriancik, et. al., 2012 & Ross, 2012). Retinoic acid isoforms can also directly impact naïve T lymphocyte differentiation by regulating their transcription factor function. Retinoic acid via RXR binding initiates interleukin-4 production by naïve T lymphocytes initiating increased T_H2 differentiation while suppressing T_H1 (Rosa Bono et. al., 2016). Through the RAR pathway, DC facilitate the differentiation of naïve T lymphocytes into T regulatory (T_{reg}) lymphocytes at the expense of T_H17 (Rosa Bono et. al., 2016). T lymphocyte impairment in vitamin A

deficiency is also due to indirect effects on antigen presenting cells ability to stimulate T helper lymphocytes. This leads to impairment of T_H2 lymphocyte function, ultimately resulting in decreased antibody production from B cells including IgM, IgA, and IgG.

1.4 Vitamin A and Immune Cell Metabolism

All immune cells express RAR and RXR and respond to retinoic acid through transcriptional regulation. However, murine cluster of differentiation (CD) 103⁺ DC express RALDH2 and are able to metabolize retinol to retinoic acid (Molenaar et. al., 2011). The resultant retinoic acid either regulates genes in CD103⁺ DC or is released from CD103⁺ DC to affect transcription in local T and B lymphocytes. Lymphocytes, including T, B, and NK cells, are not known to express RALDH2 and therefore need to rely on exogenous retinoic acid isoforms to regulate genes via RAR/RXR transcription factors.

1.5 PBMC Phenotype and Function

There are several types of leukocytes within a peripheral blood mononuclear (PBMC) isolation from human samples. These include natural killer T lymphocytes (NKT), natural killer lymphocytes (NK), B lymphocytes, T lymphocytes, monocytes, and dendritic cells (DC). There are also several cell subsets within each different type of lymphocyte.

Natural killer T lymphocytes are identified using the following cluster of differentiation markers, CD3⁺/CD56⁺/CD19⁻. NKT cells are known to recognize lipid antigens presented by CD1d that are elevated during inflammatory response (Fox et. al., 2009). B lymphocytes (CD3⁻/CD56⁻/CD19⁺) are a crucial part of the adaptive

immune response and are responsible for producing antibodies in response to infection and presenting antigens. To function properly, B lymphocytes must first be activated by cytokines produced by T helper lymphocytes (CD3⁺/CD8⁻/CD4⁺). Cytotoxic T lymphocytes (CD3⁺/CD8⁺/CD4⁻) induce apoptosis in cells that have been infected by virus and cancer cells.

Natural killer cells are categorized into one of three subsets, cytotoxic (CD3⁻/CD56^{hi}/CD16^{var}), cytokine (CD3⁻/CD56^{dim}/CD16⁺), and CD56^{dim}/CD16⁻ (CD3⁻/CD56^{dim}/CD16⁻). Cytotoxic NK cells proliferate, produce cytokines upon stimulation, and may be precursors to cytokine and CD56^{dim}/CD16⁻ NK cells (Björkström et. al., 2010). Cytokine NK cells' main function is to produce cytokines and induce apoptosis in virus infected cells and cancer cells.

Monocytes are also categorized into three subsets, classical (inflammatory; CD4⁻/CD14⁺/CD16⁻), nonclassical (patrolling; CD3⁻/CD14⁻/CD16⁺), and intermediate (CD3⁻/CD14⁺/CD16⁺). Intermediate monocytes highly express genes involved in T lymphocyte stimulation, antigen presentation, and are precursors to both patrolling and inflammatory subsets (Wong et. al., 2011). Nonclassical monocytes are highly motile cells that express genes involved in cytoskeletal rearrangement and phagocytosis to initiate immune responses. Classical monocytes secrete the broadest range of cytokines and chemokines, many of which involved in proinflammatory tissue repair.

Dendritic cells are also categorized into three subsets, mDC1 (CD14⁻/CD19⁻/CD1c⁺/CD141⁻/CD303⁻), mCD2 (CD14⁻/CD19⁻/CD1c⁻/CD141⁺/CD303⁻), and pDC (CD14⁻/CD19⁻/CD1c⁻/CD141⁻/CD303⁺). The mDC1 subset is unique because these cells express toll like receptor-3, cross-present antigen to CD8⁺ T lymphocytes, and produce high levels of

interleukin-12, promoting T_H1 responses (Eisenbarth, 2018). mDC2 primary function is to present antigen to CD4⁺ T lymphocytes. pDC secrete large amounts of type 1 interferon, promoting protection from viruses in nearby cells. These immune cells work in combination to protect the human body from bacteria, virus, and disease.

1.6 Hypothesis

Due to the importance of vitamin A in immune cell development, STRA6 is hypothesized to be expressed on all peripheral blood mononuclear cell subsets at varying intensities. Intensity of expression will be dependent on cell type as well as demographic and anthropometric data. If PBMC subsets express STRA6, then the expression will be co-localized with the lipid raft due to its intracellular cytokine signaling through the JAK/STAT pathway.

2. Methods

2.1 Participants

Human subjects were recruited via a convenience sample from University of Michigan-Flint students, faculty, staff, and residents living in the surrounding area. Subjects were verbally screened and provided written consent. The inclusion criteria included no clotting disorders, no known chronic or infectious disease, not currently pregnant, and above eighteen years of age. Subjects self-reported birthdate, race/ethnicity, and physical activity, and were measured for height, weight, and percent body fat using bioelectrical impedance (Tanita). All protocols were approved by the University of Michigan Flint Institutional Review Board.

2.2 Blood Sample Collection and Processing

A certified phlebotomy technician collected three volumes of venous blood into heparinized vacutainer tubes (BD Biosciences: San Jose, CA) using standard techniques. Heparinized whole blood was layered over an equal volume of room temperature Histopaque 1077 (Thermo Fisher Scientific; Waltham, MA). Density gradient centrifugation was performed at 400xg, 25C, 30 minutes without braking. The buffy coat was removed, fluorescence activated cell sorting (FACS) buffer [5% fetal bovine serum (FBS), 0.1% sodium azide, in $\text{Ca}^{++}/\text{Mg}^{++}$ -free Dulbecco's Phosphate Buffered Saline] (Thermo Fisher Scientific) was added to wash the cells, and then were centrifuged at 320xg, 25C, for 10 minutes to pellet. The supernatant was poured off and cells were resuspended in the residual volume. Cells resuspended in FACS buffer were diluted in trypan blue (Thermo Fisher Scientific) for hemocytometer cell counts.

2.3 ARPE-19 Cell Processing

Undifferentiated retinal pigmented epithelium-19 (ARPE-19) cells were obtained from American Tissue Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific) media supplemented with 1% FBS, penicillin, streptomycin, and sodium pyruvate for seven to ten days (Samual et. al., 2017). Accutase (Thermo Fisher Scientific) was then used to remove the adherent cells. Cells were washed with FACS buffer by centrifugation at 320xg, 25C, 10 minutes to pellet. After centrifugation, the supernatant was poured off and cells were resuspended in the residual volume. Cells were resuspended in FACS buffer and hemacytometer cell counts were completed using trypan blue.

2.4 Flow Cytometry of PBMC Subsets

Aliquots of 1-5 million cells were dispensed into 5mL round bottom tubes. Cells were then stained in the dark and on ice for thirty minutes using specific antibody cocktails (Table 1). After staining, FACS buffer was added and cells were centrifuged at 320xg, 4C, for 10 minutes to pellet. The supernatant was poured off and cells were resuspended in the residual volume. This process was repeated for a total of three washes. Cytofix/Cytoperm (BD Biosciences: San Jose, CA) solution was added and cells were incubated on ice for thirty minutes. Cells were washed with Perm/Wash buffer (BD Biosciences) and centrifugation 530xg, 4C, 10 minutes to pellet. The supernatant was poured off and cells resuspended. The cells were stored in Perm/Wash buffer 14-40 hours at 4C. Cell were centrifuged at 530xg, 4C, for 10 minutes to pellet, the supernatant was poured off, and the cells were resuspended. Cells were incubated with either STRA6-AF488 (R&D Systems; Minneapolis, MN) or the isotype (BD Biosciences) for thirty minutes. Cells were then washed three times with Perm/Wash Buffer via centrifugation at 520xg, 4C, for 10 minutes to pellet. The supernatant was poured off, cells were resuspended in the residual volume, and data were acquired on a BD Biosciences FACS Calibur or FACS Canto II flow cytometer. Each subset was acquired on the same flow cytometer for consistency. All data were analyzed following standard gating strategies using FlowJo software (Ashland, OR) (Table 2).

Table 1. Antibody cocktails for flow cytometry.

Subset	Antibody¹
PBMC	anti-CD3-Alexa Fluor488
	anti-CD56-PE
	anti-CD19-PerCp-eFluor710
T Cell	anti-CD3-Alexa Fluor488
	anti-CD8-PE
	anti-CD4-PerCp-eFluor710
NK Cell	anti-CD3-Alexa Fluor488
	anti-CD56-PE
	anti-CD16-PerCp-eFluor710
Monocytes	anti-CD3 Alexa Fluor488
	anti-CD16 PerCp-eFluor710
	anti-CD14 PE
DC	anti-CD1c FITC
	anti-CD141 PE
	anti-CD14 PerCp-eFluor710
	anti-CD19 PerCp-eFluor710
	anti-CD303 AF700

¹All antibodies were used at 1x concentration as recommended by manufacturer (BD Biosciences). Master mixes were made using FACS buffer.

Table 2. PBMC subset gating strategies

Cell Type	Subset	CD Markers
PBMC	NK	CD3 ⁻ /CD56 ⁺ /CD19 ⁻
	NKT	CD3 ⁺ /CD56 ⁺ /CD19 ⁻
	B lymphocytes	CD3 ⁻ /CD56 ⁻ /CD19 ⁺
T Cell	Cytotoxic T lymphocytes	CD3 ⁺ /CD8 ⁺ /CD4 ⁻
	T helper lymphocytes	CD3 ⁺ /CD8 ⁻ /CD4 ⁺
NK cells	Cytotoxic	CD3 ⁻ /CD56 ^{hi} /CD16 ^{var}
	Cytokine	CD3 ⁻ /CD56 ^{dim} /CD16 ⁺
	CD56 ^{dim} /CD16 ⁻	CD3 ⁻ /CD56 ^{dim} /CD16 ⁻
Monocytes	Classical	CD3 ⁻ /CD14 ⁺ /CD16 ⁻
	Nonclassical	CD3 ⁻ /CD14 ⁻ /CD16 ⁺
	Intermediate	CD3 ⁻ /CD14 ⁺ /CD16 ⁺
Dendritic Cells	mDC1	CD14 ⁻ /CD19 ⁻ /CD1c ⁺ /CD141 ⁻ /CD303 ⁻
	mDC2	CD14 ⁻ /CD19 ⁻ /CD1c ⁻ /CD141 ⁺ /CD303 ⁻
	pDC	CD14 ⁻ /CD19 ⁻ /CD1c ⁻ /CD141 ⁻ /CD303 ⁺

2.5 ARPE-19 Cells and PBMC Confocal Microscopy

After hemocytometer cell counts, volumes were aliquoted so each tube contained 1×10^6 cells. Cells were stained in the dark using a Vybrant Alexa Fluor 488 kit (Thermo Fisher Scientific), according to manufacturer instructions. Cells were incubated for twenty minutes on ice with CT-B-AF488 (BD Biosciences) to stain for the lipid raft. Cells were then washed in PBS via centrifugation at 320xg, 4C, for 10 minutes to pellet. Supernatant was poured off to waste and cells were resuspended in the residual volume. This was repeated for a total of three washes. The cells were then incubated with anti-CT-B (BD Biosciences) serum for fifteen minutes. The cells were washed three times in PBS via centrifugation at 320xg, 4C, for 10 minutes to pellet. Cells were then fixed and permeabilized by adding Cytofix/Cytoperm solution and incubated for thirty minutes. After incubation, the cells were washed three times with Perm/Wash via centrifugation 320xg, 4C, for 10 minutes to pellet. Cells were then stained with optimal concentrations of STRA6-AF647 or isotype for thirty minutes. The stain was washed out using Perm/Wash via centrifugation at 320xg, 4C, for 10 minutes to pellet. The cells were then stained with Hoechst 33342 (Thermo Fisher Scientific) for five minutes to stain for the nucleus. Cells were then washed twice, first with Perm/Wash and then with PBS via centrifugation 320xg, 4C, for 10 minutes to pellet. The supernatant was poured off to waste and the cells were loaded into vitro tubes to be viewed on a TCS SPE DMI8 confocal microscope (LEICA; Buffalo Grove, IL). Images were obtained using a 63X oil immersion objective and a frame average of four with LASX software (LEICA). Settings to capture fluorescence were; Hoechst 33342, 405nm laser, 33.3% laser power, emission filter 410-520, gain 800 volts; AlexaFluor488, 488nm laser, 33.3% laser power,

emission filter 505-590nm, gain 1250 volts; AlexaFluor647, 635nm laser, 33.3% laser power, emission filter 650-770nm, gain 1250 volts. The differential interference contrast image was also collected. Images were then processed using Fiji (ImageJ) (NIH).

2.6 Statistical Analyses

Forty subjects was the determined n after using G*Power 3.1.9.2 *a priori* linear multiple logistic regression fixed model R^2 deviation from zero power analysis with 4 predictors variables, a large effect size of 0.26, alpha 0.05, and beta 0.2. The predictor variables included age, sex, race/ethnicity, and body composition. Multiple linear regression was performed using R on the expression of STRA6 on each cell population. ANOVA with Tukey's post hoc multiple comparisons in Prism was used for cell population differences.

3. Results

3.1 Demographic and Anthropometric Analyses

Power analysis results determined 40 subjects would be required to observe a significant difference with large effect size, alpha at 0.05, beta at 0.2, for a multiple linear regression analysis. Forty-one human subjects were recruited to participate in this study. One subject had unusual PBMC scatter profiles, subset marker expression, and high STRA6 MFI in all cell populations; therefore, this subject was considered an outlier and not included in the following analysis.

Participants self-reported as being predominately white (75%, 12.5% black) females (60%) with a mean age of 28.28 ± 1.58 calculated by their birthdate (Table 3). Body composition was measured in each participant. The mean body fat percentage was 28.20 ± 1.87 . The mean body mass index was 27.45 ± 0.81 kg/m², with overall

composition in the following categories as defined by the Center for Disease Control (CDC); 2.5% underweight, 35% normal, 25% overweight, 25% obese, and 25% obese

II. 45% of participants self-reported to be athletic, defined according to Tanita as “Athlete” over 16 years old and exercised ten or more hours per week for the past six months, or a resting heart rate (HR) of 60 beats per minutes or less, or “very fit” for years, but currently exercises less than or equal to ten hours a week.

Table 3. Demographic and anthropometric data

Characteristic	Mean \pm SEM
Race/Ethnicity	75% White, 12.5% Black ¹
Sex	60% Female
Age	28.28 \pm 1.58
Height (m)	1.72 \pm 0.01
Body Weight (Kg)	81.58 \pm 2.98
Body Composition	
Body Mass Index	27.45 \pm 0.80
% in each BMI Category ²	35% Normal
	25% Overweight
	25% Obese
	12.5% Obese II
Body Fat%	28.20 \pm 1.87
Body Fat (Kg)	23.93 \pm 2.08
Lean Body Mass % ³	71.80 \pm 1.837
Lean Body Mass (Kg)	57.65 \pm 2.08
Lifestyle ⁴	45% Athletic

¹Race/ethnicity as defined by NIH. Remaining percentage was Asian

²BMI categories as defined by CDC; Normal 18.5-24.9, Overweight 25.0-29.9, Obese 30.0-34.9, Obese II \geq 35.0

³Calculated as 100% -% body fat

⁴Defined according to Tanita as “Athlete” $>16\text{yo} \pm 10$ hours/week for 6 months, OR resting HR $\sim 60\text{bpm}$, OR “very fit” for years, but currently ≤ 10 hours/week

3.2 PBMC STRA6 Expression

One million PBMC were stained separately for CD3, CD56, and CD19 to identify NKT, NK, and B lymphocytes. Of the subsets identified, NKT cells expressed significantly more STRA6, with a mean MFI of 286.63 ± 33.89 , compared to total NK cells (233.02 ± 28.69 , Tukey's $p < 0.001$) and B lymphocytes (105.93 ± 17.18 , Tukey's $p < 0.0001$) (Figure 1). The mean MFI of STRA6 on NK cells was significantly higher than the mean MFI of B lymphocytes (Tukey's $p < 0.0001$).

One million cells were stained separately for CD3 and CD4 to identify T helper lymphocytes and CD8 to identify cytotoxic T lymphocytes. Of the two cell types, CD8⁺ T cytotoxic lymphocytes expressed significantly higher STRA6 with a mean MFI of 225.68 ± 28.45 compared to CD4⁺ T helper lymphocytes, whose mean MFI was 179.91 ± 22.09 (Student's t-test $p < 0.0001$) (Figure 2).

Three million cells were stained separately for CD3, CD56, and CD16 to identify three subsets of NK cells. Cytokine NK cells had a mean MFI of 176.31 ± 23.21 which was significantly higher than the mean MFI of CD56^{dim}/CD16⁻ NK cells, 164.12 ± 21.34 (Tukey's $p = 0.0171$) (Figure 3). Cytotoxic had a mean MFI of 166.86 ± 20.41 and was not significantly different from the other two cell populations.

Three million cells were stained separately for CD3, CD16, and CD14 to identify classical, nonclassical, and intermediate monocytes. Nonclassical monocytes had a mean MFI of $1,089.81 \pm 184.73$, which was significantly higher than classical (CD14⁺/CD16⁻) (Tukey's $p = 0.0002$) and intermediate monocytes (Tukey's $p < 0.0001$) (Figure 4). Classical monocytes had a mean MFI of 454.70 ± 58.01 , which was

significantly higher than intermediate monocytes mean MFI of 371.62 \pm 43.60 (Tukey's $p < 0.0001$).

Five million cells were stained separately for CD1c, CD141, CD14, CD19, and CD303 to identify three subsets of dendritic cells. pDC dendritic cells had a mean MFI of 5,939.83 \pm 901.35, which was significantly higher than the mean MFI of mDC2 at 4,289.97 \pm 474.30 (Tukey's $p = 0.05$) (Figure 5). mDC1 had a mean MFI of 5,040.53 \pm 766.46 and was not significantly different than either of the other dendritic cell subsets.

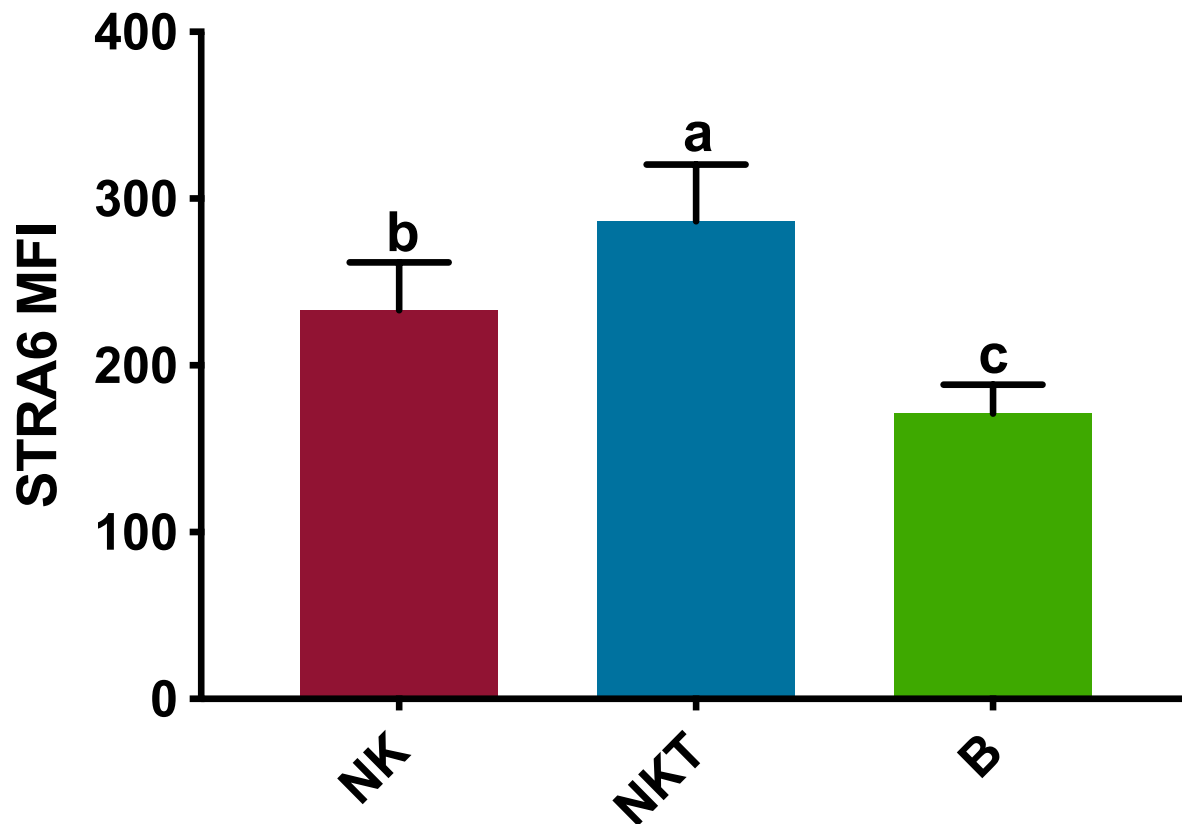


Figure 1. Expression of STRA6 on NKT, NK, and B lymphocytes. One million PBMC were stained for CD3, CD56, and CD19 to identify total NK cells (CD3⁺/CD56⁺/CD19⁻), B lymphocytes (CD3⁻/CD56⁻/CD19⁺) and NKT cells (CD3⁺/CD56⁺CD19⁻). Expression of STRA6 on NKT cells was significantly increased compared to NK cells and B lymphocytes, and NK cells expressed significantly higher STRA6 than B lymphocytes. Data represent mean \pm SEM (n=41) and bars with different letters were statistically significant at $p < 0.05$ by ANOVA with Tukey's post-hoc analysis.

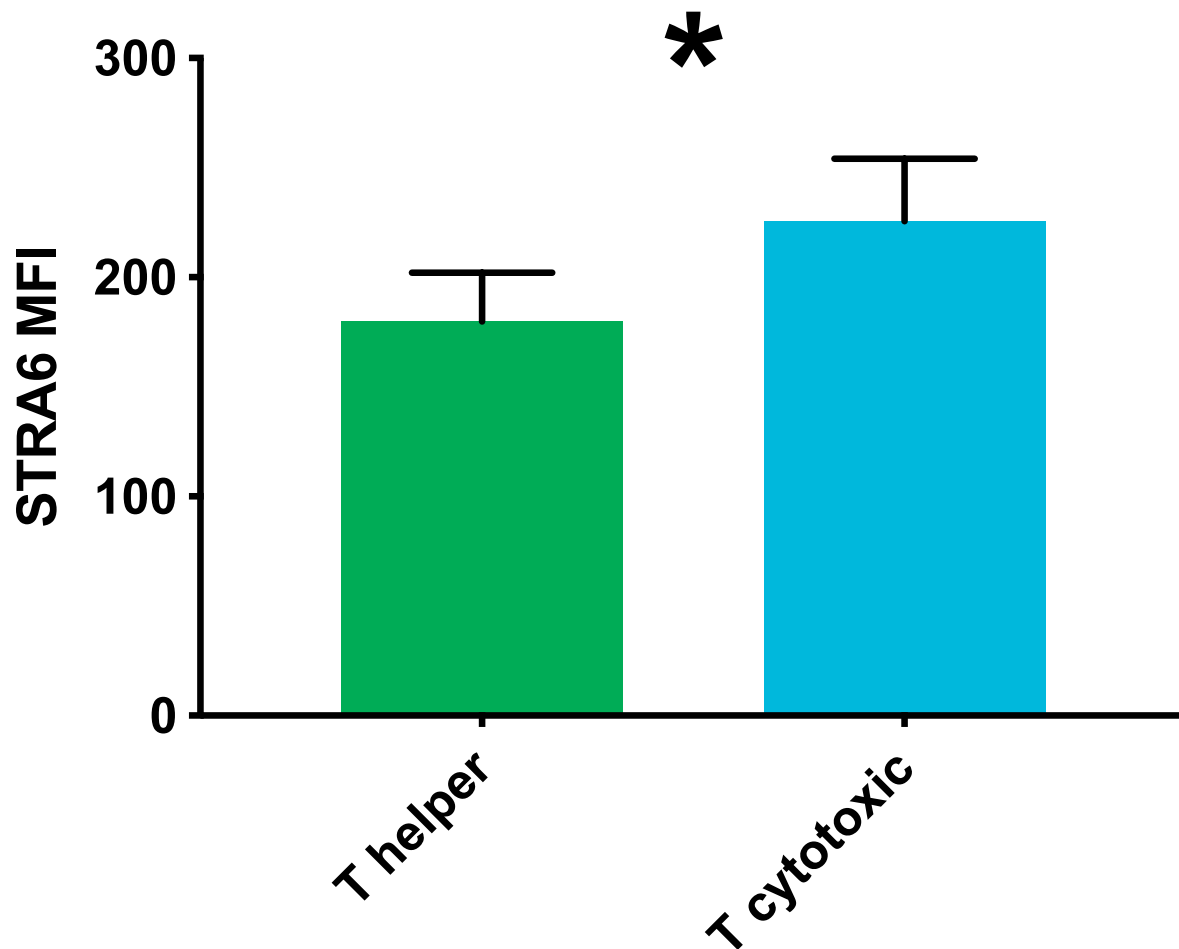


Figure 2. Expression of STRA6 on T helper and T cytotoxic lymphocytes. One million PBMC were stained for CD3, CD8, and CD4 to identify total T helper (CD4⁺) and T cytotoxic (CD8⁺) lymphocytes. Expression of STRA6 on T cytotoxic lymphocytes was significantly increased compared to T helper lymphocytes. Data represent mean \pm SEM (n=32) and bars and asterisk indicate statistically significant at $p < 0.05$ by Student's t-test.

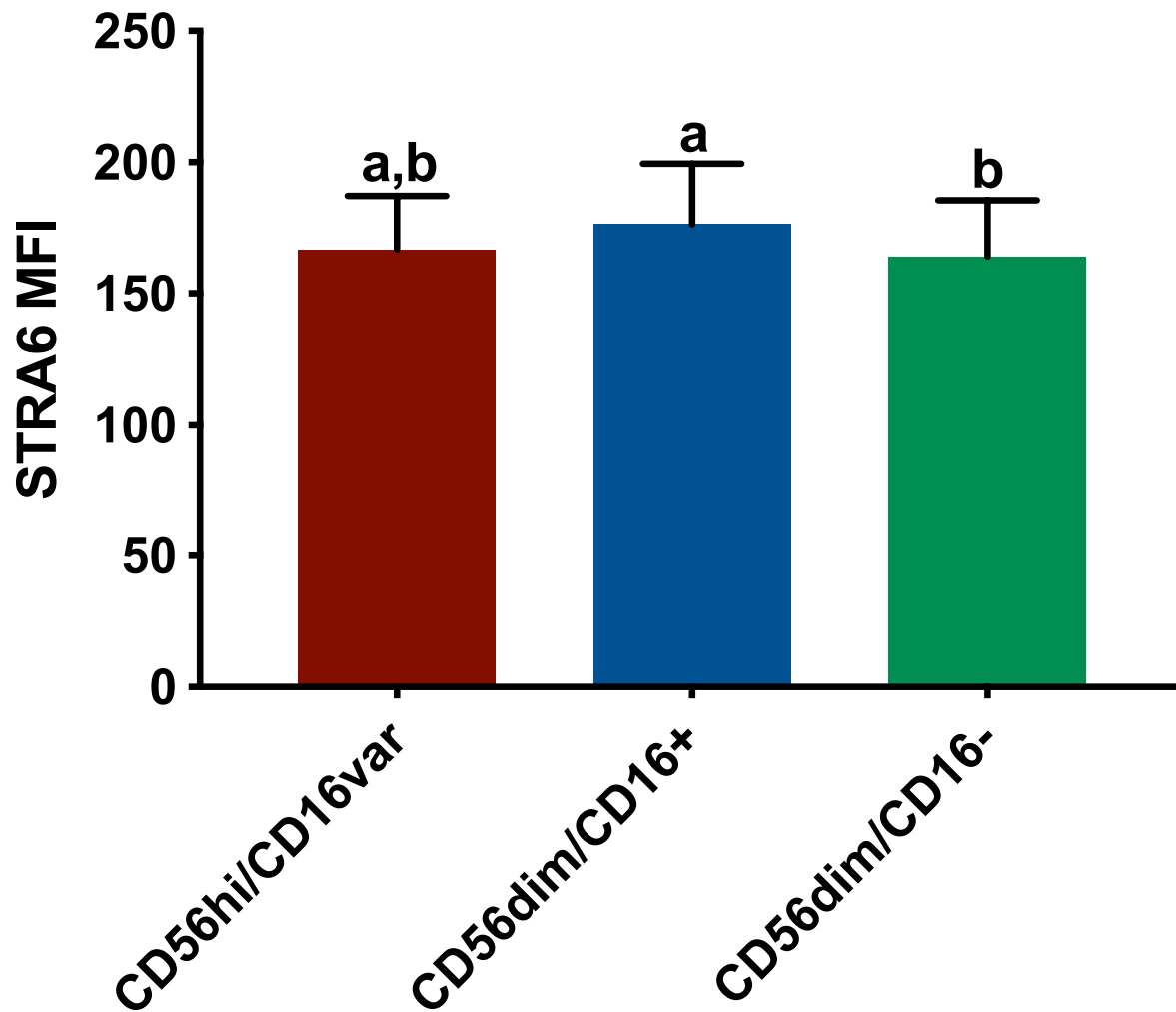


Figure 3. Expression of STRA6 on cytotoxic, cytokine, and CD56^{dim}/CD16⁻ NK cells. Three million PBMC were stained for CD3, CD56, and CD16 to identify cytotoxic (CD3⁺/CD56^{hi}/CD16^{var}) cytokine (CD3⁺/CD56^{dim}/CD16⁺) and CD56^{dim}/CD16⁻ NK cells. Expression of STRA6 on cytokine NK cells was significantly increased compared to CD56^{dim}/CD16⁻ NK cells, but not cytotoxic NK cells. Data represent mean \pm SEM (n = 40) and bars with different letters were statistically significant at $p < 0.05$ by ANOVA with Tukey's post-hoc analysis.

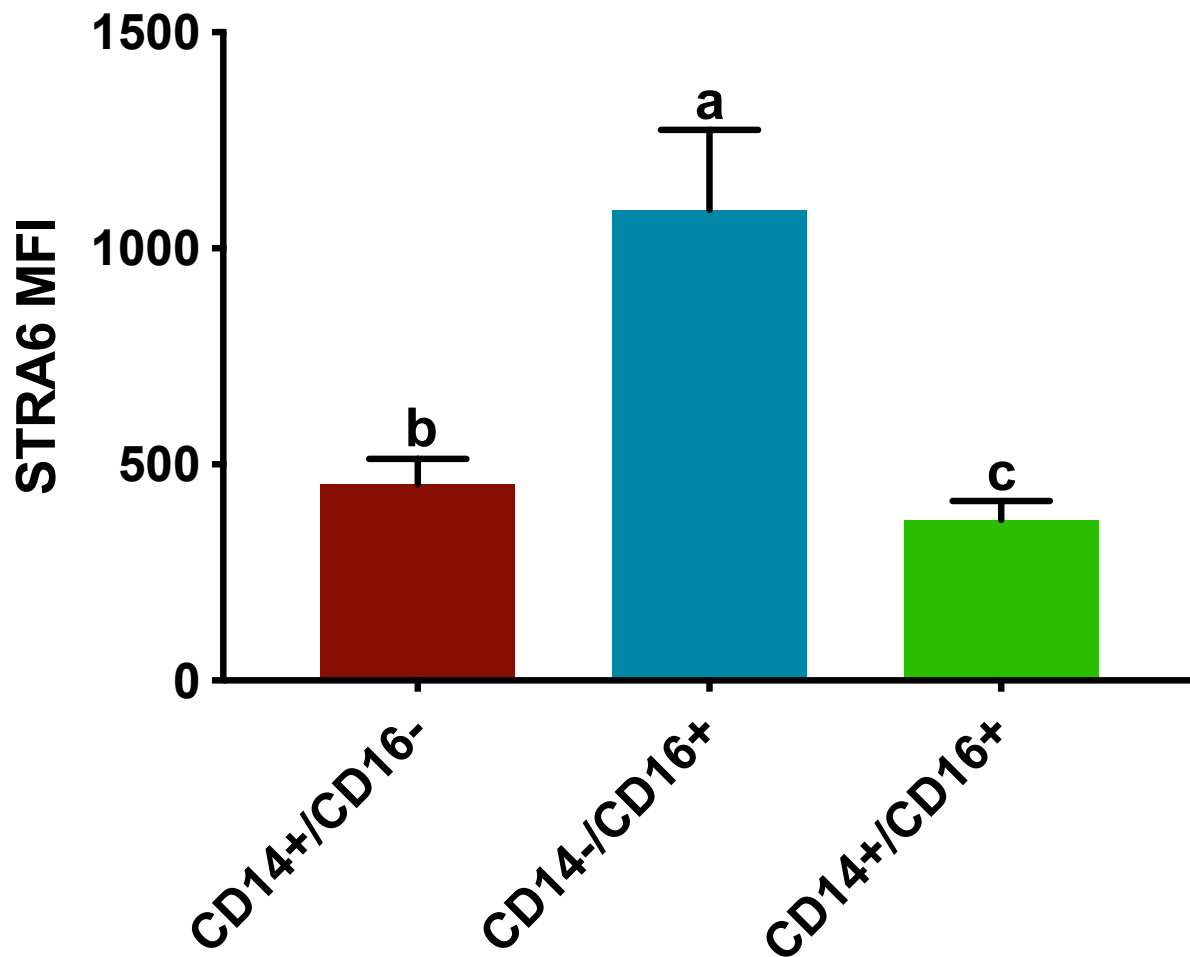


Figure 4. Expression of STRA6 on classical, nonclassical, and intermediate monocytes. Three million PBMC were stained for CD3, CD16, and CD14 to identify total classical (CD3⁺/CD14⁺/CD16⁻) nonclassical (CD3⁺/CD14⁻/CD16⁺) and intermediate (CD3⁺/CD14⁺/CD16⁺) monocytes. Expression of STRA6 on nonclassical monocytes was significantly increased compared to classical and intermediate monocytes, and expression of STRA6 on classical monocytes was significantly increased compared to intermediate monocytes. Data represent mean \pm SEM (n = 38) and bars with different letters were statistically significant at $p < 0.05$ by ANOVA with Tukey's post-hoc analysis.

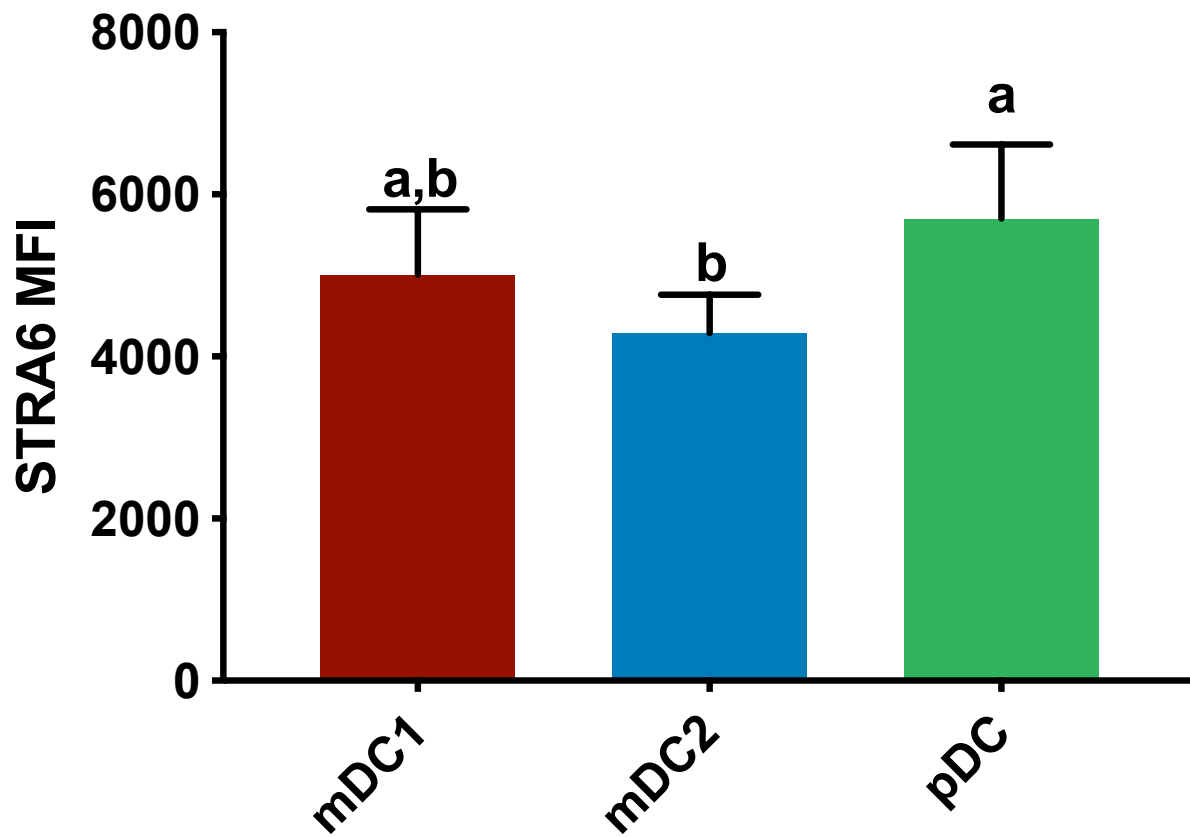


Figure 5. Expression of STRA6 on mDC1, mDC2, and pDC dendritic cells. Five million PBMC were stained for CD14, CD19, CD1c, CD141, and CD303 to identify total mDC1, mDC2, and pDC dendritic cells. Expression of STRA6 on pDC cells was significantly increased compared to mDC2, but not mDC1. Data represent mean \pm SEM (n = 37) and bars with different letters were statistically significant at $p < 0.05$ by ANOVA with Tukey's post-hoc analysis.

3.3 Predictive Factors for STRA6 Expression

We hypothesized age, race/ethnicity, body composition (body fat percentage), and sex would be predictive of STRA6 expression. However, in no model for any cell population was body fat percentage, sex, or race/ethnicity significantly predictive of STRA6 expression. Backwards mixed effects linear regression consistently indicated that age was the most predictive factor for STRA6 expression.

Although not statistically significant, age was the most predictive factor for STRA6 expression on NK cells ($p = 0.0933$), NKT cells ($p = 0.105$), and B lymphocytes ($p = 0.072$). Age was a significant predictive factor for STRA6 expression on classical monocytes and was significant alone ($p = 0.0381$) or in combination with all other factors; sex, race/ethnicity, and body fat percentage ($p = 0.0467$) (Figure 6). Age was also the best predictive factor, not significantly, for STRA6 expression on nonclassical ($p = 0.119$) and intermediate monocytes ($p = 0.0817$).

Age was the most predictive factor for STRA6 expression on all NK cell subsets; cytotoxic ($p = 0.2084$), cytokine ($p = 0.2055$), and $CD56^{\text{dim}}/CD16^-$ ($p = 0.1807$). The most predictive factor for STRA6 expression on T lymphocyte subsets was age, for both T helper ($p = 0.2215$) and T cytotoxic ($p = 0.2215$). Age was also the most predictive factor for STRA6 expression on all dendritic cell subsets: mDC1 ($p = 0.4484$), mDC2 ($p = 0.2042$), and PDC ($p = 0.2311$).

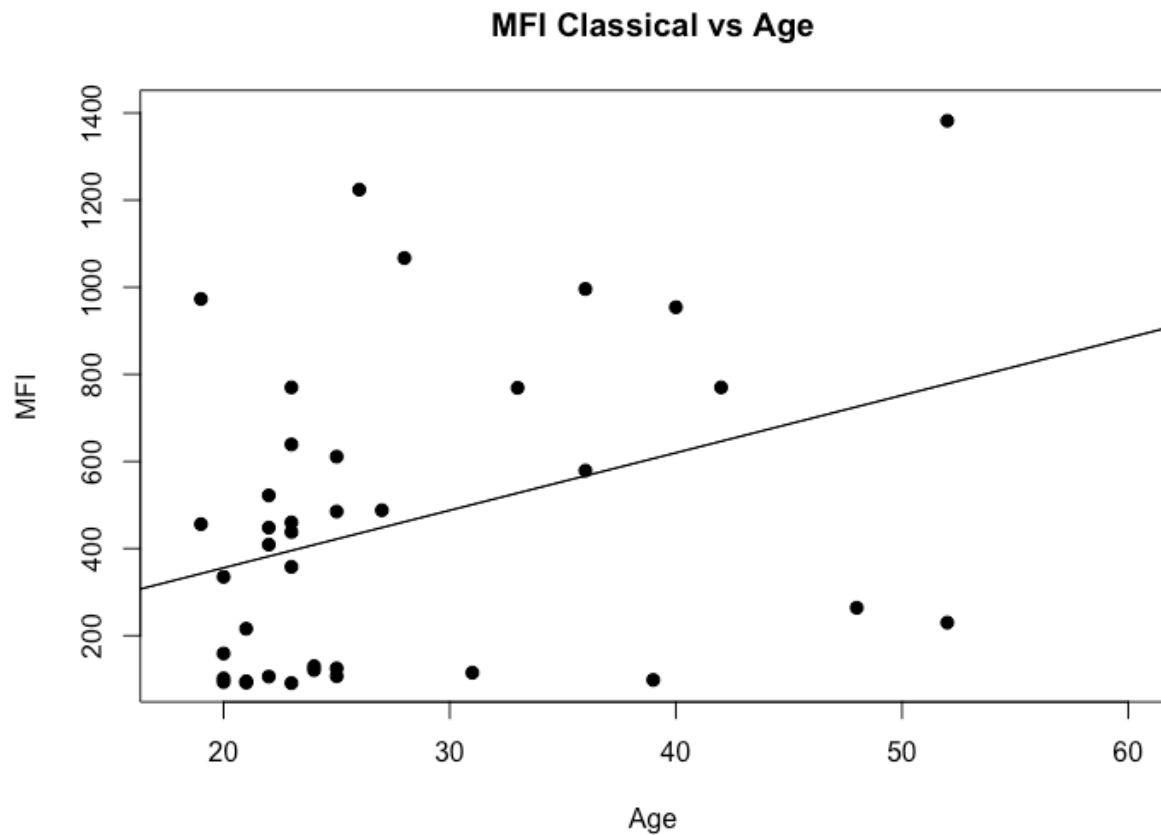


Figure 6. Age significantly predicts median fluorescent intensity on classical monocytes. Backwards mixed effects linear regression indicates that age is a significant predicative factor for median fluorescent intensity (MFI) of the STRA6-AF488 antibody expression of classical monocytes alone ($p = 0.0381$; shown) and in combination with body composition, sex, and race/ethnicity ($p = 0.0467$).

4. Discussion

All PBMC subsets expressed STRA6 at varying intensities, which is to our knowledge the first characterization of STRA6 on human immune cells. As a cell population, monocytes were found to have the greatest level of expression of STRA6 compared to NK, NKT, B lymphocytes, and T lymphocytes. Within each cell population, differences in expression were also detected amongst the identified subsets. From the subsets identified with the PBMC cocktail of antibodies, NKT cells had the greatest level of expression of STRA6. T cytotoxic lymphocytes had increased expression compared to T helper lymphocytes. Cytokine NK cells had increased expression of STRA6 compared to cytotoxic and CD56^{dim}/CD16 NK cell subsets. STRA6 expression was increased in nonclassical monocytes compared to classical and intermediate monocytes. From dendritic cell subsets, expression of STRA6 was increased on the pDC subset compared to the mDC1 and mDC2 subsets. Interestingly, although all PBMC subsets express STRA6, confocal microscopy data analysis indicates its expression may not be co-localized with the plasma membrane, and specifically lipid raft regions, in PBMC subsets. Therefore, more research is needed to elucidate the cellular trafficking of STRA6 and its role in immune cells.

Analysis of predictive demographic and anthropometric factors indicate that age is the most predictive factor in determining the level of expression of STRA6 on human immune cell subsets. In classical monocyte populations, age is a significant predictive factor of STRA6 expression, indicating an increased expression of STRA6 on classical monocytes as age increased. Therefore, these monocytes may have an increased requirement for, or response to, vitamin A in older individuals. Analyses also revealed

race/ethnicity and sex have a very low predictive power of STRA6 expression, suggesting that race/ethnicity and sex do not impact immune cells ability to uptake vitamin A. Body composition was also found to have a low predictive power on STRA6 expression on immune cells. Neither body fat percentage nor BMI were predicative of STRA6 expression on immune cells.

The low predictive power of body composition on STRA6 expression in immune cells is interesting considering the known intracellular signaling due to JAK/STAT activation. Upon holo-RBP binding to STRA6 the JAK2/STAT5 pathway is activated, resulting in the activation of SOCS3. Berry and colleagues discovered activation of this pathway impairs insulin receptor-mediated signaling, resulting in the development of insulin resistance in mice that had been injected with holo-RBP (Berry et. al., 2011). Insulin resistance is associated with an increase in abdominal fat accumulation, thus having an obvious impact on body composition (Kim & Park, 2018). The lack of body composition being a predictive factor for the expression of STRA6 support Berry and colleagues' findings that insulin resistance resulting from this pathway is due holo-RBP and not STRA6 expression. This evidence explains why body composition is a low predictive factor of STRA6 expression.

When considering predictive factors and these findings it is important to call attention to the limitations of our laboratory. PBMC included in this study were limited to those collected from human blood and may not be reflective of STRA6 expression on immune cells that have been trafficked to tissues or the gut. It is possible, and maybe even likely, that STRA6 expression of immune cells that have been trafficked out of the

circulatory system may have dissimilar expression of STRA6 compared to what is currently being reported.

5. Conclusion

Vitamin A signaling in combination with JAK/STAT signaling via STRA6 expression, now known to be present on all human PBMC subsets, allows vitamin A uptake to significantly contribute to immune cell homeostasis and may become increasingly important as one ages. Indicating a need to study differential expression of STRA6 on immune cells in chronic and infectious disease states.

6. Future Direction

Future research in this area of study should focus on completing the confocal microscopy experiments. Experiments would further investigate the physical location of STRA6 in immune cells and give a better understanding of co-localization of STRA6 with the plasma membrane and lipid raft giving better insight into function of STRA6 on immune cells. STRA6 expression should also be investigated on proliferating immune cells that are metabolically active to determine any differences in expression that may exist. Expression of STRA6 should be further investigated on T lymphocytes This would give explanation to differences that maybe be observed on T_H1 vs T_H2 or TH_{17} vs T_{reg} subsets as well as effector vs memory subsets. This might also explain, differences seen in effector and memory subsets prior to and after influenza vaccination.

References

1. Amand, M., Iserentant, G., Poli, A., Sleiman, M., Fievez, V., Sanchez, I. P., Sauvageot, N., Michel, T., Aouali, N., Janji, B., Trujillo-Vargas, C. M., Seguin-Devaux, C., ... Zimmer, J. (2017). Human CD56^{dim}CD16^{dim} Cells As an Individualized Natural Killer Cell Subset. *Frontiers in immunology*, 8, 699. doi:10.3389/fimmu.2017.00699
2. Amengual, J., Zhang, N., Kemmerer, M., Maeda, T., Palczewski, K., & Von Lintig, J. (2014). STRA6 is critical for cellular vitamin A uptake and homeostasis. *Human Molecular Genetics*, 23(20), 5402-5417. doi:10.1093/hmg/ddu258
3. Bakdash, G., Vogelpoel, L. T., van Capel, T. M., Kapsenberg, M. L., & de Jong, E. C. (2015). Retinoic acid primes human dendritic cells to induce gut-homing, IL-10-producing regulatory T cells. *Mucosal Immunology*, 8(2), 265-278. Doi:10.1038/mi.2014.64
4. Berry, D. C., Levi, L., & Noy, N. (2014). Holo-Retinol–Binding Protein and Its Receptor STRA6 Drive Oncogenic Transformation. *Cancer Research*, 74(21), 6341-6351. doi:10.1158/0008-5472.CAN-14-1052
5. Berry, D. C., Jacobs, H., Marwarha, G., Gely-Pernot, A., O'Byrne, S. M., DeSantis, D., ... Ghyselinck, N. B. (2013). The STRA6 Receptor Is Essential for Retinol-binding Protein-induced Insulin Resistance but Not for Maintaining Vitamin A Homeostasis in Tissues Other Than the Eye. *The Journal of Biological Chemistry*, 288(34), 24528–24539. doi:10.1074/jbc.M113.484014
6. Berry, D. C., Jin, H., Majumdar, A., Noy, N., & Schlessinger, J. (2011). Signaling by vitamin A and retinol-binding protein regulates gene expression to inhibit insulin

- responses. *Proceedings of the National Academy of Sciences of the United States of America*, 108(11), 4340-4345. doi:10.1073/pnas.1011115108
7. Berry, D. C., O'Byrne, S. M., Vreeland, A. C., Blaner, W. S., & Noy, N. (2012). Cross Talk between Signaling and Vitamin A Transport by the Retinol-Binding Protein Receptor STRA6. *Molecular and Cellular Biology*, 32(15), 3164–3175.
doi:10.1128/MCB.00505-12
 8. Björkström, N. K., Riese, P., Heuts, F., Andersson, S., Fauriat, C., Ivarsson, M. A., ... & Guzmán, C. A. (2010). Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. *Blood*, 116(19), 3853-3864.
 9. Breton, G., Lee, J., Liu, K., & Nussenzweig, M. C. (2015). Defining human dendritic cell progenitors by multiparametric flow cytometry. *Nature Protocols*, 10(9), 1407-1422. doi:http://dx.doi.org/10.1038/nprot.2015.092
 10. Chen, Y., Clarke, O., Stowe, S., Kim, Y., Assur, Z., Cavalier, M., & Ruiz-Godoy, R. (2016). Structure of the STRA6 receptor for retinol uptake. *Science*, 353(6302), 887-899. doi:10.1126/science.aad8266
 11. Conaway, H., Henning, P., Lerner, U. (2013) Vitamin A Metabolism, Action, and Role in Skeletal Homeostasis. *Endocrine Reviews*, 34(6), 766–797, doi:10.1210/er.2012-1071
 12. Duriancik, D. M., Lackey, D. E., & Hoag, K. A. (2010). Vitamin A as a Regulator of Antigen Presenting Cells. *The Journal of Nutrition*, 140, 1395-1399.
doi:10.3945/jn.110.124461

13. Eisenbarth, S. C. (2018). Dendritic cell subsets in T cell programming: location dictates function. *Nature Reviews Immunology*, 1.
14. Fedders, R., Muenzner, M., & Schupp, M. (2015). Retinol binding protein 4 and its membrane receptors: a metabolic perspective. *Hormone molecular biology and clinical investigation*, 22(1), 27-37.
15. Fox, L. M., Cox, D. G., Lockridge, J. L., Wang, X., Chen, X., Scharf, L., ... & Howell, A. R. (2009). Recognition of lyso-phospholipids by human natural killer T lymphocytes. *PLoS biology*, 7(10), e1000228.
16. Hall, J., Grainger, J., Spencer, S., & Belkaid, Y. (2011). The Role of Retinoic Acid in Tolerance and Immunity. *Immunity*, 35(1), 13-22. doi:10.1016/j.immuni.2011.07.002.
17. Golzio, C., Martinovic-Bouriel, J., Thomas, S., Mougou-Zrelli, S., Grattagliano-Bessi res, B., Bonni re, M., & Delahaye, S. (2007). Matthew-Wood Syndrome Is Caused by Truncating Mutations in the Retinol-Binding Protein Receptor Gene STRA6. *The American Journal of Human Genetics*, 80(6), 1179-1187. doi:10.1086/518177
18. Isken, A., Golczak, M., Oberhauser, V., Hunzelmann, S., Driever, W., Imanishi, Y., ... von Lintig, J. (2008). RBP4 Disrupts Vitamin A Uptake Homeostasis in a STRA6-Deficient Animal Model for Matthew-Wood Syndrome. *Cell Metabolism*, 7(3), 258–268. doi:10.1016/j.cmet.2008.01.009
19. Jiang, Y., Obuseh, F., Ellis, W., Piyathilake, C., & Jolly, P. (2007). Association of vitamin A deficiency with decrease in TNF- α expressing CD3-CD56+ NK cells in Ghanaians. *Nutrition research*, 27(7), 400-407. doi:10.1016/j.nutres.2007.04.016

20. Karunanithi, S., Levi, L., DeVecchio, J., Karagkounis, G., Reizes, O., Lathia, J. D., ... Noy, N. RBP4-STRA6 Pathway Drives Cancer Stem Cell Maintenance and Mediates High-Fat Diet-Induced Colon Carcinogenesis. *Stem cell reports*, 9(2), 438–450. doi:10.1016/j.stemcr.2017.06.002
21. Kawaguchi, R., Yu, J., Honda, J., Hu, J., Whitelegge, J., Ping, P., . . . Sun, H. (2007). A Membrane Receptor for Retinol Binding Protein Mediates Cellular Uptake of Vitamin A. *Science*, 315(5813), 820-825. doi:10.1126/science.1136244
22. Kim, K., & Park, S. M. (2018). Association of muscle mass and fat mass with insulin resistance and the prevalence of metabolic syndrome in Korean adults: a cross-sectional study. *Scientific reports*, 8(1), 2703.
23. LeBien, T. W., & Tedder, T. F. (2008) B lymphocytes: how they develop and function. *Blood*, 112(5), 1570-1580. doi:10.1182/blood-2008-02-078071
24. Molenaar, R., Knippenberg, M., Goverse, G., Olivier, B. J., de Vos, A. F., O'Toole, T., & Mebius, R. E. (2011). Expression of retinaldehyde dehydrogenase enzymes in mucosal dendritic cells and gut-draining lymph node stromal cells is controlled by dietary vitamin A. *The Journal of Immunology*, 186(4), 1934-1942.
25. Muenzner, M., Tuvia, N., Deutschmann, C., Witte, N., Tolkachov, A., Valai, A., Schupp, M. (2013). Retinol-Binding Protein 4 and Its Membrane Receptor STRA6 Control Adipogenesis by Regulating Cellular Retinoid Homeostasis and Retinoic Acid Receptor α Activity. *Molecular and Cellular Biology*, 33(20), 4068–4082. doi:1128/MCB.00221-13

26. Naeim, F., Nagesh Rao, P., Song, S. X., & Grody, W. W. (2013). Principles of Immunophenotyping. *Atlas of Hematopathology*, 25-46. doi:10.1016/B978-0-12-385183-3.00002-4
27. Pasutto, F., Flinter, F., Rauch, A., & Reis, A. (2017). Novel STRA6 null mutations in the original family described with Matthew–Wood syndrome. *American Journal of Molecular Genetics*, 176A, 134-138. doi:10.1002/ajmg.a.38529
28. Price, J. D., & Tarbell, K. V. (2015). The Role of Dendritic Cell Subsets and Innate Immunity in the Pathogenesis of Type 1 Diabetes and Other Autoimmune Diseases. *Frontiers in Immunology*, 6, 288. doi:10.3389/fimmu.2015.00288
29. Rosa Bono, M., Tejon, G., Flores-Santibañez, F., Fernandez, D., Roseblatt, M., & Sauma, D. (2016). Retinoic Acid as a Modulator of T Cell Immunity. *Nutrients*, 8(6), 349-363. doi:10.3390/nu8060349
30. Ross, A. C., & Sephensen, C. B. (1996). Vitamin A and Retinoids in Antiviral Responses. *FASEB*, 10(9), 979-985.
31. Ross, A. (2012). Vitamin A and retinoic acid in T cell-related immunity. *American Journal of Clinical Nutrition*, 96(5), 1166S-1172S. doi:10.3945/ajcn.112.034637
32. Samuel, W., Jaworski, C., Postnikova, O. A., Kutty, R. K., Duncan, T., Tan, L. X., Poliakov, E., Lakkaraju, A., ... Redmond, T. M. (2017). Appropriately differentiated ARPE-19 cells regain phenotype and gene expression profiles similar to those of native RPE cells. *Molecular vision*, 23, 60-89. doi:
33. Stabile, H., Fionda, C., Gismondi, A., & Santoni, A. (2017). Role of Distinct Natural Killer Cell Subsets in Anticancer Response. *Frontiers in immunology*, 8, 293. doi:10.3389/fimmu.2017.00293

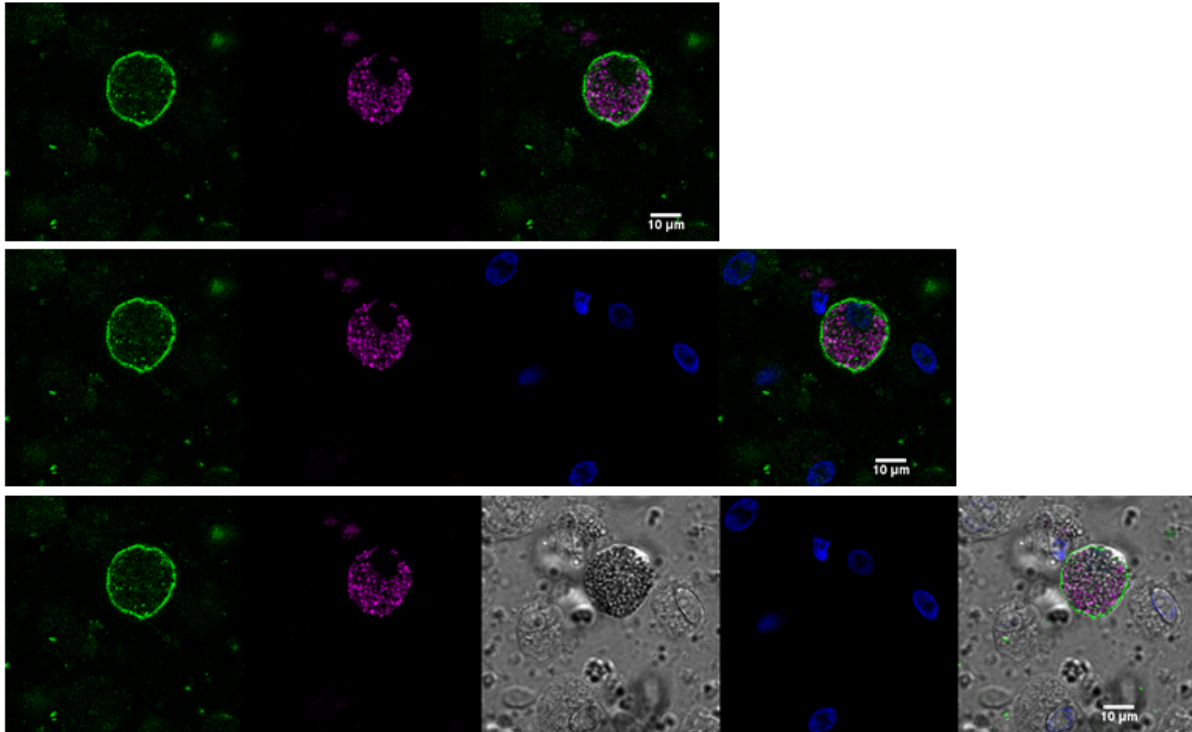
34. Stephensen, C.B., Jiang, X., Freytag, T. (2004) Vitamin A Deficiency Increases the in Vivo Development of IL-10–Positive Th2 Cells and Decreases Development of Th1 Cells in Mice, *The Journal of Nutrition*, 134(10), 2660–2666. doi:10.1093/jn/134.10.2660
35. Sun, H., & Kawaguchi, R. (2011). The Membrane Receptor for Plasma Retinol Binding Protein, a New Type of Cell-Surface Receptor. *International Review of Cell and Molecular Biology*, 288, 1–41. doi:10.1016/B978-0-12-386041-5.00001-7
36. Tallone, T., Turconi, G., Soldati, G., Pedrazzini, G., Moccetti, T., & Vassalli, G. (2011). Heterogeneity of human monocytes: An optimized four-color flow cytometry protocol for analysis of monocyte subsets. *Journal of Cardiovascular Translational Research*, 4(2), 211-219. doi:10.1007/s12265-011-9256-4
37. Terra R, Wang X, Hu Y, Charpentier T, Lamarre A, et al. (2013) To Investigate the Necessity of STRA6 Upregulation in T Cells during T Cell Immune Responses. *PLoS ONE* 8(12): e82808. doi:10.1371/journal.pone.0082808
38. Valiathan, R., Lewis, J. E., Melillo, A. B., Leonard, S., Ali, K. H., & Asthana, D. (2012). Evaluation of a flow cytometry-based assay for natural killer cell activity in clinical settings: Flow cytometry-based assay for NK cell activity. *Scandinavian Journal of Immunology*, 75(4), 455-462. doi:10.1111/j.1365-3083.2011.02667.x
39. Wong, K. L., Tai, J. J. Y., Wong, W. C., Han, H., Sem, X., Yeap, W. H., ... & Wong, S. C. (2011). Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. *Blood*, 118(5), e16-e31.
40. Zhong, M., Kawaguchi, R., Ter-Stepanian, M., Kassai, M., & Sun, H. (2013). Vitamin A Transport and the Transmembrane Pore in the Cell-Surface Receptor for Plasma

Retinol Binding Protein. *PLoS ONE*, 8(11), e73838.

doi:10.1371/journal.pone.0073838

41. Zhu, J., & Paul, W. E. (2008). CD4 T cells: fates, functions, and faults. *Blood*, 112(5), 1557-1569. doi:10.1182/blood-2008-05-078154

Appendix



Appendix 1. Representative confocal microscopy images of differentiated ARPE-

19 cells. Panel A1-3, Cholera toxin subunit B AlexaFluor 488. Panel B1-3, STRA6

AlexaFluor 647. Panel C1, merged images. Panel C2, Hoeschst. Panel C3, DIC image.

Panel D2, merged images. Panel D3, Hoeschst. Panel E3, merged images. Alexa

Fluor488 excited by the 488nm laser, emission collected 505nm-590nm. Alexa Fluor647

excited by the 635nm laser, emission collected 650-770nm. Hoechst excited by 405nm

laser, emission collected 410-520nm. STRA6 expression is intracellular and not co-

localized with the lipid raft or plasma membrane.