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Comparing Antibody-Coated Immune Beads with Flow Cytometry to Measure ß-2-Microglubulin+ Murine Spleen Cells

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COMPARING ANTIBODY-COATED IMMUNE BEADS WITH FLOW CYTOMETRY TO MEASURE β-2-MICROGLUBULIN^{+} MURINE SPLEEN CELLS.

A thesis submitted in partial fulfillment

of the requirements for the degree of

Master of Science.

By

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B.S., King Saud University, 2011

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Amal Fahad Alajman ENTITLED Comparing Antibody-Coated Immune Beads with Flow Cytometry to Measure β-2-Microglobulin^{+} Murine Spleen Cells. BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Alajman, Amal Fahad. M.S., Microbiology and Immunology Graduate Program, Wright State University, 2016. Comparing Antibody-Coated Immune Beads With Flow Cytometry to Measure β-2-Microglubulin+ Murine Spleen Cells.

In diseases like AIDS, Multiple Myeloma, Multiple Sclerosis (MS), Type 1 Diabetes, and Systemic Lupus Erythromatosus the detection of major histocompatibility complex type 1 molecules (MHC 1) can be a helpful component of disease diagnosis and prognosis. Most somatic mammalian cells display to varying degrees major histocompatibility complex (MHC) class 1 antigen on the cell surface. MHC1 molecules consist of a polymorphic alpha α chain and a monomorphic beta chain, beta 2 microglubulin (β2m). The β2M composition is fairly constant within a species whereas the alpha chain is not. The polymorphic alpha chain is encoded by an MHC complex gene that is specific for each member of the species. The consistency of the β2M protein within a species makes it a superior target to the variable α chains when attempting to quantify MHC1 molecules**.** Currently, there are no reagents available to identify the MHC1 antigen within an outbred population such as human or laboratory animals other than defined in bred mouse strains. In this study, the presence of MHC1 molecules on spleen cells from an outbred mouse strain (ICR strain) was evaluated by looking for monomorphic β2m molecules. The two methods, which were used to obtain the numbers of $\beta 2m^+$ mouse spleen cells, were flow cytometry and the antibody-latex bead method. Similar numbers of β2m- positive cells were obtained using these two methods (7-9%). These results demonstrate that the antibody-coated beads are a suitable, less expensive and time saving alternative to determine the numbers of $\beta 2m^+$ positive cells when compared to flow cytometry.

HYPOTHESIS

The hypothesis of our study is that the using antibody coated latex beads will give comparable results to those of flow cytometry in detecting cells bearing the light chain of MHC I.

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LIST OF ABBREVIATIONS

AIDS= acquired immunodeficiency syndrome

BiP= immunoglobulin binding protein

BSA= bovine serum albumin

CD =Crohn's disease

CD4= cluster of differentiation 4

CD8= cluster of differentiation 8

CML= Carboxylate modified Latex beads

CTL= cytotoxic T lymphocyte

DC= dendritic cell

DMEM= Dulbecco's Modified Eagle's Medium

ER= endoplasmic reticulum

ERK= extracellular signal- regulated kinases

HIV= human immunodeficiency virus

HLA= human leucocyte antigen

ICR= imprinting control region mouse

IgG= immunoglobulin molecules

IL-10= interleukin 10

IL-6= interleukin 6

IMWG= international myeloma working group

INF-Y= interferon gamma

ISS= international staging system

LMP2, and LMP 7= two subunits of the protostome

MEK= Mitogen-activated protein kinase

MES= 2-N- morpholino-ethanesulfonic acid

MHC= major histocompatibility complex

MM= Multiple Myeloma

MS= multiple sclerosis

NK= natural killer cell

NKT= natural killer T cell

NMDAR= N-methyl-D aspartate receptor

PBS= phosphate buffer saline

PLC= peptide loading complex

RA= rheumatoid arthritis

Raf= proto-oncogene serine/threonine-protein kinase

SLE= systemic lupus erythematosus

STAT3= Signal transducer and activator of transcription 3

T1D= type 1 diabetes

TAP= transporter associated with antigen processing

TCR= T cell receptors

TPN= tapasin

UC=ulcerative colitis

WBCs= white blood cells

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Introduction

This study is a continuation of the work of Sana Alhawsawi who used immunohistochemistry to detect the expression of the polymorphic alpha chain and β -2-microglobulin (β2m) of the major histocompatibility complex I (MHCI) in different mouse tissues (spleen, stomach, small and large intestine). She found no difference in expression using antibodies specific for the alpha-chain verses the β -2-microglobulin. The goal of the present study was to quantitate the expression of β -2-microglobulin on mouse spleen cells using flow cytometry and direct counting of cells attached to antibody-coated latex beads.

Flow cytometry detects single cells through laser detector beams. The procedure of the flow cytometry starts by using fluorescent-labeled antibodies specific to cell-surface markers that are selected to show the cell population of interest (Jahan-Tigh, Richard R., et al. 2012). This entire process lasts approximately 10 hours. The cost of flow cytometry ranges from $30,000 to $150,000 (Matthews, 2006). Developed and developing countries could both benefit from a faster and more affordable method to detect cell populations of interest.

The beads method involves microscopically counting cells attached to latex beads. In this study, latex spheres were coated with a β -2-microglubulin rabbit polyclonal antibody and used to enumerate the numbers of single positive β -2-microglubulin^{+} cells in a suspension of mouse spleen cells.

Super Active Latex Beads

There are five types of Super active latex beads: carboxylate modified beads (CML), chloromethyl beads, aldehyde/amidine beads, aldehyde/sulfate beads, and aliphatic amine. The functional groups of super active latex beads have a very high density for covalent coupling of proteins to the particles. The super active layer consists of a three- dimensional layer that raises the colloid stability of the particles and makes it easier for the protein to bind to the particles. This type of bead maintains the protein structure more than the distortion of proteins caused by absorption onto a rigid surface ("Super Active Latex Beads," n.d.).

Carboxylate-modified beads are composed of latex polymer particles that have highly charged and relatively hydrophilic surfaces with a pka of 5-9. These features create a perfect site for covalent bond formation with the antiβ-2 macroglobulin rabbit polyclonal antibody coating and present less distortion of the antibody structure ("Super Active Latex Beads," n.d.).

Literature Review

There are two major defensive systems against different kinds of pathogens: Innate and adaptive immune systems (Abele and Tampe, 2004). Genes of the host germ line encrypt the innate immune system while the genes of the host somatic line encrypt the adaptive immune system (Abele and Tampe, 2004).

Two types of cells are responsible for antigen recognition in the adaptive immune system: B and T cells. The B cells have evolved in humoral immune response. T cells associate with major histocompatibility complex (MHC) molecules in recognition of antigenic peptides (Abele and Tampe, 2004).

The major histocompatibility complex (MHC)

The major histocompatibility complex (MHC) is essential part of the immune system in all vertebrates by presenting peptides to T cells. The MHC gene is divided to two groups, MHC class I, and MHC class II. MHC molecules can mediate the interaction of white blood cells (WBCs), which are also known as leukocytes with each other or with other cells in the body. MHC genes specify the human leukocyte antigens (HLA) (Hughes and Yeager, 1998).

The MHC class I

MHC class I molecules are located on every nucleated somatic cells (Reche and Reinherz, 2003). MHC class 1 molecules present the antigens from cytosolic (intracellular) proteins to T cells that express the CD8 cell-surface glycoprotein (Bjorkman and Parham, 1990). Consequently, the process of MHC class I presentation is called the cytosolic or endogenous pathway. Virus specific cytotoxic T lymphocytes

(CTL) bind to somatic cells that present MHC class I molecules on cell surface for peptides that derived from viral antigens and eradicate infected cells (Hewitt, 2003).

MHC class I antigens contain two polypeptide chains, variable heavy (α chain) and light chains (β2microglobulin). The α chain has three domains α1, α 2, and α 3. The variable heavy chain (α chain) is polymorphic and encoded by human genes called HLA-A, HLA-B, and HLA-C (Figure 1) (Bernal et al., 2012).

The two chains (α and β2-microglobulin) are bound together by a non-covalent bond via the interaction between β2m and the α3 domain. The α3 domain binds to the CD8 co-receptor on T cells. On the other hand, the α1-α2 heterodimer bind to T cell receptor (TCR) on cytotoxic T cell (Bernal et al., 2012). Peptides that attached to MHC class I glycoproteins are 8-10 amino acids long (Gil-Torregrosa et al., 1998).

The β2m chain is monomorphic and is encoded by the β2m gene. The composition of β2m is constant in every cell of a given species. The β2m protein is small enough to be filtered by the glomerular membrane and be completely reabsorbed by the proximal tubule back into the blood. Hence, serum levels have a significant impact on glomerular function in disease conditions. If glomerular function is damaged, serum levels increase in reverse ratio to glomerular filtration rate (Karlsson et al., 1979). In some diseases including Multiple Myeloma, Multiple Sclerosis (MS), AIDS, Type 1 Diabetes, and Systemic Lupus Erythromatosus the expression of the β2m chain in major histocompatibility complex type 1 molecules (MHC 1) can be a useful factor in disease diagnosis and prognosis (Fernando et al., 2008). The polyclonal antibody against β2m is easy to use, inexpensive, and is readily accessible in non-specialized laboratories (Delgado et al., 2009).

The MHC class II

MHC class II molecules are usually found on antigen presenting cells such as dendritic cells, macrophages, mononuclear phagocytes, and some endothelial cells, and B cells (Reche and Reinherz, 2003). MHC II molecules present antigens that derive from extracellular proteins to T cells expressing the CD4 cell-surface glycoprotein (Bjorkman and Parham, 1990). The pathway of MHC class II of antigen presentation is called the endocytic or exogenous pathway.

MHC class II molecules are similar to MHC class I molecules, in that they are also heterodimers, but MHC class II molecules consist of two polymorphic peptides chains (α and β chains). Both of these chains consist of two domains, α1, α2, β1, and β2 that are encoded within the HLA gene (Figure 1). Since the antigen -binding groove of MHC class II glycoproteins is open at both ends, it binds to peptides between 15 and 24 amino acids in length. In contrast, the antigen binding groove on class I molecules is closed at each end and binds smaller peptides (8-12 amino acids) (Ting and Trowsdale, 2002).

The expression of MHC class II is stimulated by INF-γ at the transcriptional level (Ting and Trowsdale, 2002). The expression of MHC-II glycoprotein is activated via the initiation of the protein tyrosine kinases JAKI and JAK2 signaling cascade, leading to tyrosine phosphorylation, and STAT1 dimerization. Subsequently, STAT1 dimers are transported to the nucleus where they attached to IFN-γ-activated sequence (GAS) elements in the promoters of IFN-γ genes (Giroux et al., 2003).

INF- γ is a cytokine that plays a critical role in the innate and adaptive immunity against viral, bacterial and protozoa infectious agents. In the immune system, IFN-γ directly prevents viral replication. IFN-γ also regulates antigen presentation, proliferation, and differentiation of lymphocytes. Furthermore, high levels of IFN-γ serve as an indicator of T helper 1 (Th1) activity in stimulating the phagocytic actions of macrophages (Billiau et al., 1998)**.** Natural killer cells (NK) and natural killer T (NKT) cells produce INF- γ as part of the innate immune response, while the CD4 Th1 and CD8 cytotoxic T lymphocyte (CTL) as part of adaptive immunity (Ting and Trowsdale, 2002).

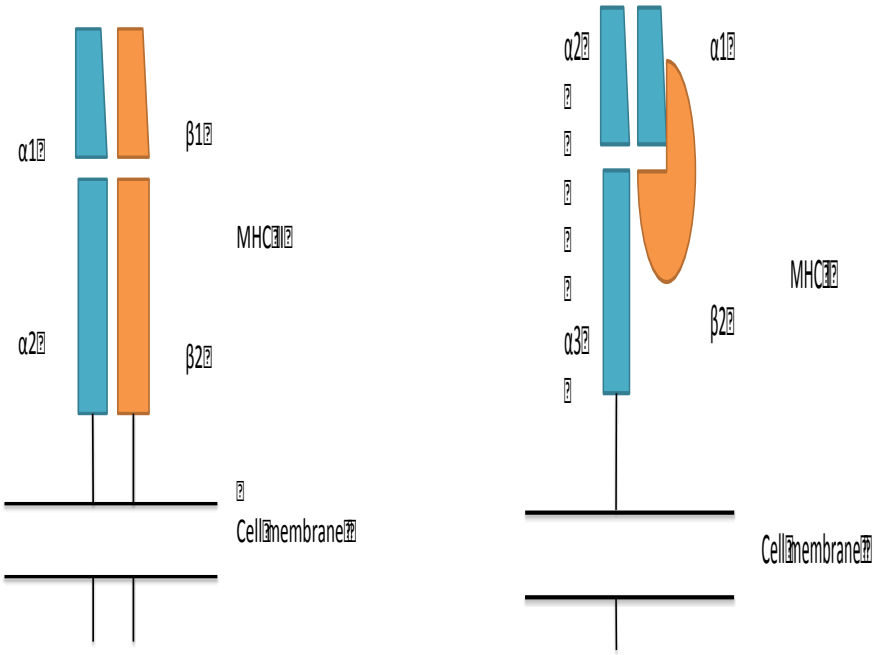

Figure 1. Schematic diagram that shows the differences in the structure of two classes of major histocompatibility complexes (MHC). MHC class1 contains three α-domains and oneβ-2microglubulin molecule. The peptide-binding groove is found between α1 and α2 domains. MHC class II has two α-domains and two β-domains. The peptide-binding groove is found between α1 and β1 domains (Modified from Zhong et al., 2011).

MHC class I pathway for antigen presentation

The peptides binding to the MHC class I are generated by the 20 S proteasome (Lehner et al., 1998) and the assembly of MHC class I molecules occurs in the endoplasmic reticulum (ER) lumen by the formation of β-2-microglubulin dimers (Figure. 2) (Ortmann et al., 1997) Two subunits LMP2 and LMP7 are essential for degradation of cytosolic proteins to small peptides (Lee et al., 1999). The transporter associated with antigen processing (TAP) facilitates the translocation of the peptides from the cytosol into the ER lumen (Neumann and Tampe, 1999).

Two stages are involved in the formation of MHC1 glycoprotein in endoplasmic reticulum (ER) in: the early phase and the later phase. The folding of the alpha chain α with the β2m chain occurs in the early stage. The late phase involves the formation of the 'peptide-loading complex' (PLC). In the early stage, monoglycosylated ($Man_9GlcNAc_2Glc_1$), which is sugar that chemically associated at position 86 of α chain with conserved asparagine. This facilitates the binding between the trans-membrane domain of α chains in MHC class 1 and chaperone calnexin (Antoniou and Powis, 2008). The structural domain of calnexin (glycan protein) promote the binding of MHC class 1-α chains with the extended proline-rich domain and helps the formation of a 'folding cage' for the early phases of assembly. However, the proline-rich domain recruits oxido-reductase ERp57, which is one member of the protein disulphide isomerase family that can decrease, oxidize or isomerize disulphide bonds. In the late stage, MHC class I α chains binds to the immunoglobulin-binding protein (BiP). BiP binds temporarily, or for a long period either to newly synthesized or misfolded proteins in the MHC I α chains (Antoniou and Powis, 2008). After that, the calnexin is transported by another soluble ER

lectin, calreticulin, which involves prolonged proline- rich domain that recruits ERp57 (Antoniou and Powis, 2008). The calreticulin binds to TAP to form the peptide loading complex (PLC) with the help of Tapasin (TPN), which is an accessory molecule of MHC I**.** PLC is very critical to assisting appropriate MHC I loading before the entire complex is transfered to the cell surface**.** The peptide-loading complex (PLC) coordinates peptide transport and recognition in addition to the efficient loading of high-affinity peptides onto MHC I glycoproteins (Blees et al., 2015). When MHC class I molecules dissociate from TAP, they are transferred to the cell surface by cargo vesicles for transfer to the Golgi. Then, MHC I molecules display their antigen cargo to Cytotoxic T lymphocytes CTLs (Figure 2) (Hewitt, 2003).

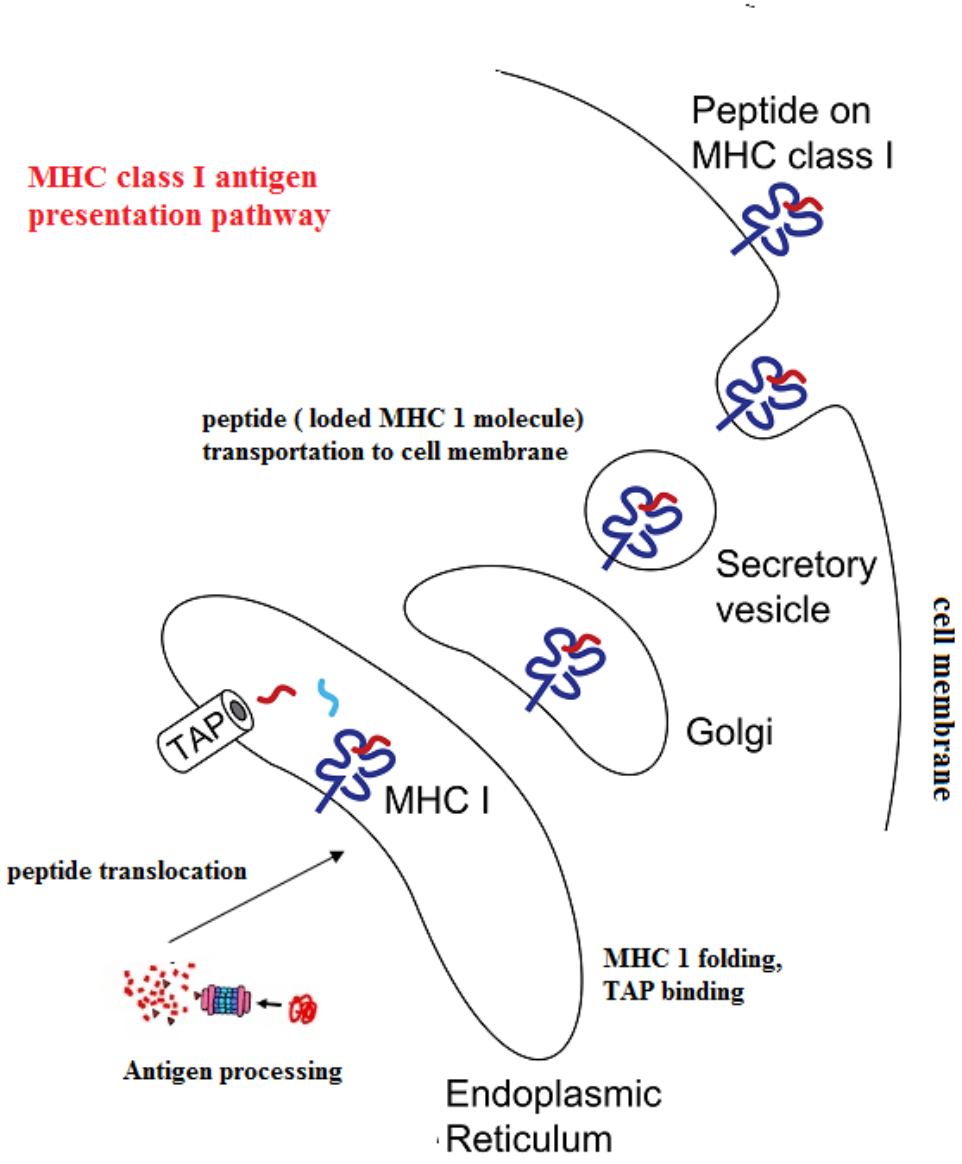

Figure 2.The MHC class I antigen presentation pathway.

First, proteasome generate the proteins in the cytosol. Then, peptides translocate into the ER lumen by TAP. ER chaperones calnexin, calreticulum and ERP57 facilitates the MHC class I molecules folding and assembly in the ER. When Peptides bind to MHC class I molecules, it dissociates from TAP and then is transported to the plasma membrane (Modified from Hewitt, 2003).

MHC class I on Human and Mouse

In humans, the α chains of MHCI are different than that found in mice. The α gene of MHCI is known as human leukocyte antigen (HLA) and displays three-class loci (A, B and C) known as HLA-A, HLA-B, and HLA-C (Pick et al., 2012). While in mice, the MHC I α chain is coded by the H-2 gene. The MHC I H-2 gene presents three loci (K, D, and L). In each locus, both alleles are expressed, so these genes are extremely polymorphic. For example, HLA-B gene has about 500 known alternative genes, over 200 variants of HLA-A genes, and about 100 variants of HLA-C genes (York and Rock, 1996). In humans, the major histocompatibility complex class I is found on the chromosome 6 while in mice it is located on the chromosome 17 (Relle and Schwarting, 2012). In humans, β2m is found on chromosome 15 while in mice is located on chromosome 2. One allele is found in humans, whereas seven alleles are found in mice (York and Rock, 1996).

Biological roles of MHC I

Beside the well-known role of human MHC I glycoprotein in the immune response, some studies have indicated that human MHC I plays a role in some autoimmune and inflammatory diseases. MHC I seems to be important in the pathogenesis of multiple sclerosis (MS), Crohn's disease (CD), type 1 diabetes (T1D), ulcerative colitis (UC), systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) (Fernando et al., 2008). MS affects the central nervous system and is a chronic inflammatory syndrome and T1D is a chronic autoimmune disease described by T cell-mediated impairment of pancreatic cells, which results in insulin insufficiency. SLE, also

known as lupus, is an autoimmune disease that can affect multiple organ systems. Lupus is a genetic disease linked to the genes of the major histocompatibility complex (MHC)(Relle and Schwarting, 2012). CD and UC are inflammatory bowel diseases of the gastrointestinal tract. Rheumatoid arthritis (RA) is a chronic inflammatory polyarthritis which often will have systemic symptoms (Fernando et al., 2008). In inflammatory bowel disease (IBD) patients, the activity of β2m may enhance the activation of macrophages and T-lymphocytes (Yılmaz et al., 2014). In autoimmune diseases, the immune response of the body is abnormal in that it attacks antigens and tissues that are normally present in the body (Relle and Schwarting, 2012). The role of human MHC I in autoimmune diseases is not well understood, but the main cause for autoimmune diseases could be due to the disequilibrium alleles at multiple loci in the human MHC I (Fernando et al., 2008).

MHC I has an important role in transplantation complications, including graft rejection in human liver. In healthy liver tissue of humans, the expression of both chains of MHC 1 is very low (Steinhoff, 1990). In a liver transplant, the recipient's immune system targets the donor cells causing graft rejection. During rejection and after liver transplantation, the level of human MHC I protein is increased notably in the plasma membrane of the hepatocytes, epithelia, endothelia, and Kupffer cells (Steinhoff et al., 1990). The MHC I molecule has to be compatible in both donor and recipient cells in order for grafts to be accepted (Carcia et al., 2012).

In brain development, the function of MHC class I in human is similar to its function in mice. The genes of both MHC class I and β_{2m} are expressed in humans and mice during cerebellar cortex development (Lv et al., 2014). The expression of H-2Kb/Db and HLA-B/C molecules increase during the growth of the brain in mice and

humans, respectively, but they both decrease after birth (Lv et al., 2014). These findings emphasize the role of MHC class I in plasticity and synaptic remodeling during cerebellar development.

Recently it has been discovered that MHC I is expressed on neurons (Cebrián et al., 2016). There is evidence that expression of MHC I is necessary for the development of the brain structure involved in memory (Cebrián et al., 2016). Furthermore, an overexpression of neuronal MHC I also appear to play a role in neurodegenerative disorders like Parkinson's disease (PD) (More et al., 2013).

β2-microglobulin

All nucleated cells synthesize β2microglobulin polypeptide and it is found in most cells. It forms the light chain of MHC class I (Chelazzi and Senaldi, 1986). It has a low molecular weight 11,800 kDa and has structure that is similar to the constant region of immunoglobulin molecules (IgG). This protein can be isolated from human urine (Manicourt et al., 1978). β2m is not significantly polymorphic and is similar in all mammals (York and Rock, 1996). β2m is a non-glycosylated protein and consists of 100 amino acids. The level of normal serum β2m is usually less than 3mg/L, but in elderly people it gradually increases. Kidneys play an important role in β2m catabolism as evidence by the high serum levels of β2m in the renal failure patients (Chelazzi and Senaldi, 1986). High serum levels of β2m are present in neoplastic and non- neoplastic diseases, and acquired immunodeficiency syndrome (AIDS). Also, β2m is useful marker in the evaluation of the multiple myeloma (Chelazzi and Senaldi, 1986). Many studies show that the level of serum β2m may be elevated abnormally in patients with different types of malignant tumors, as compared to controls (Forman, 1982).

Serum β2 microglobulin and diagnosis of human disease

Serum β2 microglubulin has been determined as an important prognostic marker in several diseases such as hematologic malignancies and non-hematologic disorders (Yang et al., 2006) .The levels of β2 microglubulin were elevated in lymphoma proliferative disorders including multiple myeloma, and chronic lymphocytic leukemia (Henne et al., 1996). In 1982, Bataille et al found that the levels of serum β2m were significantly elevated in patients with multiple myeloma at time of diagnosis comparable to normal individuals (bataille et al., 1982). Also, the levels of serum β2m in multiple myeloma seem to correlate well with survival time. Patients with the highest level of serum β2m have the shortest survival (Bataille, and Grenier, 1987). In addition, there is a correlation between the levels of serum β2m and the stage of the multiple myeloma disease. In the late stage of the disease, patients have higher levels of β2m than in the early stage of the disease (Chelazzi and Senaldi, 1986). The elevation of serum β2m in multiple myeloma indicates it functions to increase inflammation (Prizment et al., 2014).

In the immune system, the propagation of B cells and the production of antibodies are tightly regulated. In multiple myeloma, usually the chromosomal translocation occurs between the immunoglobulin heavy chain gene (on chromosome 14, locus q32) and an oncogene (often 11q13, 4p16.3, 6p21, 16q23 and 20q11). This results in dysregulation of the oncogene, which is the initiating consequence in the pathogenesis of myeloma. (Kyle and Rajkumar, 2004). In addition, the serum of patients with renal failure will have high levels of β2 microglubulin. In patients with healthy kidneys, the normal serum levels of β2 microglubulin are 1.5 to 3-mg/L while in renal failure, the levels increase by 60-fold

and more (Winchester et al., 2003).

The levels of serum β2 microglubulin are elevated in human Immunodeficiency virus (HIV) infection, acquired immune deficiency syndrome (AIDS), and autoimmune disorders such as rheumatoid arthritis. However, the high level of β2 microglubulin can activate or inhibit the immune system. For example, in HIV patients, the high level of serum β2 microglubulin is associated with progression to AIDS while the levels of serum β2 microglubulin correlates with poor prognosis in the hematologic malignancies (Xie et al., 2003).

Disease conditions associated with elevated levels of serum β2 microglubulin include multiple sclerosis, meningeal dissemination, and sarcoidosis (Adachi, 1991). Increase the serum levels of β2m are due to that the β2 microglubulin is shed from the surface of nucleated cells into serum during these diseases. These syndromes are involved with activation of the immune system and/or increased cell turnover (Gooptu et al., 2014).

Staging of multiple myeloma

Multiple myeloma is a malignancy of B- lymphocytes. These malignant lymphocytes express more β2m (Kumar et al., 2008). International myeloma working group (IMWG) has described a staging system of multiple myeloma known as international staging system (ISS) based on two factors: β2m, and serum albumin (Raja, 2012). The level of serum β2m is normally less than 2mg/L (Xie et al., 2003), but in multiple myeloma it increases up to 5.5 mg/L (Raja, 2012). International staging system (ISS) has been described three stages of Multiple Myeloma (Raja, 2012). In stage I, the level of Serum β2 microglobulin is <3.5-mg/L and albumin ≥3.5 g/DL and the survival

duration is an average of 62 months. In stage II, the survival duration is 44 months and the β2 microglobulin is ≥5.5 mg/L and the patients survive up to 29 months (Raja, 2012). The high levels of serum β2m in multiple myeloma are associated with increased cell proliferation (Karlsson et al., 1979).

β2-microglobulin as a negative regulator of the immune system

Dendritic cells (DCs) are key cellular immune elements in peripheral tissues. When DCs encounter pathogens, they are activated and travel to the secondary lymphoid organs, where they stimulate a specific T-cell response (Xie et al., 2003). DCs are the most effective antigen presenting cells (APCs) and can stimulate different cells including, naïve $CD4^+$ and $CD8^+$ T cells and B cells. DCs can initiate both the primary and the secondary immune response. DCs are the key targets for some conditions that involve an alteration T cells activity, such as transplantation, autoimmune disease, some cancers, and immunodeficiency. However, high levels of β2-microglobulin have a negative impact on the immune system. Cells that are treated with β2-microglobulin had decreased antigen presentation capability and enhance tumor growth and survival by producing cytokines, such as IL-6, IL8, and IL-10. These cytokines induced the secretion of weaker IL-2 and INF-ϒ that activate T-cell responses, and compromised DC ability to mount a type I T-cell response. β2-microglobulin treated cells inhibit the Raf/MEK/ERK signal transduction cascade and activate STAT3, a protein that has a crucial role in cell growth, proliferation, and survival (Xie et al., 2003).

Materials and Methods

Animal model.

Spleens were harvested from three month old ICR female and male mice by Dr. Emily Dudly and placed in Dulbecco's Modified Eagle's Medium (DMEM) (HyClone, Fisher Scientific, Pittsburgh,Pa). The spleen was macerated between the frosted ends of two suspended microscope slides. The spleen cells were in 5 ml 1X PBS and centrifuged for 5 min at 1500rpm. To sediment the cells, the cells were re-suspended in 5ml 1x PBS and quantified using both the beads method and flow cytometry.

The use of antibody-coated beads to detect β2microglubulin on cells

By using these antibody-coated beads, the cell suspension or the β2m marker can be counted under light microscopy. The beads method requires three reagents: labeling, blocking, and staining reagents. The labeling reagent, the antiβ-2 macroglobulin rabbit polyclonal antibody (Santa Cruz Biotechnology, INC) was coated on 1.4 μm carboxylate modified super active latex beads (Life Technologies, Grand Island, NY). 20% normal rabbit serum (Sigma-Aldrich, St.Louis, MO) is used as blocking agent (with 3% BSA and 5% tween). The blocking reagent limits the non -specific binding. The staining reagent can lyse red blood cells and contains 0.025% crystal violet stain and 2% acetic acid in distilled water.

The passive adsorption method was used on the labeling reagent to coat the beads. Attachment of antibodies to carboxylate modified super active latex beads occurred by physical adsorption. The MES buffer (Fisher Scientific, Pittsburg, PA) (0,025M,PH6, wash buffer (phosphate buffered saline, 0.1M, PH 7.2), and storage buffer (phosphate

buffered saline, 0.1M, PH 7,2, 0.1 glycine, 0,1% NaN3) were used. The high value of the pH of MES buffer is identical to the isoelectric point of the β-2 microgubulin. Therefore, it will increase the protein density on the particle surface ("Passive Adsorption Protocol," n.d.).

MES buffer Preparation (2- N-Morpholino-EthanesulfonicAcid)

48.8 mg of MES was weighted and added to 5 ml of double distilled water. Then, 1 N NaOH was added in order to increase the PH value (Appendix 1).

Storage Buffer preparation

100 mg glycine was mixed with 100 mg sodium azide, then the volume was brought to 10 ml PBS (Appendix 1).

The quantities of Antibody and super latex beads

The following equation determines the amount of antibody required for coating beads:

$$weight\ of\ the\ antibody = \frac{weight\ the\ antibody\ for\ the\ total\ particle\ weight}{diameter\ of\ the\ particle\ in\ \mu m}$$

(Appendix 1).

The preparation of the super latex beads labeling with antibody

We diluted 2.5 ml latex beads in 10 ml MES buffer. Then, we centrifuged the compound for 20 min to 3000rpm. Then, re-suspends the pellet in 10 ml MES buffer. And we centrifuged it again at same condition. We re-suspended the pellet in 5 ml MES buffer. After that, we added the calculation amount of the antibody and Incubated the mixture at room temperature overnight. Then, we centrifuged the latex/ antibody mixture and re-suspended the pellet in 10 ml phosphate buffer saline (PBS). We centrifuged the

pellet 3 times for 20 min at 3000 rpm and Re- suspended the final latex of β2m in 5 ml storage buffer. The latex was stored at 4 °C ("Passive adsorption protocol"n.d.) (Appendix 2).

Counting β2microglubulin by beads method

For each sample we used two 12 ×75 mm tubes: test tube# 1; label with B+L and test tube#2; label with S. We pipetted 100 µl of agent S into the test tube that labeled with S. (Figure 3.A). Then, we put 100 µl of spleen cells suspension into the test tube labeled with B+L. (Figure 3.B). We added 10 µl of the blocking agent (B) to the spleen cells suspension in the tube labeled with B+L. (Figure 3.C). Then, we added 10 µl of the Labeling agent (L) to the B+L test tube. (Figure 3.D). We added 10µl from the mixture in the B+L test tube to the S tube. (Figure .3.E). By using the hemocytometer (Figure. 3.F). Under the light microscope, cells that had three or more latex spheres attached were counted as having β2m ("Manual CD4 count kit" n.d.) (Appendix 3).

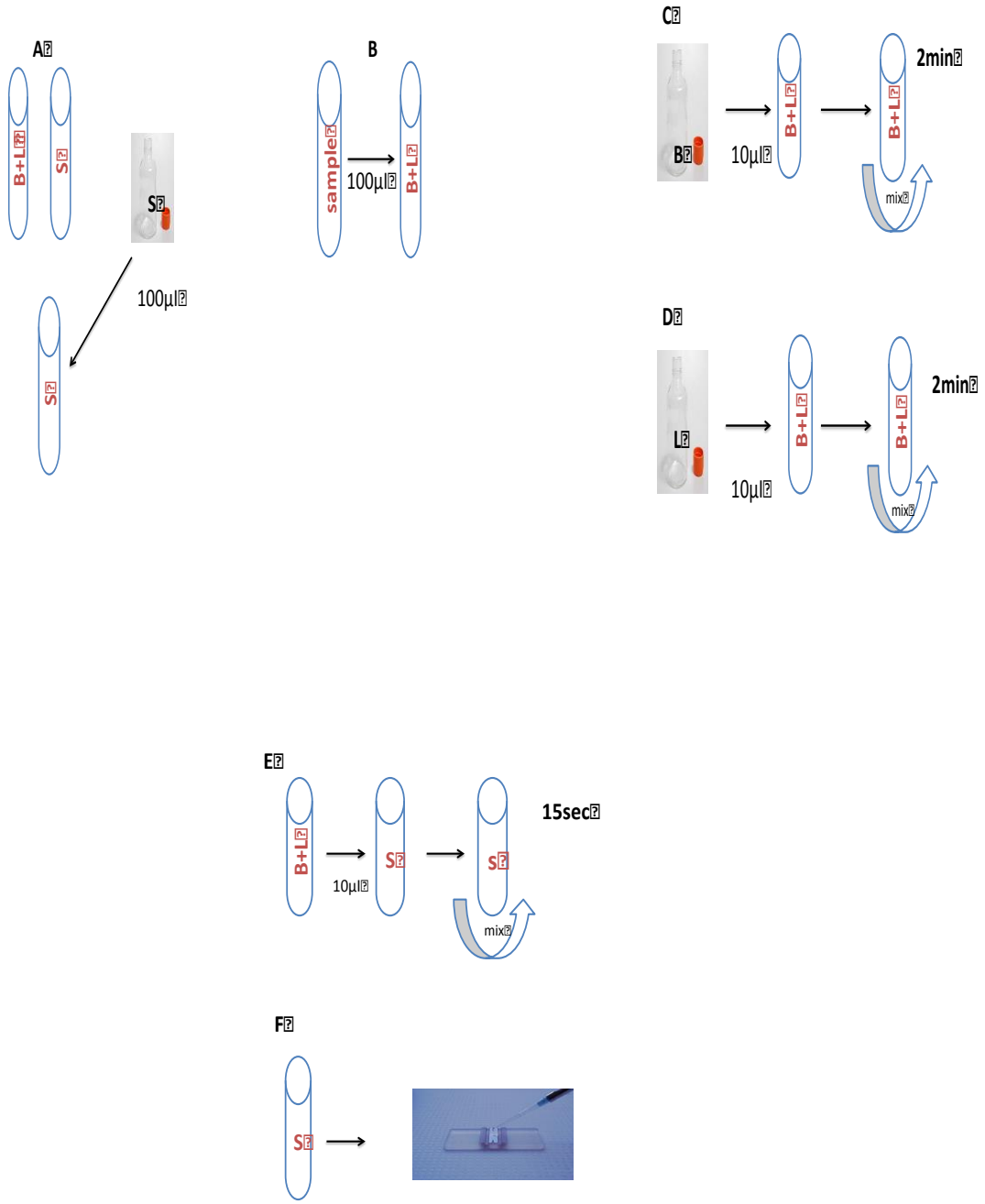

Figure 3.The steps of the bead method. (A-F). Figures demonstrate the steps of the bead method. The letter B + L represents blocking reagent + labeling reagent and the S letter represents staining reagent. Adapted from ("Manual CD4 count kit" n.d.).

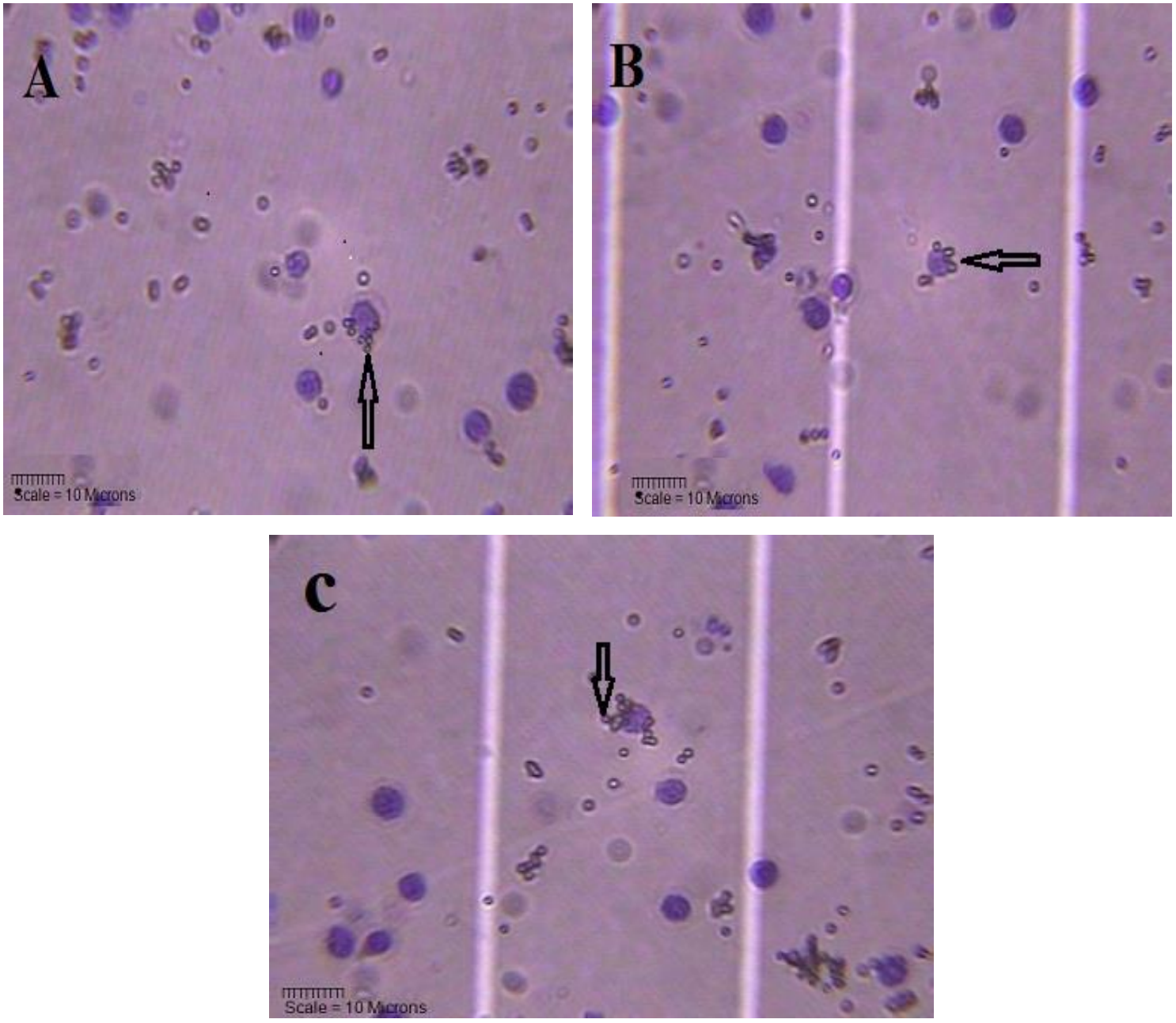

Figure 4.The attachment of three or more beads to cells. Images under light microscopy demonstrate the attachment of three or more beads to $\beta 2m^+$ cells.

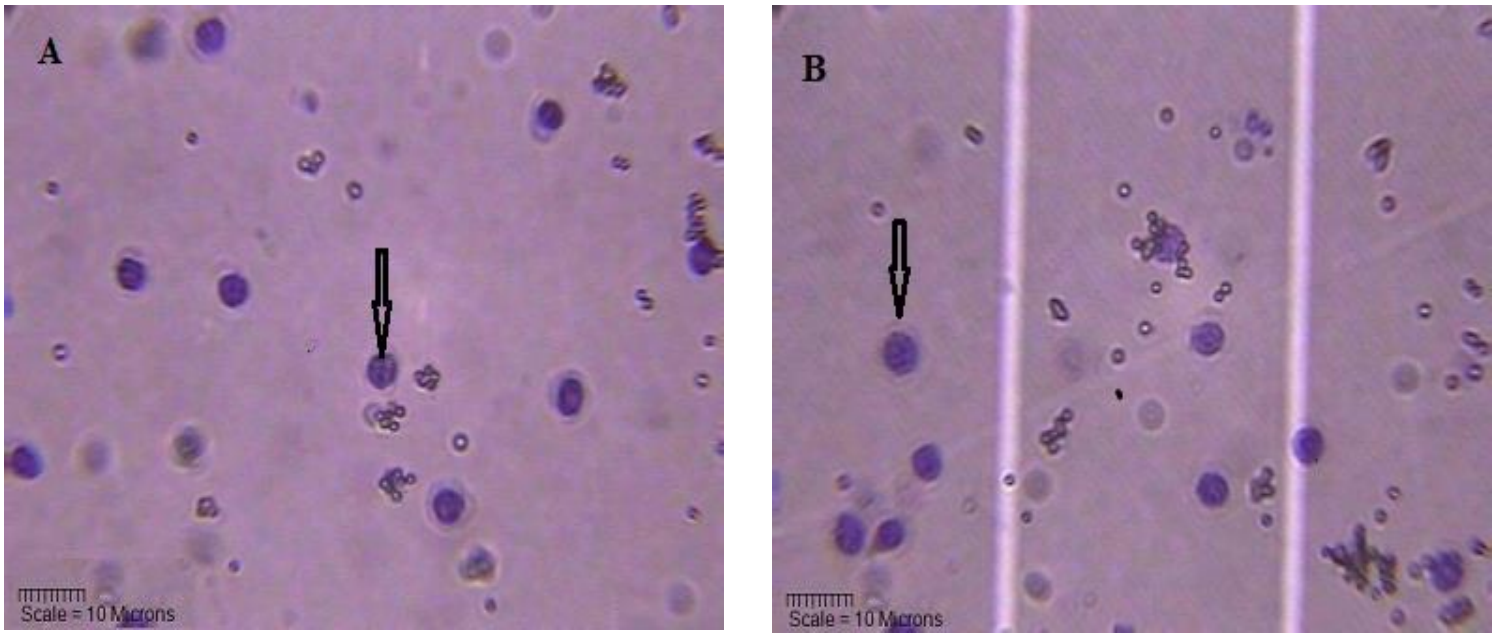

Figure 5.No attached beads.

Images under microscope show no beads attached to the $\beta 2m^{+}$ cells.

Absolute Count

The cells that have no attached beads (figure 3), one bead attached, or two beads attached were excluded from count. One or two attachments of the latex beads to the antibody can be by chance and it is non-specific. Three attachments of the latex beads to the β2m antibody are more definitive and indicate the binding is not accidental. The results of the control experiment indicated that cells never exceeded two beads. The next formula demonstrates the absolute counting for β2m+ cells in mice spleen:

$$\beta 2m^{+} cells \,/\, \mu l =$$

$$\frac{\beta 2m^{+} cells\ numbers\ \times chamber\ depth\ correction \times sample\ dilution\ correction}{surface\ area(mm^{2})}$$

(Appendix 4).

Counting β-2microglubulin by Flow Cytometry

Flow cytometry uses laser beams to analyze the chemicals and physical proprieties of any particles in a fluid. It is commonly used for cell counting, sorting, and biomarker detection. The process and reagents for surface markers can be very costly. In this study, the flow cytometry were used to count the number of $\beta 2m^{+}$ cells. The indirect antibody was used. For the isotype control, normal rabbit serum (Sigma Aldrich, St.Louis, MO) was used. The primary antibody used was the anti-β2m rabbit polyclonal antibody (Santa Cruz Biotechnology, Dallas, Texas) and the secondary antibody was goat anti- rabbit IgG (FITC)(Santa Cruz Biotechnology, Dallas, Texas). The purpose of the isotype control was to measure the level of non-specific background signal caused by

primary antibodies. This control was used to distinguish non-specific background signals from specific antibody signals.

The spleen cells suspension was washed three times with 1% BSA (bovine serum albumin) and centrifuged at a speed of 1200 rpm for 5 minutes. After that, 3%BSA was used for 30 minutes for the purpose of blocking non-specific staining and was incubated at room temperature. Then, the cells were washed again three times with 1% BSA and centrifuged at a speed of 1200 rpm for 5 minutes. The fluorochrome conjugated primary antibody specific for β2m was added and incubated with the cells for 15 to 45 min 4°C in the dark 5 µg antibody / ml with 100 µl of 3% BSA for each sample. Then, cells were washed three times with 1% BSA and centrifuged at a speed of 1200 rpm for 5 minutes. The secondary conjugated antibody was added and incubated with the cells for 15 to 45 min 4°C in the dark (Add 0.1 to 10 µg antibody/ml then diluted with 100 ml of 3% BSA). Then, the cells were washed and centrifuged again with 1% BSA 3X (1200rpm, 5min). Re-suspend the cells on cold PBS, 10% FCS, and 1% sodium azide until analysis within 24 hours. The results from flow cytometry were analyzed using the FCS Express program (Figure 6).

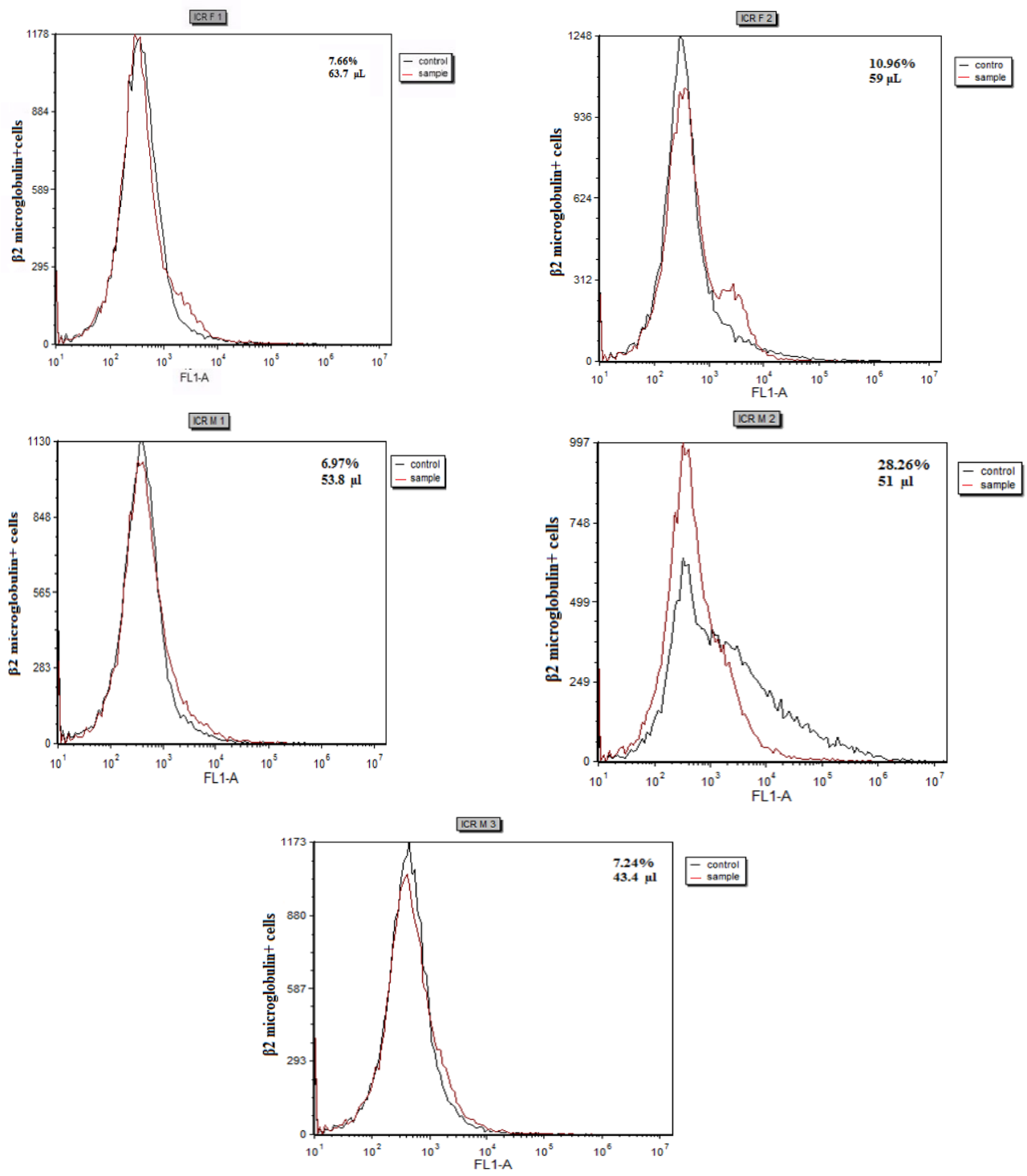

Figure 6: Flow Cytometry Analysis of β2-microglubulin polyclonal Antibody (A-E). It shows the analysis of flow cytometer of ICR female mouse spleen cells suspension using β2-microglubulin polyclonal antibody at 20,000 events. The number of β2microglubulin^{+} cells per sample = 20,000/ volume at which 20,000 was detected (Dataanalysis in Table 1-3).

Results

The number of $\beta 2m^+$ cells was enumerated by using two different methods; flow cytometry and bead method in spleen cells from two three-months-old ICR females and three three-month-old ICR males. The overall number of cells in all spleens of ICR female and male mice was around 10 million. The results of both methods flow cytometry and bead methods are similar and no significant differences between them. It is important to note that the bead method results were higher than flow cytometry in terms of $\beta 2m^+$ cell counts. The results were analyzed by using Sigma Plot. The numbers of $\beta 2m^+$ cells were between 400-500 cells/ μI.

Table 1 demonstrates the total numbers of $\beta 2m^+$ spleen cells individual samples from five ICR female and male mice by using flow cytometry and bead method. One-way ANOVA shows that the differences in the mean values between the two methods are not great enough and there is not a statistically significant difference (P = 0.058) (Mean of the flow cytometry $\beta 2m^+$ = 375 ± 25, and mean of the bead method $\beta 2m^+$ = 466 ± 32, mean ± SEM). Table 2 indicates the total number of $\beta 2m^+$ spleen cells per sample. The absolute number of spleen cells was approximately 10 million in all samples.

In addition, the percentage of the total numbers of single $\beta 2m^+$ cells to the total number of spleen cells is presented in Table 3. There were no significant differences between the two methods. The percentage of total $\beta 2m^+$ cells to the total spleen cells counts was between 7 and 9%.

We excluded ICR M 2 sample from tables 4, 5, and 6 from our data due to the statically insignificant curve of the isotype control.

Table 1

Comparison of β2m$^+$ cell numbers determined by flow cytometry and immunobeads.

Mouse	Flow cytometry	Bead method
ICR F 1	313/μI	467/ μI
ICR F 2	340/μI	394/ μI
ICR M 1	373/μl	408/μl
ICR M 2	392/μl	576/μl
ICR M 3	461/μl	489/μl
Mean ± SEM	375 ± 25/μl	466 ± 32/ μl

In Table 1, β2-microglubulin$^+$ spleen cell counts from female and male ICR mice were determined using flow cytometry and the immune bead method. Note that the numbers are not significantly different from one another when pairwise comparisons were made using one-way ANOVA.

Table 2

Numbers of Single β2-micoglubulin^{+} Cells Per Sample.

Mouse	Flow cytometry	Bead Method
ICR F 1	626,000	934,000
ICR F 2	680,000	788,000
ICR M 1	746,000	816,000
ICR M 2	784,000	1,152000
ICR M 3	922,000	978,000
Mean ± SEM	751,600±505,07	933,600±650,76

In Table 2, the results are shown for total β2-micoglubulin^{+} cells in each sample as determined by flow cytometry and immune bead. These results were not significantly different between the two methods. (One-way ANOVA pairwise comparisons).

Table 3

Percentage of Single β2microglubulin^{+} Cells / Total Spleen as determined by flow cytometry and immunobead counting.

Mouse	Flow cytometry	Bead Method
ICR F 1	6	9
ICR F 2	6	7
ICR M 1	7	8
ICR M 2	7	11
ICR M 3	9	9
Mean ± SEM	7± 0,5	8± 0,6

In Table 3, no significant difference can be seen in the percentage of β2 microglubulin^{+} Cells / Total Spleen cells was found (One-way ANOVA, pairwise comparisons).

Table 4

Modified comparison of β2m$^+$ cell numbers determined by flow cytometry and immunobeads

Mouse	Flow cytometry	Beads
ICR F1	313	467
ICR F2	340	394
ICR M 1	373	408
ICR M 3	461	489
Mean± SEM	371±32	439±22

In Table 4, β2-microglubulin$^+$ spleen cell counts from female and male ICR mice were determined using flow cytometry and the immune bead method. Note that the numbers are not significantly different from one another when pairwise comparisons were made using one-way ANOVA.

Table 5

Modified numbers of β2-micoglubulin^{+} Cells per Sample.

Mouse	Flow cytometry	Beads
ICR F1	626,000	934,000
ICR F2	680,000	788,000
ICR M1	746,000	816,000
ICR M3	922,000	978,000
Mean± SEM	743,500± 643,60	879,000±457,12

In Table 5, the results are shown for total β2-micoglubulin^{+} cells in each sample as determined by flow cytometry and immune bead. These results were not significantly different between the two methods. (One-way ANOVA pairwise comparisons).

Table 6

Modified percentage of β2microglubulin^{+} Cells / Total Spleen as determined by flow cytometry and immunobead counting.

Mouse	Flow cytometry	Beads
ICR F1	6	9
ICR F2	6	7
ICR M1	7	8
ICR M3	9	9
Mean± SEM	7±0,7	8±0,4

In Table 6, no significant difference can be seen in the percentage of β2 microglubulin^{+} Cells / Total Spleen was found (One-way ANOVA, pairwise comparisons).

Discussion

In this study, we evaluated the expression level of MHC I in murine spleen cells. Since the MHC I α chain is encoded by variant HLA-A gene, and the β chain (β_2-microglobulin) is consistent (Bernal et al., 2012), the main purpose of the study was to detect the MHC I molecules in mouse spleen cell using an antibody against the β2m protein. The aim of the current study was to quantitate the expression of β2m on mouse spleen cells using flow cytometry and direct counting of antibody-coated latex beads for comparison with the results of Sana Alhawsawi who found no difference in expression of spleen cells from C47BL/6 mice staining positive for the alpha-chain of MHCI compared to the β2m (S. Alhawsawi, MS Thesis, Wright State University, 2015). In the present study, the numbers of $\beta 2m^+$ cells in mouse spleen suspensions from outbred ICR Swiss mice were also similar using flow cytometry (typical method) and counting antibody-coated latex beads. In this study, 7-9% of the spleen cells tested positive for β2m-labled cells. This number is low because these cells are obtained from healthy mice. In Alhawsawi's study, she found approximately 10% or less normal mouse spleen cells staining for β2m. However, in spleens of mice that were infected with mouse parvovirus class I, 80% or greater of the spleen cells stained positive for the β2m chain and for the polymorphic alpha chain of C57Bl/6 mice.

Flow cytometry detects biomarkers, counts and sorts cells by using laser beam and fluidics. The flow cytometry technique is more expensive than the bead method. Flow cytometry also demands a highly trained person. The reagents of flow cytometry

are costly from $30,000 to $150,000 ("Flow cytometry," n.d.). Furthermore, the process takes approximately 10 hours. These factors indicate that we need an alternative method to enumerate the $\beta 2m^+$ cells that is cheaper, easier to use, faster, and more accessible. In contrast, the reagents of the bead method are not costly when compared to the cost of flow cytometry. In the bead method, monoclonal antibody-coated- latex beads associate with the surface of the cells that express β2m. When anti-β_2m coated -latex beads bind to the cell that displays β2m cell surface antigen, this binding forms a cell-latex bead rosette that is easily recognized using light microscopy.

In this study, no statistically significant differences were seen using the bead and flow cytometry method for enumerating single $\beta 2m^+$ cells. However, there was a little difference in the absolute numbers of $\beta 2m^+$ cells in both methods. The results will be more precise when using negative isotype control for beads method. In addition, the numbers of β2m+ cells in beads method were higher compared to the flow cytometry due to the sequence of the experiments. The results of this study suggest that the bead method can be an alternative method to flow cytometry in identifying cell surface antigens.

These results support those of Allabidi (2014) in which the number of $CD4^+$ and $CD8^+$ T lymphocytes in ICR Swiss mouse spleens yielded similar results using both methods. In addition, the present study supports the observations of Othman (2015) in which he found similar numbers of single mouse spleen cells displaying two surface markers ($CD5^+$ and $CD19^+$) using flow cytometry and antibody-coated latex beads.

Future studies

The bead method could be used to diagnose and monitor the progress of treatment of metastatic embryonal carcinomas. Several studies have shown that in embryonal carcinomas the expression of CD30 is elevated at both protein and mRNA levels. After chemotherapy treatment, $CD30^{+}$ cells are eliminated due to chemotherapy (Berney et al., 2001). This demonstrates that CD30 could be a factor for stem cell regulation in early embryonic development.

Multiple myeloma is a neoplastic disease of the plasma cells in which the plasma cells (PC) proliferate abnormally in the bone marrow (Kumar et al., 2008). One characteristic of multiple myeloma is osteolytic bone destruction. Since the levels of β2m are highly expressed in patients with multiple myeloma (Raja, 2012), we can detect the MHC class I expression by examining the number of $\beta 2m^{+}$ cells to monitor the disease progression. Future studies can be done, by using western blot. Western plot will detect the expression of specific intracellular proteins in samples while flow cytometry can be used to detect the surface marker in suspended cells.

Immunohistochemistry (IHC) is a technique that can detect proteins in tissue by antibody-antigen binding (Ramos-Vara and Miller, 2014). Since the levels of serum β2m are highly expressed in inflammatory bowl diseases such as ulcerative colitis (UC), Crohn's diseases (CD), and rheumatoid arthritis (RA) (Fernando et al., 2008), we can detect the expression of antiβ-2 macroglobulin rabbit polyclonal antibody by using indirect immunohistochemistry to determine patient's prognosis. In cases such as metastases or carcinoma of unknown origin, immunohistochemistry can be helpful for

cell identification. IHC such as that described in this paper can also help distinguish between benign and malignant tumors.

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Appendix

Appendix 1

MES buffer Preparation (2- N-Morpholino-EthanesulfonicAcid)

We weighted 48,8 mg of MES and added it to 5 ml of double distilled water. Then, we tittered the PH value by using electrode with 1 N NaOH in order to increase the PH from 4 to 6. After that, we transferred the MES buffer to the volumetric flask and diluted it to the final volume of 10 ml of PBS ("Passive Adsorption Protocol," n.d.).

Storage Buffer preparation

We weighted 100mg glycine, 100mg sodium azide. Then we mixed them and bring the volume to 10 ml with PBS. We used glycine in order to fill any reactive site on the surface that does not cover by protein. Bovine serum albumin (BSA) can be used to decrease non-specific binding. On the other hand, the NaN3 is worked as biocide ("Passive adsorption protocol," n.d.).

The quantities of Antibody and super latex beads

The following equation determines the amount of antibody required for coating beads:

$$weight\ of\ the\ antibody = \frac{weight\ the\ antibody\ for\ the\ total\ particle\ weight}{diameter\ of\ the\ particle\ in\ \mu m}$$

For example, in this study, we coated 100 mg of 1.4 μm latex beads with 2 mg of β2m antibody, so the weight of β2m = 2mg⁄1.4 μm = 1.4 mg of β2m antibody is needed to coat 100 mg of the super latex beads ("Passive adsorption protocol" n.d.).

Appendix 2

The preparation of the super latex beads labeling with antibody

1. 2.5 ml latex beads were diluted in 10 ml MES buffer.
2. Centrifuge the compound for 20 min to 3000rpm.
3. Remove the supernatant and re- suspend the pellet in 10 ml MES buffer.
4. Centrifuge again and remove the supernatant.
5. Re-suspend the pellet in 5 ml MES buffer. (The latex suspension is probably ~ 20mg/ml).
6. Add the calculation amount of the antibody.
7. Incubate the mixture at room temperature overnight.
8. Centrifuge the latex/ antibody mixture.
9. Re-suspend the pellet in 10 ml phosphate buffer saline, PH=6.
10. Centrifuge the pellet 3 times for 20 min at 3000 rpm.
11. Re- suspends the final latex of β2m in 5 ml storage buffer (the concentration at 2% solids).
12. Store the latex at 4 °C ("Passive adsorption protocol"n.d.).

Appendix 3

Counting β2 microglubulin by beads method

1. Two 12 ×75 mm tubes were used for each sample.
    a. Test tube# 1; label with B+L.
    b. Test tube#2; label with S. (as in Fig3.A)
2. Pipette 100 µl of agent S into the test tube that labeled with S. (Fig3.B).
3. Put 100 µl of spleen cells suspension into the test tube labeled with B+L. (Fig3.C).
4. Add 10 µl of the blocking agent (B) to the spleen cells suspension in the tube labeled with B+L. (Fig3.D)
5. Immediately, after adding the B agent, gently mix the B+L test tube for 2 min.
6. Add 10 µl of the Labeling agent (L) to the B+L test tube. (Fig3.E).
7. After adding the L agent, mix the B+L test tube for 2 min.
8. Place 10µl from the mixture in the B+L test tube into the S tube. (Fig.3.F).
9. Mix the test tube gently by hand for 15 sec.
10. Put the sample from step 9 on both chambers of the 0,1 mm deep hemacytometer.
11. By using the light microscope, cells that had three or more latex spheres attached to the cells were counting as β2m ("Manual CD4 count kit" n.d.).
12. Under the light microscope, we count the cells that had three or more large latex spheres attached as $\beta 2m^{+}$ cells.

Appendix 4

Absolute Count

The cells that have no attached beads (figure 3), one bead attached, or two beads attached were excluded from count. One or two attachments of the latex beads to the antibody can be by chance and it is non-specific. While, three attachments of the latex beads to the β2m antibody are more specific and it is not by chance.

The next formula demonstrates the absolute counting for β2m+ cells in mice spleen:

$$\beta 2m^{+} cells \,/\, \mu l =$$

$$\frac{\beta 2m^{+} cells\ numbers\ \times chamber\ depth\ correction \times sample\ dilution\ correction}{surface\ area(mm^2)}$$

$$chamber\ depth\ correction = \frac{1}{chamber\ depth} = \frac{1}{0.1} = 10\ for\ 0.1\ mm\ deep\ chamber.$$

Correction for sample Dilution=

A) 100 µl whole sample

+10 µl reagent L

+10 µl reagent B = 100⁄120

B) Secondary dilution=

10 µ of 100⁄120 primary dilution in 100 µl reagent C =

$$\frac{10\mu l \times \left(\frac{100}{120}\right)}{110\mu l} = \frac{1}{13.2}$$

Therefore the correction for sample dilution =

$$1 \div \frac{1}{13.2} = 13.2$$

Surface area = 18 mm^2 for two sides of 0.1 mm deep chamber ("Manual CD4 count kit," n.d.)

For example, if the count $\beta 2^+$ cells in both sides of 0.1 mm chamber were 50 the $\beta 2m^+$ cells count can be calculated by applying the next formula:

$$\frac{50 \times chamber\ depth\ correction(10) \times sample\ dilution\ correction(13.2)}{surface\ area(18mm^{2)}}$$

$$50 \times 7.3 = 365\ \beta 2m^{+} cells\ /\ \mu l.$$