THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF VETERINARY AND BIOMEDICAL SCIENCES

THE EXPRESSION OF SURFACE MARKERS CD14 AND CD33 ON RED BLOOD CELL PROGENITOR CELLS

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A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Immunology and Infectious Disease
with honors in Immunology and Infectious Disease

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ABSTRACT

Stress erythropoiesis is an alternate pathway for erythrocyte development, triggered by periods of stress including anemia, oxygen poor in-utero development, and other conditions that lead to hypoxia. This pathway allows for the rapid production of red blood cells (RBCs) via the expansion and differentiation of stress erythroid progenitors (SEPs). SEPs express unique cell surface markers that differ from those of mature erythroid markers. Upon secretion of erythropoietin (EPO), SEPs are triggered to differentiate into stress BFU-Es. A strong understanding of the cell surface markers that differentiate SEPs from mature erythrocytes, as well as those that differentiate stress erythrocytes from steady state erythrocytes, is important for expanding research on erythropoiesis and for improving therapies for relates diseases, like anemia. The research presented in this thesis was conducted to investigate the expression of two distinct cell surface markers, CD14 and CD33, in a population of immature stress progenitors, Kit+Sca1+ cells. Data was also collected for the expression of these markers in more mature, Kit+Sca1- cells. The results show that the expression of these markers is dynamic, changing from cell expansion to cell differentiation. Overall, the unique expression patterns of CD14 and CD33 show a potential use for delineating SEPs from stress BFU-Es, although additional studies must be conducted to determine the relationship between these two cell markers.

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Lastly, I would like to thank my family for their continuous support through the entirety of my academic life. My mother has supported my decision to pursue a career in medicine since I was in middle school and I strongly believe that her faith in me has served as my motivation, leading me to where I am today.

Chapter 1

Introduction

Erythropoiesis

Erythropoiesis is the process that gives rise to mature red blood cells (RBCs), or erythrocytes. Erythrocytes are rich in oxygen-carrying hemoglobin, a protein that delivers oxygen from the lungs to peripheral tissues and then transports carbon dioxide from those tissues to be expelled from the body (1). Hemoglobin is a four-subunit protein, in which each subunit contains a heme group with a high affinity for oxygen. Each hemoglobin has 2 alpha globin proteins, 2 beta globin proteins, and 4 heme molecules, while each heme molecule has a central ferrous iron atom to which the oxygen binds for transport in the bloodstream (1).

The development of mature RBCs occurs in a step-wise process that works constantly in a healthy functioning human, generating 2 million RBCs per second (2). Steady state erythropoiesis occurs in the bone marrow (BM), beginning from multipotent hematopoietic stem cells (HSC) (3). In the first stage of erythropoiesis, HSCs differentiate through three cell types: a common myeloid progenitor, a megakaryocytic-erythroid progenitor, and a burst-forming unit-erythroid (BFU-E) (2). The differentiation to BFU-Es make up the engagement phase of erythropoiesis. The BFU-E is committed to erythroid production and differentiates into a more mature colony forming unit-erythroid (CFU-E) (2). Once the sequence reaches the point of committed erythroid-precursors, the terminal phase of erythropoiesis begins (3). This takes place in erythroblastic islands within the BM.

The second stage of erythropoiesis involves another series of differentiating cells. CFU-Es differentiate into proerythroblasts, followed by basophilic erythroblasts, polychromatic erythroblasts, orthochromatic erythroblasts, and finally, reticulocytes (2). The second stage is home to the critical enucleation that allows for the production of mature RBCs. The nucleus, along with many organelles, are removed as the orthochromatic erythroblasts differentiate into reticulocytes, the first enucleated cell of erythropoiesis (3). This also generates pyrenocytes, which consists of the ejected nucleus surrounded by a plasma membrane, that are digested and removed by macrophages (3).

In the last stage of erythropoiesis, the reticulocytes complete maturation, removing any remaining membrane bound organelles, and completing the terminal phase of erythropoiesis (3). Finally, the reticulocytes differentiate into an erythrocyte, which takes on the classical biconcave shape (2).

Erythropoiesis is a complex sequential pathway that requires a significant regulatory system. The hormone erythropoietin (EPO) plays a significant role in erythropoiesis regulation. It is secreted into the bloodstream from the kidneys in response to hypoxic conditions (2). This would allow for increased production of RBCs, thus delivering oxygen to the deprived peripheral tissues (2). Therefore, EPO is a positive regulator of erythropoiesis. EPO signaling acts in concert with the action of transcription factors, such as hypoxia inducible factor and GATA factors (2). GATA 1 is a key transcription factor that is needed for erythroid development. It also regulates the erythroid gene expression program. GATA 2, a member of the GATA family, acts on more immature progenitor cells, playing a role in the development of other hematopoietic lineages.

Stress Erythropoiesis

A second pathway for erythrocyte development is utilized during periods of stress, such as anemia, and other conditions that lead to a hypoxic environment (4). Fetal development is another example of when the oxygen poor in-utero environment requires a more robust erythroid response. This is known as stress erythropoiesis. Research has shown that this process occurs outside the BM and is referred to as extra-medullary erythropoiesis (4). Stress erythropoiesis allows for a rapid increase in RBC production in response to oxygen deprivation in the tissues. These stress conditions trigger the expansion of a population of specialized erythroid progenitors that respond to the secretion of EPO, leading to the enhanced differentiation of erythroid progenitors (4).

Stress erythropoiesis utilizes a unique set of erythroid progenitors that differ from those that give rise to RBCs via steady state erythropoiesis (4). Stress erythroid progenitors develop in the spleen; however, these cells originate in the BM. Unlike BM erythroid progenitors, which develop from multi-potential progenitors or megakaryocyte-erythroid progenitors, spleen stress erythroid progenitors (SEPs) are derived directly from short-term hematopoietic stem cells (ST-HSCs) that migrate to the spleen in response to anemic stress.

Research has shown that stress erythropoiesis is also regulated by hedgehog, bone morphogenetic protein 4 (BMP4), stem cell factor, and hypoxia (4). Once ST-HSCs migrate to the spleen, Hedgehog and BMP4 signaling restrict the ST-HSCs to the erythroid lineage, although the immature SEPs maintain the ability to self-renew. SEPs begin to proliferate following lineage restriction. These cells express specific stem cell markers, yet lack expression of mature erythroid markers. SEPs begin to commit to differentiate into stress BFU-Es upon

signaling of serum EPO. These cells begin to express mature erythroid markers, while losing stem cell markers. Stress BFU-Es differ from steady state BFU-Es in that stress BFU-Es, observed when cells are plated with low oxygen, produce a greater number of erythrocytes.

Overall, hedgehog also plays a critical role in the positive regulation of stress erythropoiesis, as it has been shown to control the conversion of BM erythroid progenitors to stress erythroid progenitors in the spleen (4). This is the signal that allows for the replenishment of the spleen progenitors, as discussed previously. Once the progenitors are successfully converted to stress progenitors, they can respond to other stress erythropoiesis regulatory signals that continue the process of RBC production.

Anemia

Anemia is a common condition that creates hypoxic stress, activating the stress erythropoiesis pathway. More than 3 million Americans are diagnosed with anemia, creating a demand for effective treatments (5). Anemic patients are deficient in RBCs and therefore, lack a sufficient number of cells to carry oxygen to peripheral tissues. Some anemic patients may be iron-deficient, significantly inhibiting the ability of RBCs to successfully bind oxygen; however, many other types of anemia exist that are caused by a myriad of underlying health conditions.

Aplastic anemia is characterized by the destruction of BFU-Es (5). This impedes steady state erythropoiesis in the BM, as well as the migration of erythroid progenitors to the spleen. Hemolytic anemia is characterized by the destruction of mature RBCs (5). An example of this type of anemia is sickle cell anemia, a genetic disorder in which the mutated beta globin protein polymerizes and causes the erythrocyte to adopt an unnatural crescent shape that results in premature cell death (5). Sickle cell erythrocytes also cause vaso-occlusive disease when they

become lodged in capillaries and veins. Lastly, anemia can be a result of non-blood related diseases. For example, kidney disease can interrupt EPO secretion, inhibiting the activation of the erythropoiesis pathway (5).

Anemia may be treated with dietary supplements, surgical intervention, medications, or blood transfusions (5). Specific treatment options are dependent on the cause of the specific anemia. While these methods improve the quality of life for anemic patients, they come with risk of complications (5). For this reason, there is a need to better understand the regulatory signals controlling erythropoiesis for the emergence of anemic-focused gene therapies.

Anemia is a debilitating disease that causes significant morbidity and mortality, thus negatively impacting quality of life. This disease has two different root causes. It can be caused by intrinsic defects in erythroid differentiation or it can be a result of secondary pathology associated with infection or inflammation. Anemia is currently treated via blood transfusions or with erythropoiesis stimulating agents (ESAs) such as recombinant human EPO. Blood transfusion therapy is effective in the short-term, but it leaves patients at risk for infection or a harmful inflammatory response. Long-term transfusion therapy for chronic anemia allows for the potential development of allo-antibodies or iron overload. Other difficulties associated with transfusion therapy include the requirement of a compatible pathogen-free blood source and the management of the blood source's shelf life. ESAs act by stimulating erythropoiesis, but it is not an effective treatment method for all types of anemia. ESAs can negatively interfere with chemotherapy regimens and can act as immunomodulatory compounds, potentially complicating other pathologies. Therefore, there is a significant need for the development of alternate therapies for anemia. A stronger understanding of the mechanisms by which the body responds to anemic stress could aid in identifying target areas for new treatments for anemia.

Thesis Research Background

Previous research carried out in the Paulson laboratory has successfully defined the stages of stress erythropoiesis using an in vitro culture system, which allows for the separation of the expansion and differentiation stages. Flow cytometry has been used to identify specific progenitor cell populations using Kit, Sca1, CD34, and CD133 cell markers. In order to increase our stock of cell surface markers, additional markers were tested on their ability to further delineate the progenitor populations. The cell surface markers tested were CD14 and CD33. These two markers were identified as being expressed in SEPs by Siyang Hao, a graduate student in the Paulson laboratory. Hao conducted microarray analysis of both rapidly dividing and slow dividing immature SEPs. The research showed that CD14 and CD33 were expressed at higher levels in slow dividing immature SEPs. In this thesis I analyzed the expression of CD14 and CD33 on SEP populations expanded in in-vitro cultures.

Chapter 1

Materials and Methods

Stress erythropoiesis Cultures

Two C57BL/J6 mice were utilized in this thesis research. Murine bone marrow cells were isolated and cultured in stress erythropoiesis expansion media (SEEM) for 5 days. SEEM contains Gibco IMDM (Invitrogen) supplemented with 10% fetal bovine serum (Gemini Bio-Products), 1% penicillin-streptomycin, 10 μg/ml insulin, 200 μg/ml transferrin, 2mM L-glutamine, 0.01g/ml bovine serum albumin, 7 μl/L 2-mercaptoethanol, 30 ng/ml GDF15 (Novoprotein), 15 ng/ml BMP4 (R&D systems), 50 ng/ml SCF (Goldbio) and 25 ng/ml SHH (GoldBio). The media was then switched to stress erythropoiesis differentiation media (SEDM) for an additional 3 days. SEDM contains Gibco IMDM and 3U/ml EPO, as well as all supplements added to SEEM. SEDM cell cultures were incubated at 2% O_2, 5% CO_2.

Flow cytometry and cell sorting

The cells were collected on different days of expansion and differentiation. Cells were labeled with indicated antibodies, listed in supplemental table S1. A zombie yellow fixable viability dye (BioLegend) was used to determine dead cells. Flow cytometry was conducted using a LSR-II Fortessa Flow Cytometer (BD Biosciences) and the resulting data was analyzed using FlowJo software.

Table 1. Antibodies Used

Antigen	Fluorochrome	Company	Catalogue #
Kit (c-kit)	Brilliant Violet 421	BioLegend	1058282
CD14	PE-Cy7	BioLegend	123315
Sca1 (Ly-6A/E)	FITC	BioLegend	108106
CD33	PE	Thermofisher	12-0331-82

Chapter 2

Results

Cell Surface Marker CD14

The CD14 cell surface marker is a surface antigen protein expressed by monocytes and macrophages (6). CD14 is critical for the recognition of bacterial lipopolysaccharide (LPS) and for mediating an innate immune response to a bacterial pathogen (7). It serves as a co-receptor for TLR2, TLR6, and TLR4 (6). Activation of the CD14 receptor leads to the expression of NFkB, the secretion of cytokines, and the initiation of an inflammatory immune response (6). CD14 acts through the MyD88 signal transduction pathway to trigger the immune response (6). CD14 plays a role in triggering phagocytosis in the apoptotic cells of the innate immune system, including monocytes and neutrophils (7). Overall, the CD14 marker is important for the identification of myeloid lineage cells (7).

Cell Surface Marker CD33

The CD33 cell surface marker is a sialic-acid-binding immunoglobulin-like lectin (Siglec) that binds to both alpha-2,3- and alpha-2,6-linked sialic acids glycans on the surface of pathogens (8). It is important for mediating cell-cell interactions. The CD33 receptor has a cytoplasmic tail containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that are phosphorylated by Src-family kinases upon activation, acting through downstream proteins such as PTPN6 and SHP-1 (8). Overall, CD33 is an important marker for cells of the myeloid lineage

and it has been demonstrated that antibodies against CD33 can successfully target normal and abnormal hematopoiesis sites (9). CD33 is also of clinical significance as it is expressed on the surface of leukemic blast cells in patients with acute myeloid leukemia (AML) (9).

Previous research has demonstrated that SEPs isolated from a population of slow dividing $PKH26^{Hi}$ cells expressed higher levels of CD14 and CD33 than the population of fast dividing $PKH26^{low}$ cells. This observation suggests that the CD14 and CD33 surface markers may be useful for delineating sub-populations of SEPs. To test this, we expanded SEPs in vitro and examined the expression of CD14 and CD33 markers in a Kit+Sca1+ immature population and a Kit+Sca1- mature population. Mouse BM cells were expanded in SEEM for 5 days, followed by SEDM for 3 days. Expansion cells were harvested after 3 (E3) and 5 (E5) in SEEM. Differentiating cells were harvested after 1 (D1) and 3 (D3) days in SEDM. Flow cytometry was used to analyze the expression of CD14 and CD33, as well as Kit and Sca1.

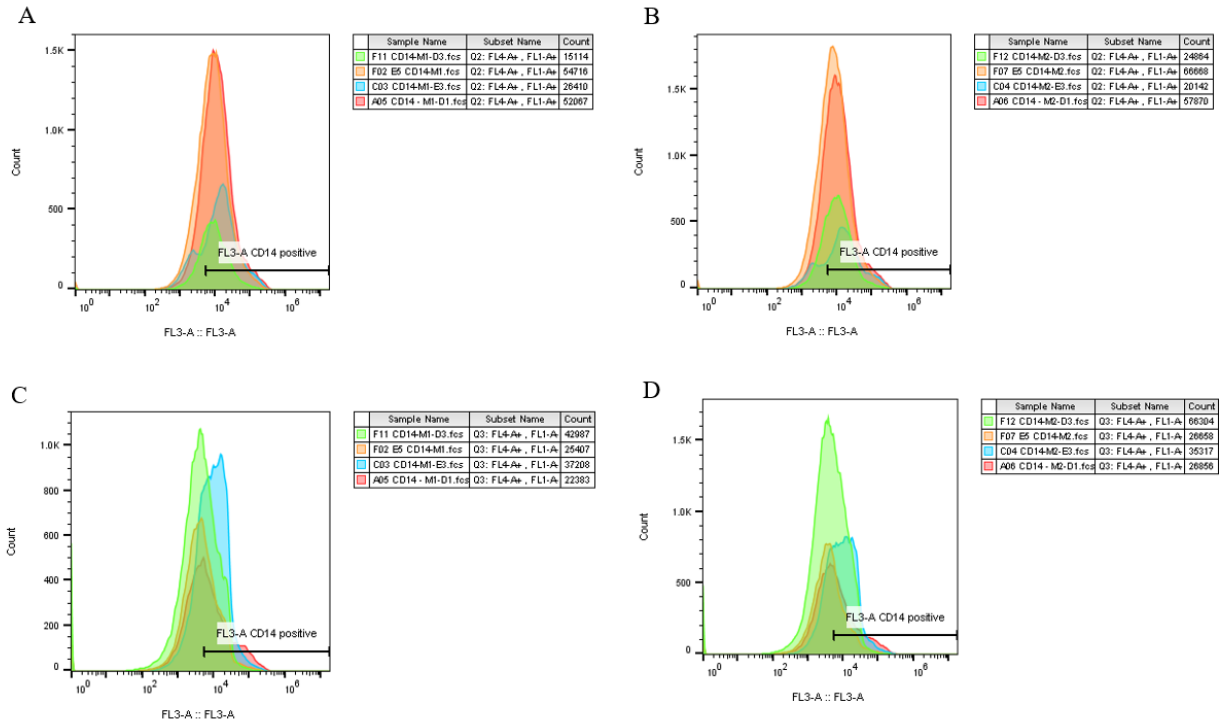

Figure 1. Change in CD14 Kit+/Sca1+ and Kit+/Sca1- surface markers

A) Cell count of Kit+/Sca1+ expression for Mouse 1. B) Cell count for Kit+/Sca1+ expression for Mouse 2. C) Cell count for Kit+/Sca1- for Mouse 1. D) Cell count for Kit+/Sca1- for Mouse 2. Expansion day 3 is represented in blue. Expansion day 5 is represented in orange. Differentiation day 1 is represented in red. Differentiation day 3 is represented in green. Figure 1 expression was quantified using an Accuri C6 Flow Cytometer and data analysis was done using FlowJo 10.6.1.

The cells quantified in Figure 1 show the percentage of cells that express the CD14 surface marker. Figure 1A and 1B present data for Kit+/Sca1+ cells, or immature stress progenitors. Figure 1C and 1D present data for Kit+/Sca1- mature cells. The data shows that the CD14+ expressing Kit+/Sca1+ cell count increases from expansion day 3 to expansion day 5 in both mouse 1 (1A) and mouse 2 (1B). In that same population of cells, the cell count decreases from differentiation day 1 to differentiation day 3 in both mouse 1 (1A) and mouse 2 (1B). The results for CD14+ expressing Kit+/Sca1- cells present inverse trends in data. The CD14+

expressing Kit+/Sca1- cell count was highest at expansion day 3 and differentiation day 3 for both mouse 1 (1C) and mouse 2 (1D). In this population, cell count decreased from expansion day 3 to expansion day 5 in mouse 1 (1C), but not mouse 2 (1D), while cell count increased from differentiation day 1 to differentiation day 3 in both mice.

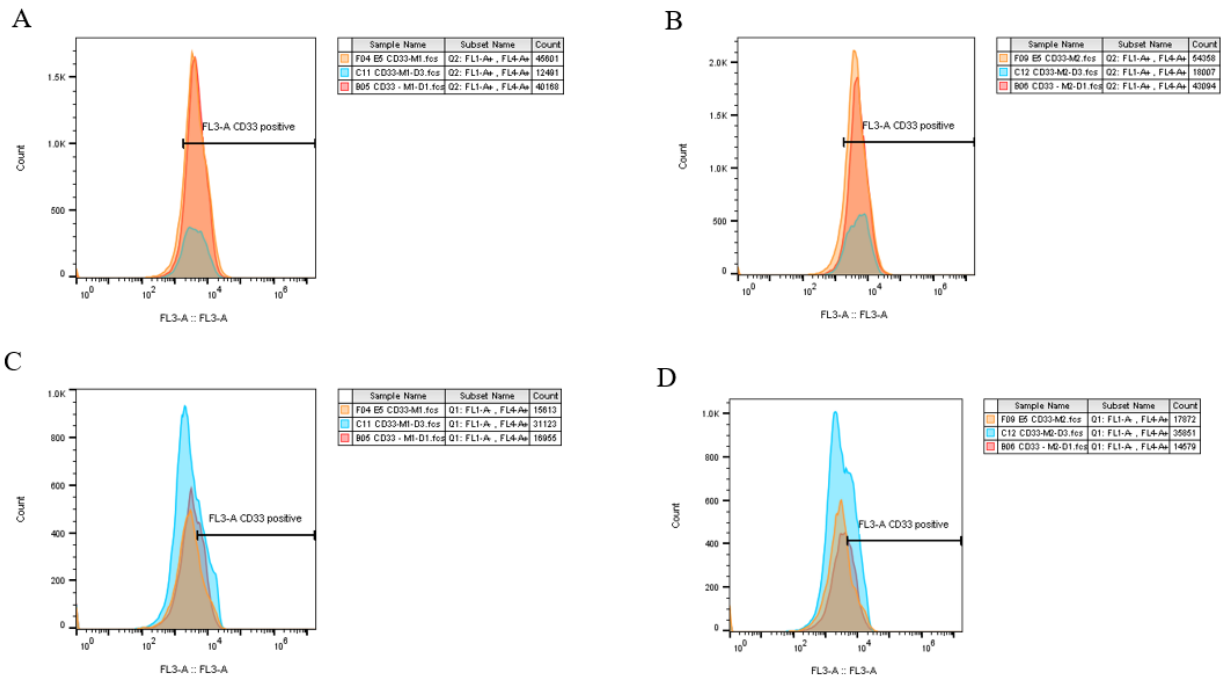

Figure 2. Change in CD33 Kit+/Sca1+ and Kit+/Sca1- surface markers

A) Cell count of Kit+/Sca1+ expression for Mouse 1. B) Cell count for Kit+/Sca1+ expression for Mouse 2. C) Cell count for Kit+/Sca1- for Mouse 1. D) Cell count for Kit+/Sca1- for Mouse 2. Expansion day 3 is represented in red. Expansion day 5 is represented in orange. Differentiation day 3 is represented in blue. Figure 2 expression was quantified using an Accuri C6 Flow Cytometer and data analysis was done using FlowJo 10.6.1.

The cells quantified in Figure 2 were analyzed for the expression of the CD33 surface marker. Figure 2A and 2B present data for Kit+/Sca1+ cells, or immature stress progenitors. Figure 2C and 2D present data for Kit+/Sca1- mature cells. The data shows that there is little

change in the CD33+ expressing Kit+/Sca1+ cell count between expansion day 3 and expansion day 5 for both mouse 1 (2A) and mouse 2 (2B). However, the cell count for this population decreased approximately three-fold from the expansion days to differentiation day 3 in both mice (2A and 2B). The data for the CD33+ expressing Kit+/Sca1- cells show inverse results. There is little change in the cell count for this population between expansion day 3 and expansion day 5 for both mouse 1 (2C) and mouse 2 (2D); however, the cell count of CD33+ expressing Kit+/Sca1- cells increased significantly from the expansion days to differentiation day 3 in both mice (2A and 2B).

Chapter 3

Discussion

The results show that the expression of CD14 and CD33 surface markers vary throughout the stages of expansion and differentiation in culture. In the Kit+/Sca1+ cell population, CD14 expression increases during expansion, yet decreases during differentiation once the cells are switched to SEDM with the addition of EPO. The inverse is true in the Kit+/Sca1- cell population, as CD14 expression decreased in expanding cells and increased in differentiating cells. However, the data was not precise enough to determine if the Kit+/Sca1- cells highly expressing CD14 were truly erythroid progenitors. The Kit+/Sca1- cells could represent bone marrow myeloid progenitors during their early life or represent monocytes or other progenitors in their later life. To confirm that the cell population consists of stress erythroid progenitors, additional research must be conducted to investigate the capability of the Kit+/Sca1+ cells to differentiate into stress BFU-Es. Furthermore, additional research can be carried out to investigate the role of LPS in Kit+/Sca1+ cell growth, being that CD14 is an LPS receptor.

The observed expression pattern for surface marker CD33 varied from that of CD14. Most notably, for the Kit+/Sca1+ cell population, CD33 expression maintained relatively constant during expansion, but decreased during differentiation. This shows that the addition of EPO marks a point of change in expression, as seen with the CD14 marker as well. CD33 is a surface protein that binds sialic acid glycans; therefore, it is possible to attribute the change in CD33 expression to differing interactions between SEPs and monocytes and macrophages. Additional experiments can be carried out in the future to investigate this hypothesis by studying the changes in adhesion molecules on monocytes and macrophages.

The next step to expanding on this research would be to track the expression of both CD14 and CD33 simultaneously on Kit+/Sca1+ cells. The data presented in this thesis has shown that CD14 and CD33 have opposite trends of expression. Analyzing this relationship more in-depth could help to illuminate their respective roles in erythropoiesis. A strong understanding of the erythropoietic stages in which each marker is present can also be utilized by other researchers for tracking SEPs.

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- Highly selective membership (total enrollment of 90 students); each year 30 rising sophomores are selected for the three-year program based on their academic achievements, leadership potential, and commitment to critical thinking exhibited during the applicant's first year at Penn State
- The academy prepares students to address difficult issues facing the world via classes, leadership seminars, engagement in community programs, field trips, and a mentorship program
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The mission of the Schreyer Honors College is to shape people who shape the world.

- Admission to the Schreyer Honors College is highly selective
- Approximately 300 students achieve an honors education with academic excellence and integrity each year
- Completion of an honors education helps the student develop a global perspective and opportunities for leadership and civic engagement
- Schreyer Honors College students represent 5 percent of all Penn State undergraduates

- Scholars earn distinction upon graduation
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Undergraduate Researcher, *Paulson Laboratory*, The Huck Institutes of Life Sciences, The Pennsylvania State University 2017 – Present

- Study the intricacies of the cell signaling pathway that lead to stress erythropoiesis in the spleen.
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Undergraduate Researcher, *Hudson Laboratory*, The Huck Institutes of Life Sciences, The Pennsylvania State University Spring 2019

- Study the attraction of ticks to humans by conducting field work in woodland areas in State College, PA.

Undergraduate Researcher, *Hudson Laboratory*, The Huck Institutes of Life Sciences, The Pennsylvania State University 2017 – 2018

- Study the interactions between parasites and host mice to better understand fluctuations in the mice population throughout a given year and to stabilize the population within the ecosystem.

Awards:

- Dean's List, The Pennsylvania State University Fall 2016
- Dean's List, The Pennsylvania State University Spring 2017
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Scholarships and Grants:

- Schreyer Honors College Academic Excellence Scholarship Fall 2016 – Present
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- Graham Open Doors Honors Scholarship — Spring 2019
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- Bicksler Scholarship — Fall 2019 – Spring 2020
- Hayes Trustee Scholarship in Agriculture — Fall 2019 – Spring 2020
- B. and L. Gall Open Doors Scholarship — Fall 2019 – Spring 2020
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Volunteer Service and Work Experience:

Member – Global Medical Brigades USA (Global Volunteer Organization) 2016 – Present

- The Pennsylvania State University chapter of the non-profit global health organization

Member – Remote Area Medical (RAM) 2018 – Present

- The Pennsylvania State University chapter of the non-profit organization
- Providing mobile medical clinics to areas in need

Teen Ambassador, *Pajama Program* 2014 – 2016

- Organized and led school-wide fundraisers to collect funds, pajamas, and books for children in low- income families, children without homes, and children in orphanages.
- Collaborated with Teen Ambassadors from each of the five boroughs of New York City
- Volunteered at the New York center to read to children

Internship – Dr. Nick G. Faraci, Dr. Jason Perlman, Comprehensive Pediatrics 2015 – 2016

- 380 clinical hours (Unpaid)
- Observed physician-patient interaction
- Transferred confidential patient files to environmentally safe digital charts

Volunteer - Schreyer Honors College Spend a Summer Day Tour Guide Summer 2017 – Present

Volunteer - Schreyer Honors College Spend a Summer Day Panelist Summer 2018 – Present

Volunteer – Tutor for Single Parent Households 2014 – Present

- Algebra, geometry, and trigonometry

Volunteer – Dyker Heights Athletic Association 2012 – 2016

- Soccer and softball coach for children ages 4 to 12
- Held open soccer clinics for all children of the program

Unpaid Work – Glitzy Chicks Makeover and Spa Parties 2014 - Present

- Party Point Person – acts as liaison between the office and customer, organizes staff, responsible for the operation of the event
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Membership in Professional and Scientific Societies:

Alpha Epsilon Delta, Penn State Beta Chapter 2016 – Present

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Skills:

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- Other Laboratory Training: Agarose gel electrophoresis, Polymerase Chain Reaction (PCR), light microscopy, molecular cloning
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Relevant Experiences:

Teaching Assistant – Race and Ethnic Relations, Dr. Wayne Gersie Fall 2017 - Present

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- Communicate with students
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Shadow – NJ Pain, Spine & Sports Associates, Dr. Faheem Abbasi Summer 2019

- Observed doctor-patient interactions
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- Worked alongside medical scribe to elicit and record patient history

URISE – Undergraduate Research Society, The Pennsylvania State University Fall 2016

- Undergraduate Research in Science and Engineering Program
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- Observed live surgery
- Examined human organ tissue under a microscope