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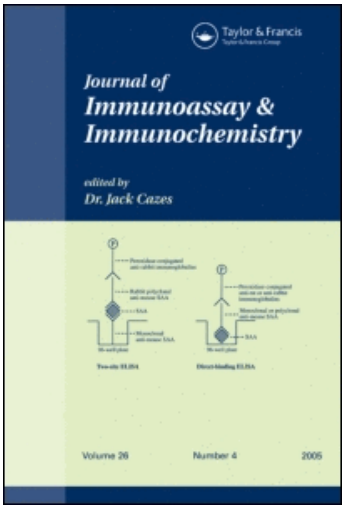

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Impact of Different Cell Isolation Techniques on Lymphocyte Viability and Function

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Impact of Different Cell Isolation Techniques on Lymphocyte Viability and Function

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Abstract: The outcome of immunological assays is markedly influenced by the method of isolation of lymphocytes. It is, therefore, important to comparatively assess various techniques of isolation of lymphocytes, an aspect thus far not thoroughly addressed. In particular, the potential of isolation techniques to influence cell recovery, viability, and function has not yet been evaluated. These studies were designed to determine the effect of different mechanical tissue dissociation methods on the viability and function of lymphocytes. Following spleen and thymus removal, the lymphoid organs were dissociated by one of four different tissue dissociation techniques: metallic screen, sheer force slide, commercial stomacher, or plunger-screen. Cells were then enumerated and a trypan blue exclusion technique and 7-amino-actinomycin D (7-AAD) were both employed to assess viability. Mitogen-induced lymphocyte proliferation was measured using the Alamar Blue™ assay. Cell viability and lymphocyte surface antigen expression were assessed using flow cytometry. No significant

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differences in lymphocyte viability, morphology, or surface antigen expression were observed among the different techniques. Likewise, cellular apoptosis and necrosis were comparable across all the techniques. However, mitogen induced splenic T-cell proliferation was higher in cells collected using the metallic screen and plunger-screen isolation methods as compared to the sheer force slide or commercial stomacher procedures. These data suggest that cell recovery, morphology, and viability are not affected by isolation techniques. However, lymphocyte function, as assessed by mitogen induced proliferation, was negatively affected by the sheer force slide or commercial stomacher isolation techniques.

Keywords: Mouse, Spleen, Thymus, Lymphocyte isolation, Apoptosis, Cell viability, Surface marker expression

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INTRODUCTION

The murine thymus and spleen are routinely collected for the evaluation of immune endpoints due to their ease of identification and removal. Additionally, these tissues serve as a good reservoir for populations of lymphocytes. Evaluation of the thymus, a primary lymphoid organ, commonly includes cell recovery, cell surface marker phenotyping, and short-term response of lymphocytes to specific mitogens in culture. The spleen, a secondary lymphoid organ, contains mature populations of T-cells and B-cells, as well as other leukocytes. Splenic lymphocytes are similarly evaluated based on cell recovery, viability, and cell surface marker expression, proliferation, and cytokine production.

Numerous mechanical cell dissociation techniques currently exist to isolate lymphocytes. However, it is not known how the quality, viability, or function of the recovered cells are influenced by the technique of dissociation and isolation. It is well-accepted that the choice of an isolation technique can affect similar endpoints in non-leukocytes.

For example, hepatocyte isolation by a combination of enzymatic digestion and mechanical dissociation resulted in higher yields of hepatocytes than solely mechanical dissociation.[1] Enzymatic digestion also more accurately reflected cell conditions in vivo than mechanical dissociation.[2] The method of hepatocyte dissociation also affects viability and function of the cells. For instance, collagenase based hepatocyte isolation techniques had less of an effect on cell metabolism than mechanical techniques.[3] Mechanical disruption includes tissue pulverizing and grinding motions, which causes damage to cytoplasm[4] and may increase apoptotic or necrotic cell death.

Regarding digestion of lymphoid tissue, studies of murine Peyer's patch using enzymatic digestion or mechanical dissociation have shown that enzymatic digestion produces higher recoveries of accessory cells without compromising function. Peyer's patch dissociation is thought to be facilitated by enzymatic dissociation, while spleens are not, due to the Peyer's patches being surrounded by areas rich in accessory cells.[5] When leukocytes were

isolated from central nervous system tissue, dispase enzyme digestion altered lymphocyte and macrophage cell-surface antigen expression.[6] In another study comparing enzymatic digestion to mechanical disruption in lung tissue, researchers reported no significant differences in lymphocyte surface antigen expression, NK, and monocyte distribution.[7] Regardless, mechanical dissociation is the most commonly employed technique to evaluate immune endpoints in the thymus and spleen.

Apoptosis, programmed cell death, involves a well-defined series of molecular and morphologic events that lead to disintegration of the cell. Characteristics of apoptosis include chromatin condensation and changes at the membrane level. Apoptosis is distinct from necrotic death by avoiding unnecessary inflammation or tissue damage.[8] Different dissociation techniques may affect lymphocyte apoptosis or necrosis; however, such has not been examined.

The ability of viable T-cells to proliferate upon challenge is related to the in vivo functional capacity of the cells to offer immune protection. Lymphocyte proliferation, in response to mitogens, is used to assess lymphocyte function in vitro. Lymphocyte proliferation is sensitive to a variety of cytokines, the production or secretion of which may be altered by dissociation technique.

In this study, we evaluated the influence of four different mechanical dissociation techniques on the recovery, viability, and function of murine lymphocytes. We hypothesized that mechanical dissociation techniques may quantitatively or qualitatively affect data being collected. This, in turn, could affect study results reported by different investigators, simply because of cell collection techniques employed.

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EXPERIMENTAL

Animals

Female BALB/c mice, 4–6 weeks of age, were bred and obtained from the Biology Animal Facility, Virginia Polytechnic Institute and State University (VPI & SU). Mice were fed a commercial pellet diet, provided water *ad libitum*, and were housed under controlled conditions of temperature (22°C), humidity (40–60%), and lighting (12 hr light/dark cycle). Animal maintenance, care, and use were, at all times, in accordance with VPI & SU Animal Care Committee (ACC) guidelines.

Thymus and Spleen Collection and Lymphocyte Isolation

Mice were euthanized by cervical dislocation, placed in dorsal recumbency, and the thymus and spleen collected aseptically using dissection scissors and curved forceps. As previously described, upon removal, thymuses and spleens were placed in separately labeled Petri dishes (Corning, NY) containing 8 mL cold incomplete RPMI-1640 media (MediaTech, Herndon, VA) (9).

The Petri dishes were maintained on ice prior to tissue dissociation. Each thymus and spleen was mechanically dissociated using one of four different methods; the metallic screen dissociation, sheer force slide dissociation, plunger-screen dissociation, or commercial stomacher dissociation. These techniques are described below.

Metallic Screen Dissociation Technique

Each thymus or spleen was grasped with the curved forceps and gently dissociated into the Petri dish containing cold RPMI, using a circular motion, across a sterile metallic sieve (Sigma, St. Louis, MO). Once the tissue had been dissociated the cells were pipetted through the metallic screen to remove the connective tissue matrix.[9,10] The cell suspension was then transferred aseptically into a sterile 15 mL centrifuge tube.

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Sheer Force Slide Dissociation Technique

As described by Molano et al., each thymus or spleen was placed between two frosted autoclaved slides (Corning, NY). The slides containing the tissue were gently pressed together in opposing directions to dissociate the tissue.[11,12] As the organ tissue began to dissociate, the slides were dipped frequently into 8 mL of incomplete media in a Petri dish until only the connective tissue remained. The cell suspension was transferred aseptically into a sterile 15 mL centrifuge tube.

Commercial Stomacher Dissociation Technique

Following the technique described by Do et al., each thymus or spleen was placed in to a separate sterile Stomacher™ bag containing 8 mL of incomplete media, and then placed into a laboratory homogenizer (Stomacher, Tekmar, Cincinnati, OH).[13–15] After a one minute disruption in the stomacher, tissues appeared as a cloudy suspension containing cells and connective tissue. The resulting cell suspension was transferred aseptically into a sterile 15 mL centrifuge tube.

Plunger-Screen Dissociation Technique

Each thymus or spleen was placed onto a sterile metallic sieve screen. The plunger from a sterile 3 mL syringe was firmly pressed against the tissue, and a circular motion was used to disrupt the tissue through the metal sieve screen.[16–18] Once the tissue had been dissociated, the cell suspensions were pipetted through the metallic screen, following dissociation, to remove connective tissue. The cell suspension was transferred aseptically into a sterile 15 mL centrifuge tube.

Thymic Cell Isolation

Once all mechanical dissociations were complete, the tubes containing thymic cell suspensions were centrifuged (7 min, 290 × g and 7°C) and supernatant poured off. The cells were then washed twice in 5 mL incomplete RPMI-1640 media and centrifuged (7 min, 290 × g and 7°C), and then placed on ice. The cells were then resuspended in 5 mL of complete RPMI-1640 media containing 10% FBS (Sigma), 2 mM L-glutamine (ICN, Costa Mesa, CA), 50 IU/mL penicillin (ICN), and 50 μg/mL of streptomycin (ICN), and maintained on ice.

Splenic Cell Isolation

Once all mechanical dissociations were complete, the tubes containing splenic cell suspensions were centrifuged (7 min, 290 × g and 7°C) and supernatant poured off. The cells were then washed once with 5 mL incomplete RPMI-1640 media and centrifuged (7 min, 290 × g and 7°C), and the supernatant was poured off. The cells were then resuspended in 1 mL incomplete RPMI-1640. To each tube, 2 mL of ACK lysis buffer (pH 7.29) was added, to lyse red blood cells, and tubes were incubated for 5 min at 23°C. After lysis incubation, the cells were resuspended in 5 mL of incomplete RPMI-1640 and washed twice (7 min, 290 × g and 7°C). The splenic leukocyte-rich cells were then resuspended in 5 mL complete RPMI-1640 media containing 10% FBS (Sigma), 2 mM L-glutamine (ICN, Costa Mesa, CA), 50 IU/mL penicillin (ICN), and 50 μg/mL of streptomycin (ICN), and maintained on ice.

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Cell Enumeration

Cells were enumerated using a CASY1 model TTC cell counter (Scharfe System GmbH, Germany) as described in our previous studies.[19] Briefly, 10 μL of cell suspension were taken from each sample and added to 10 mL of PBS and mixed by gentle inversion. Gates were set between 5 and 10 μm with a dilution factor of 5005 for both thymic and splenic cells. All cell suspensions were adjusted to 5.0×10^6 cells/mL in culture media, and maintained on ice until use.

Trypan Blue Exclusion

Cells undergoing either apoptotic or necrotic death can be distinguished from viable cells by enhanced uptake of trypan blue.[20] Cell viability was assessed using a hemacytometer (American Optical Corporation, NY) and the trypan blue assay by adding 100 μL of cell suspensions (5.0×10^6 cells/mL) to 900 μL of trypan blue stain (Sigma). Approximately 10 μL of each suspension

were added to a hemacytometer. Live and dead cells were enumerated under a light microscope. Percent viability was assessed for the thymus and the spleen at the time of isolation, and at 24 hr and 48 hr.[21]

Cell Viability, Apoptosis, and Necrosis with 7-AAD

To cells stained with monoclonal antibodies, 100 μL aliquots of 7-AAD (1 μg/well, Molecular Probes, OR) was added to each well containing 5×10^5 cells after 3 hr, 24 hr, and 48 hr culture. Cellular viability and death were measured as described.[9] Cells were incubated on ice in the dark for 30 min and then analyzed on an EPIC XL flow cytometer (Coulter, FL). The molecular probe, 7-AAD, provides assessment of stages of cell death as viable, early apoptotic or late apoptotic/necrotic populations.[22] Cells were then identified in one of three gates. In Gate 1, viable cells did not take up the stain and displayed low fluorescence and moderate forward scatter. In Gate 2, early apoptotic cells appeared with moderate fluorescence and moderate to low forward scatter. In Gate 3, late apoptotic/necrotic cells projected a high fluorescence and low forward scatter. From each sample, 5000 events were collected and analyzed using the Immuno-4 software program.

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Cell Surface Marker Expression

Thymic and splenic cells were incubated for 3 hr at 37°C. Con-A (10 μg/mL)-activated cultured thymus cells were incubated for 24 hr, while cultured splenic cells were incubated for 24 hr and 48 hr in a humidified incubator (37°C and 5% CO_2). Cells were stained with specific monoclonal antibodies and analyzed by flow cytometry as per our previous studies.[9] Mouse PE-anti-CD4 (clones H129.9 and OX-35, Pharmingen, CA) and FITC-anti-CD8 (clones 536.7 and OX-8, Pharmingen, CA) monoclonal antibodies (0.5 μg/5×10^5 cells) were added to thymic cells. Mouse B-cell marker PE-anti-CD45 (B220; clone RA-6B2) and mouse T-cell marker FITC-anti-CD90 (Thy 1.2; clone 30-H12, Pharmingen, CA) were added to splenic cells. The cell antibody suspensions were incubated on ice for 30 min, and then were washed and centrifuged (10 min, 200 × g and 7°C). Cell suspensions were resuspended in 100 μL of PBS, washed, centrifuged, (10 min, 200 × g and 7°C), and evaluated by flow cytometry. Cells were analyzed on an EPIC XL flow cytometer. From each sample, 5000 events were collected and analyzed using the Immuno-4 software program.

Lymphocyte Proliferation with Alamar Blue™

Mitogen-induced lymphocyte proliferation and viability was determined using the Alamar Blue™ dye, a redox indicator.[23] The pattern of mitogen-induced

proliferation assay as determined by Alamar Blue™ is comparable to ^{3}H-Thymidine incorporation assay.[24] Briefly, 100 μL aliquots of Concanavalin A (Con-A) (Sigma) (0, 1, 5, and 10 μg/mL) in complete media were added to specific wells, in triplicate. Duplicate plates were set up for the splenic cell suspension. Following 24 hr incubation, 20 μL of Alamar Blue™ dye (10% of incubation volume) were added to each well of the culture plates. Plates were incubated for an additional 48 hr then measured using a CYTOFLUOR II (PerSeptive Biosystems), a fluorescence multi-well plate reader. In the presence of the Alamar Blue™ dye, cellular proliferation induces a chemical reduction of the dye, changing the color from blue to red. The intensity of this red color reflects the extent of cellular proliferation. Excitation wavelength was 550 nm with a fluorescence emission at 590 nm. Data were reported as Δ fluorescence. Calculations were based on the mean specific absorbance, in triplicate. The value of the specific Δ fluorescence was defined as the specific absorbance of the stimulated cells minus the specific absorbance of the cells in media only.[25]

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Cytospin Preparation for Morphologic Analysis

A 100 μL sample of each thymus and spleen cell suspension (5.0×10^6 cells/mL) was placed into a cytospin chamber containing 100 μL of PBS. Each cytospin chamber consisted of a slide holder, slide, slide filter, and cell cup apparatus. The cytospin chambers were vertically placed into a Cyto-Tek centrifuge (Sakura Finetechnical, Tokyo, Japan) and centrifuged (5 min, 28 × g and 23°C). Cytospin chambers were then removed and the slides were allowed to air-dry overnight. The slides were then stained with a modified Wright-Giemsa stain using a Hema-Tek 2000 (Bayer Corporation, Tarrytown, NY) automated stainer as described in Witonsky et al.[16]

Statistical Analysis

Data were expressed as arithmetical mean ± standard error of the mean (SEM). Group size was 4 for all experiments and four replicates were performed for each group. A Student's statistical *t*-test was used to determine significant difference among groups. Results are described as significantly different at $p < 0.05$.

RESULTS

Cell Recovery is not Affected by Dissociation Technique

Lymphocyte cellular recoveries were not affected by the dissociation technique employed. Thymic cell recoveries from fresh collections averaged 154×10^6 cells (Table 1A). Splenic cell recoveries from fresh collections averaged 71.5×10^6 cells (Table 1B). Viable cell recoveries were

Table 1. Splenic and thymic cells were collected and enumerated using a CASY 1 model TTC cell counter with electronic gates set between 5–10 μm. Samples were taken after incubation periods of 3 hr and 24 hr for both thymus and spleen, and 48 hr for spleen only. Cells were diluted to 5.0×10^6/mL and cultured for the 24 hr and 48 hr recovery analysis. Data are reported as cell recoveries ± SEM. Results are the compilations of four experiments (n = 4 mice/group)

Technique	Fresh ($\times 10^6$)	24 hr culture ($\times 10^6$)	48 hr culture ($\times 10^6$)
A. Thymus			
Metallic screen	148.85 ± 12.89	4.05 ± 0.57	N/A
Sheer force slide	155.90 ± 35.12	3.32 ± 0.41	N/A
Commercial stomacher	165.03 ± 16.29	3.84 ± 0.11	N/A
Plunger-screen	147.43 ± 19.13	3.54 ± 0.32	N/A
B. Spleen			
Metallic screen	72.48 ± 6.87	2.64 ± 0.31	4.09 ± 0.87
Sheer force slide	76.74 ± 7.58	3.26 ± 0.34	3.60 ± 0.34
Commercial stomacher	65.17 ± 5.09	3.41 ± 0.20	3.05 ± 0.46
Plunger-screen	71.70 ± 8.62	3.05 ± 0.39	3.46 ± 0.51

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also measured at 24 hr and 48 hr to assess the influence of the different mechanical dissociation techniques. As expected with mortal cell lines, the number of cultured cellular recoveries declined over time. However, the rate of decline did not differ across groups (data not shown).

Lymphocyte Culture Viability via Trypan Blue Exclusion

Regardless of the mechanical dissociation technique, cell viability at the time of dissociation was determined to be greater than 90% for thymus and 80% for the spleen. After 24 hr incubation, thymic cultures decreased to approximately 70% (Figure 1A) and splenic cultures decreased to approximately 65% viability across all groups (Figure 1B). By 48 hr of culture, the average percent viability of splenic cells in all cultures had decreased approximately to 60% (Figure 1B).

Percentage of Apoptotic and Necrotic Cells by 7-AAD did not Differ Across Groups

The percentage of apoptotic and necrotic cells was consistent across all groups at all time intervals. Three hours after collection, the average live, early apoptotic, and late apoptotic/necrotic cells were approximately 70%, 18%, and 10%, respectively (Figure 2A). After 24 hr of culture, the percentage of live cells dropped to 40%. Early apoptotic and late apoptotic/necrotic cells

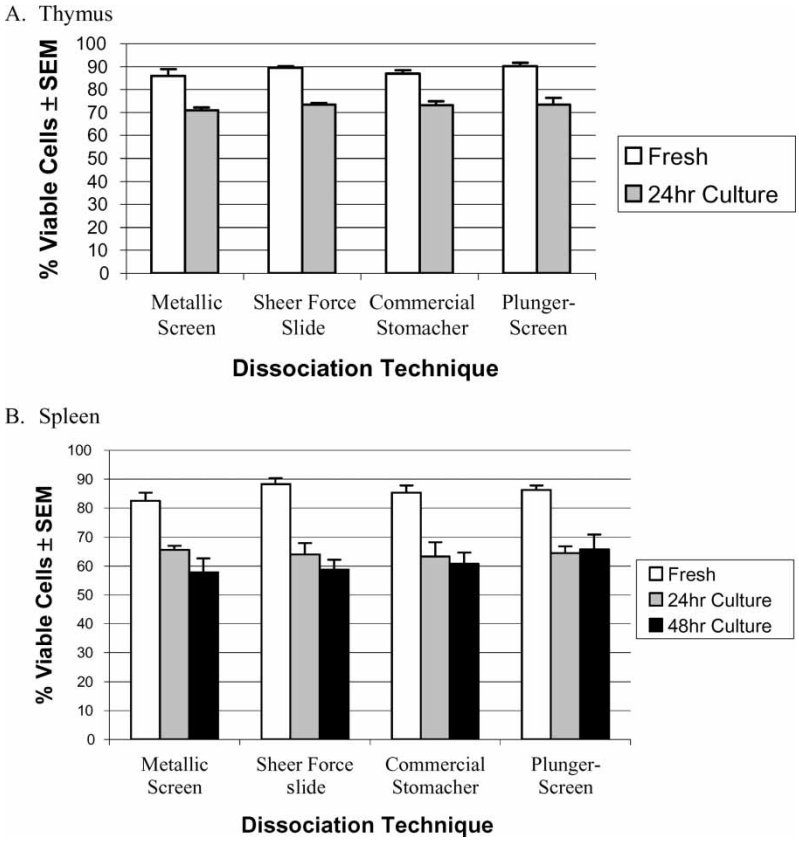

Figure 1. Percent viabilities of cell suspensions were evaluated at fresh, 24 hr for thymus and spleen, and 48 hr in culture for spleen only. Cells were diluted 1 : 10 and enumerated on a hemacytometer. Data are reported as viable cells ± SEM. Results are the compilations of four experiments (n = 4 mice/group).

were approximately 40% and 20%, respectively (Figure 2B). After 48 hr of culture, the number of live cells dropped to 32%, while early apoptotic and late apoptotic/necrotic cells were approximately 41% and 26%, respectively (Figure 2C).

Cell Surface Marker Expression was not Affected by Mechanical Dissociation Technique

Splenic B-cell and T-cell numbers were consistent over all dissociation groups. Thymic surface antigen expression for $CD4^+CD8^+$, $CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^-CD8^-$ were approximately 75%, 15%, 8%, and 5%, respectively. Cell populations were also consistent across all dissociation

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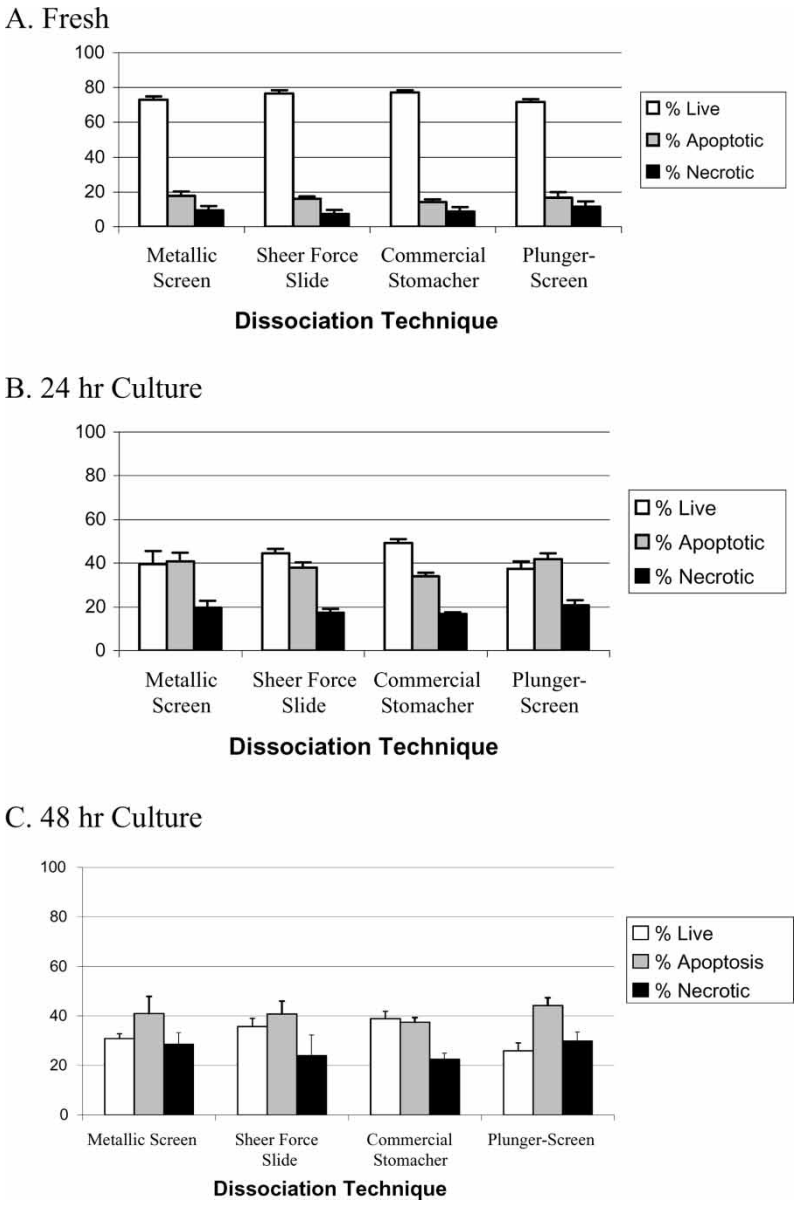

Figure 2. Viability determination of splenic lymphocytes cultured for 3 hr, 24 hr, and 48 hr. Cultures were analyzed by flow cytometry analysis with 7-AAD. Analysis detected normal live, apoptotic, and necrotic function of the cells at 24 hr and 48 hr culture. The results show a decrease in lymphocyte populations over 24 hr and 48 hr culture periods. Data are reported percent cells $\pm$ SEM. Results are the compilations of four experiments (n = 4 mice/group).

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groups (Figure 3A). Percent B-cells for spleen dissociations were approximately 35% and percent T-cells were approximately 25% (Figure 3B).

Con-A-induced Proliferation Varied According to Dissociation Technique

One hundred microliter aliquots of Con-A (0, 1, 5, 10 μg/mL) in complete media were added to specific wells containing splenic cells (100 μL/well). For all groups, the peak proliferation Δ fluorescence occurred at Con-A 10 μg/μL. By 72 hr of incubation, Con-A at optimal concentration of 10 μg/μL, the Δ fluorescence varied between the groups. The metallic screen and plunger screen techniques showed a Δ fluorescence of 400 units. These dissociation techniques had significantly greater Δ fluorescence than the sheer force slide or commercial stomacher techniques with Δ fluorescence of 200 units (Figure 4).

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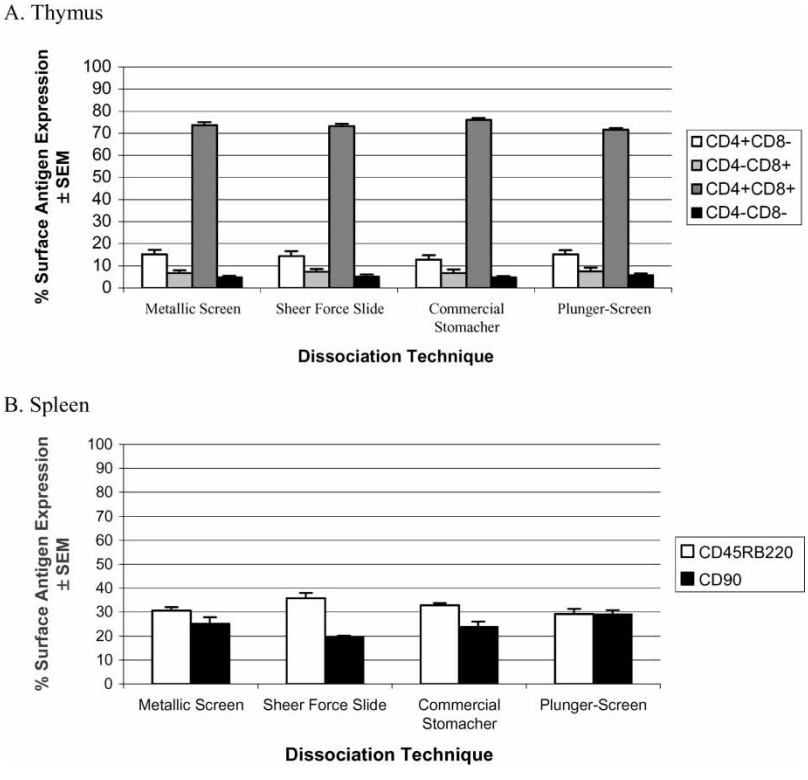

Figure 3. Lymphocyte surface antigen expression was analyzed using flow cytometry. Data are reported as lymphocyte expression ± SEM. Results are the compilations of four experiments (n = 4 mice/group).

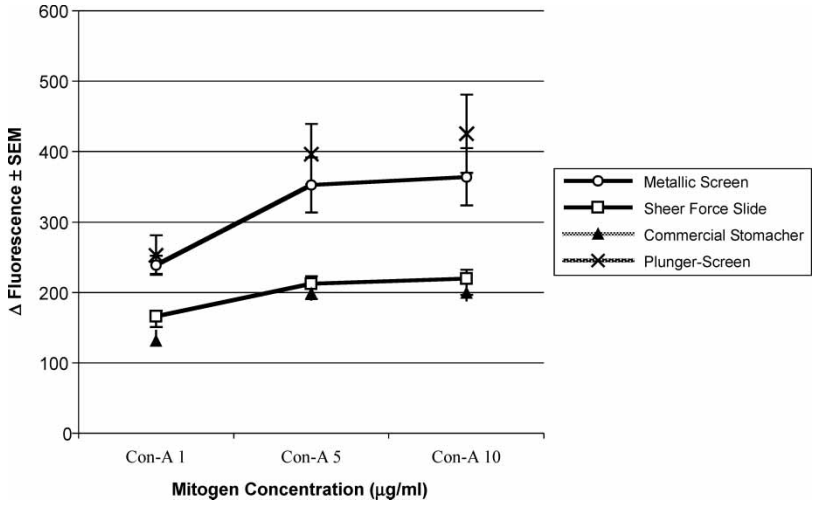

Figure 4. Mitogen-induced lymphocyte proliferation was assessed using Alamar Blue™ dye. Splenic cell suspensions were cultured for 72 hr in Concanavalin A 1, 5, and 10 μg/mL. Data are reported as lymphocyte Δ proliferation ± SEM. Results are the compilations of four experiments (n = 4 mice/group).

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No Differences in Cell Morphology Between Test Groups

Cytological analysis of the murine lymphocytes showed normal non differing morphology across all test groups. There was approximately equal evidence of apoptosis at 24 hr and 48 hr of culture denoted by increased granularity, shrinkage, and blebbing. By 48 hr culture, the lymphocytes showed increased granularity (data not shown).

DISCUSSION

The thymus and spleen are routinely collected for immunological analysis of developing cells (thymocytes) and mature cells (splenocytes). Many techniques currently exist for dissociating these tissues. However, the potential impact of these different dissociation techniques on cell viability and function are not currently known. This study compared four different mechanical dissociation techniques: metallic screen dissociation, sheer force slide dissociation, commercial stomacher dissociation, and plunger-screen dissociation, to assess potential differences in lymphocyte recovery, viability, and function.

The results from this study yielded some unexpected observations. The four mechanical dissociation techniques evaluated did not appear to affect many of the immune endpoints examined. Cell recoveries were comparable across all groups at all time intervals. Likewise, cell surface marker expression

of major lymphocyte surface antigens examined did not differ across groups tested. Lymphocyte viability was evaluated by both trypan blue exclusion assay and flow cytometry probe, 7-AAD. Trypan blue exclusion assay is typically employed to provide a rough estimate of viability and is typically less sensitive than analysis with flow cytometry.[25] Thus, trypan blue exclusion assay cannot distinguish early apoptotic cells, since the cell membrane must be greatly compromised in order for the dye to be taken up into the cells. Using trypan blue exclusion assay, cell viability following mechanical dissociation was similar across all groups. Further, the rate of decline in viable cell cultures was proportional over time in all test groups. To confirm the trypan blue exclusion assay results, 7-AAD was used to provide a more accurate characterization of viable, early apoptotic, and late apoptotic/necrotic cells. As reported in other studies, the percent of viable cells across all groups was lower compared to the trypan blue exclusion assay.[26] However, the percent viable cells, albeit lower, did not differ across all groups measured. Similar findings were observed in early apoptotic and late apoptotic/necrotic cells. In summary, these two cell viability assays indicated that cell viability and apoptosis/necrosis were similar across the four mechanical dissociation techniques.

We next examined whether lymphocyte surface antigen expression was altered by the mechanical dissociation techniques employed. Percent B-cells (CD45RB220) and T-cells (CD90) were comparable across all splenic dissociation techniques. Percent $CD4^+CD8^-$, $CD8^+CD4^-$, $CD4^+CD8^+$, and $CD4^-CD8^-$ cells were similar across all thymic dissociation techniques.

Importantly, measurable differences in the Con-A-induced lymphocyte proliferation were observed depending on the dissociation technique employed. Con-A binds to cell membrane glycoprotein residues acting as a ligand and causing agglutination of cells. The mitogen binds selectively to glucopyranosides, mannopyranosides, and fructofuranosides on the cell-surface glycoprotein residues.[27] Since cell recovery, viability, and death do not appear to be influenced by the technique, it may be that Con-A's ability to bind to cells was altered by the commercial stomacher or sheer force slide techniques. Alternately, different dissociation techniques may differentially affect cytokine production or secretion, which in turn alters in vitro proliferation. The trend toward different proliferation capacities is evident at each concentration of Con-A administered. Average Δ fluorescence at Con-A concentration of 5 and 10 μg/mL showed proliferation response for the metallic screen or plunger-screen dissociation techniques to be, on average, higher than with the commercial stomacher or sheer force slide dissociation techniques. A possible explanation for this observation is that the commercial stomacher or sheer force slide mechanical dissociation techniques may alter the glycoprotein residues or the residue stereochemistry.[28] Thus, these changes may decrease Con-A's ability to bind, decreasing Con-A's agglutinating ability and mitogen potential. A caveat to this study is that we did not evaluate cytokine synthesis, response, or expression analysis. Whether these points

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are sensitive to the method of lymphocyte isolation, as well as changes in glycoprotein stereochemistry, warrants further investigation.

In summary, the choice of which mechanical technique to use, in dissociating murine lymphoid tissue to obtain lymphocytes, would appear to depend on which immune endpoints are to be evaluated. These studies demonstrate thymic and splenic cell recovery, viability, and cell surface marker expression are not significantly altered by any of the four mechanical dissociation techniques examined. This does not appear to be the case with the mitogen-induced functional lymphocyte proliferation assay. Con-A, a polyclonal T-cell mitogen, had a consistently higher proliferative response in culture, with mature splenic lymphocytes obtained with either the metallic screen or plunger-screen dissociation technique. Based on our familiarity with the Alamar Blue™ assay in comparison to the ^{3}H-thymidine assay, this would constitute a significant difference in the thymidine proliferation response as well.[24] Further, this would suggest that the type of mechanical dissociation technique employed might also influence the potential response of other functional immune assays (i.e., mixed lymphocyte response (MLR), antibody production, cytotoxic lymphocyte (CTL) assay). Additional studies comparing different mechanical cell dissociation techniques may be warranted to optimize these other assays.

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