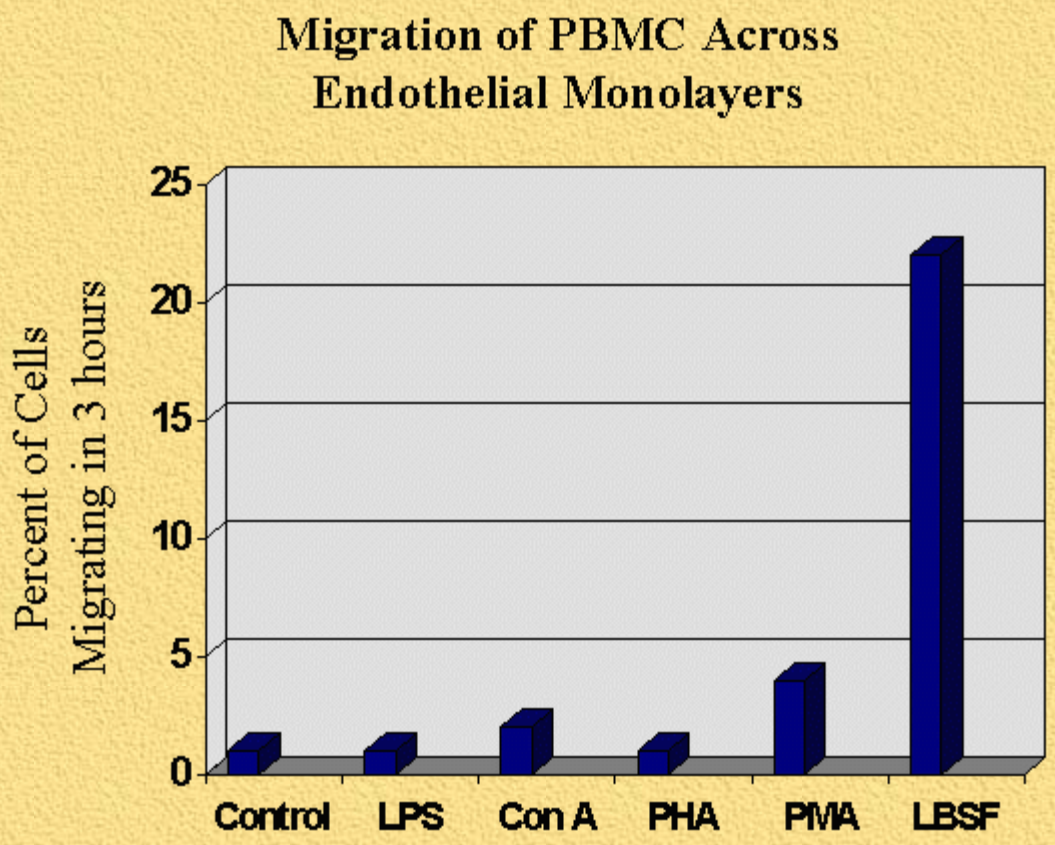


**Figure 1**

Effect of various activating factors on the migration of bovine lymphocytes. 1% LBSF was added to the migration assay with the bovine PBMCs of the upper well of transwell migration assay, migration was allowed to proceed for 3 hours, and cells passing through the 3um filter were then counted microscopically. Graphs show percentages of cells added to top well migrating through a 3 um pore size filter with a confluent monolayer of endothelial cells.

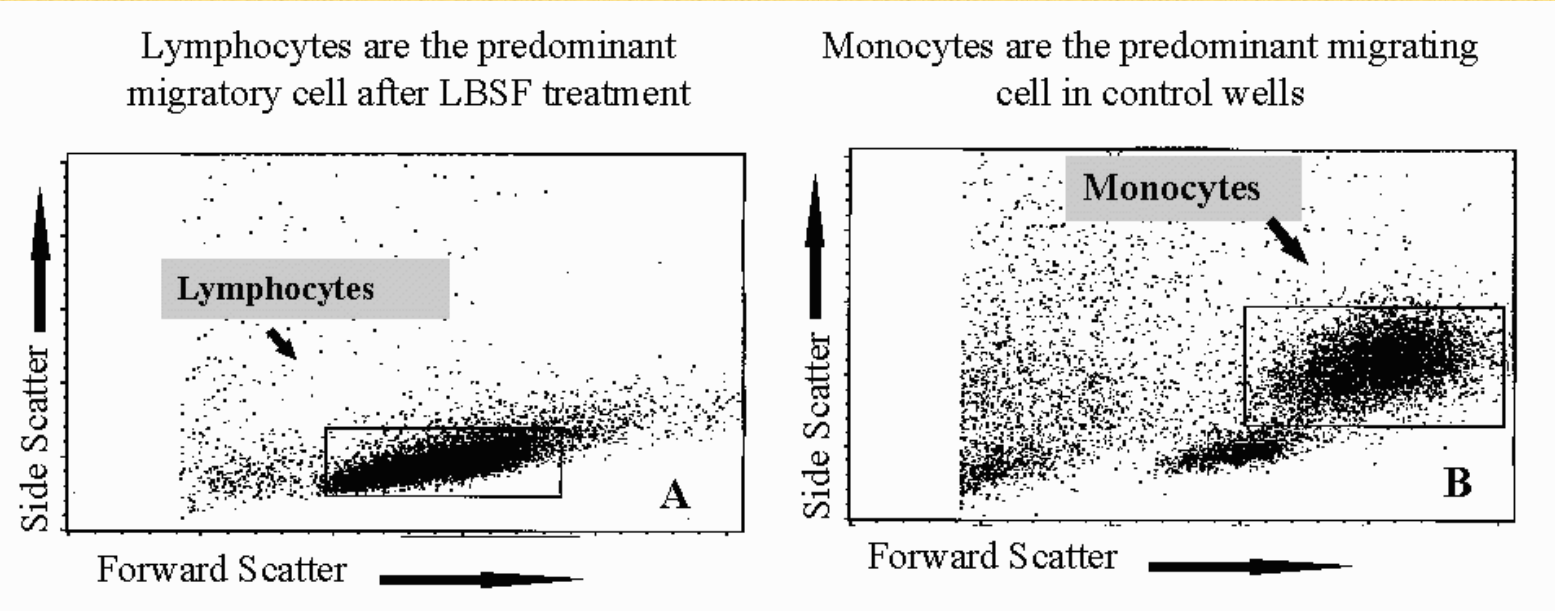


**Figure 2**

LBSF specifically stimulates the migration of lymphocytes. 1% LBSF was added with the PBMCs in the migration assays. Following three hours migration, migratory cells were analyzed by forward and side light scatter profiles. Assays stimulated with LBSF were found to contain mostly Lymphocytes (A). Control assays receiving no activating factors were found to contain mostly monocytes (B).

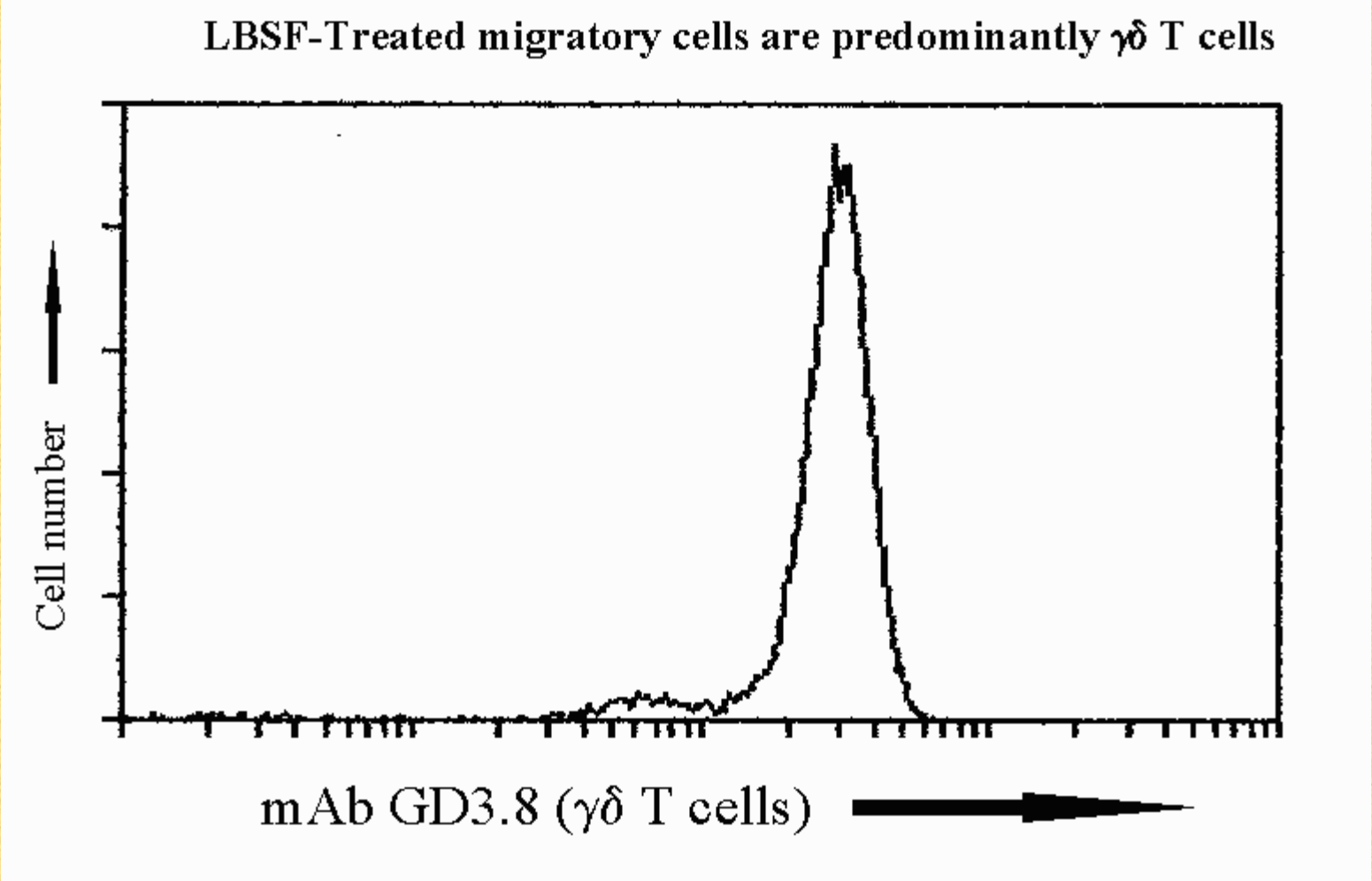


**Figure 3**

LBSF selectively stimulates the migration of gamma/delta T cells. Flow cytometric analysis of migratory lymphocytes showed up to 99% of the migrating lymphocytes in LBSF stimulated assays were gamma/delta T cells.

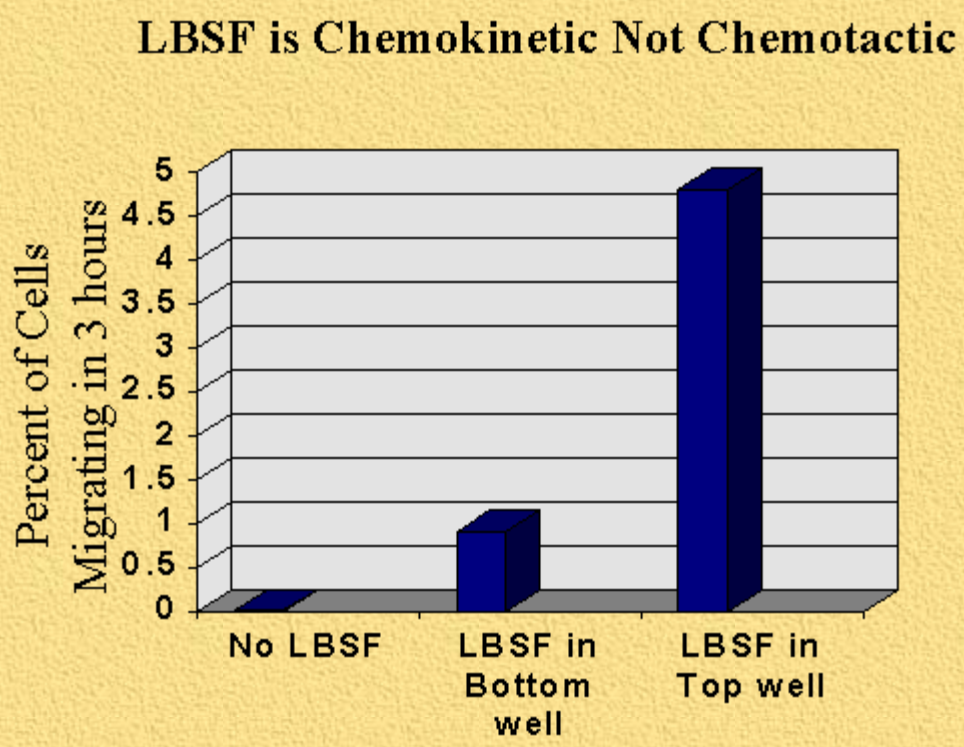


**Figure 4**

LBSF is chemokinetic, not chemotactic. A 1% solution of LBSF was added to the top or bottom well of a transwell migration chamber, and the effect on PBMC migration was measured 3 hours latter.

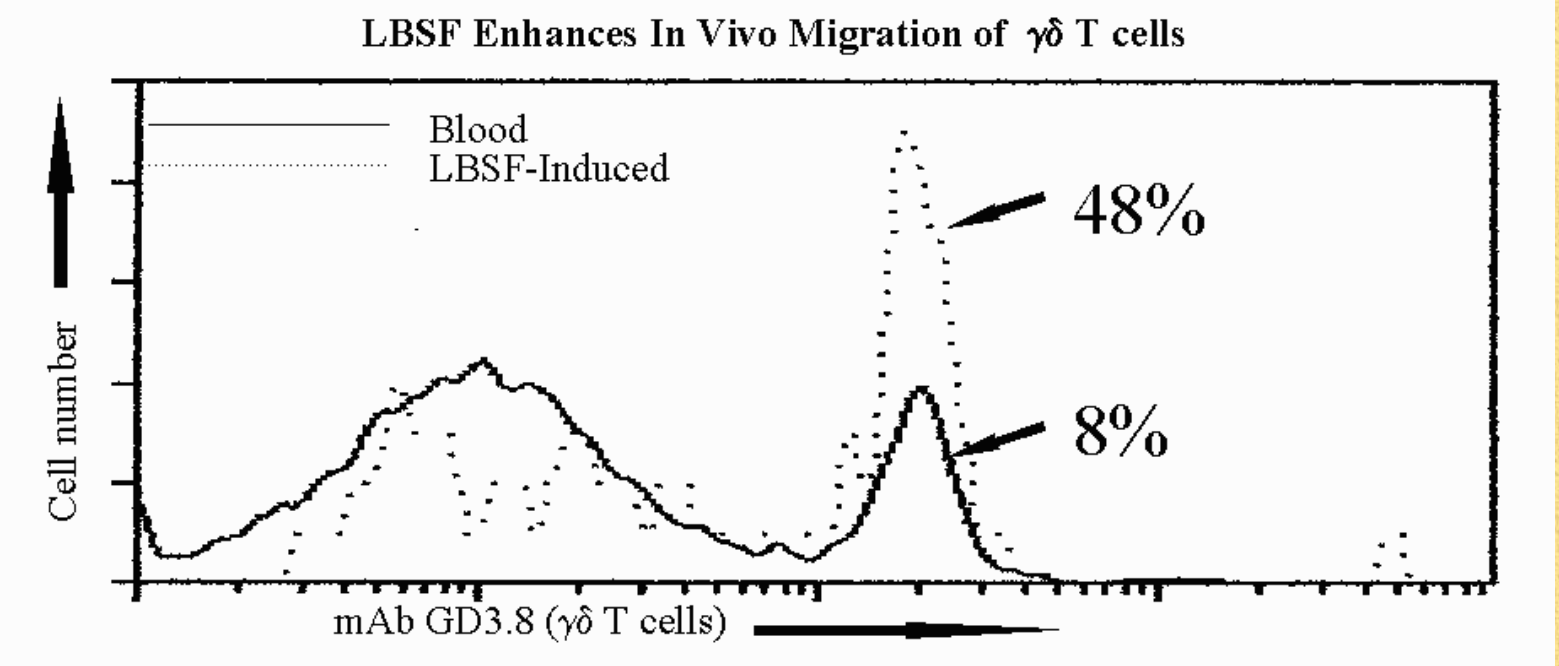


**Figure 5**

LBSF induces gamma/delta T cell specific migration in vivo. The effect of LBSF in vivo was analyzed by measuring the influx of gamma/delta T cells into a hollow, fluid filled, plastic wiffle ball, inserted under the dermis, following the injection of LBSF. The percent of gamma/delta T cells in the inflammatory site was compared to the percent in the blood of the same animal. Briefly a 7 cm wiffle ball was surgically implanted under the skin of 2-4 month old calves. Two weeks post implantation, LBSF was injected into the fluid filled ball. Eighteen hours latter, fluid was aspirated from the center of the ball and the accumulated cells analyzed by flow cytometry.

G00020

# Generation of a supernatant fluid by stimulated lymphocytes that causes selective migration of bovine gamma delta T cells through endothelial monolayers


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**Abstract:**
In this abstract, we describe the generation and functional activity of a unique, unidentified factor found in the supernatant fluid of activated bovine peripheral blood mononucular cells. When stimulated by this supernatant fluid, the migration of bovine gamma delta T cells across 3 um pore filters, as well as endothelial monolayers, is dramatically and selectively increased. The supernatant fluid has been shown to be highly chemokinetic, but not chemotactic, for bovine gamma delta T cells. Furthermore, the active component does not bind heparin and is very heat stabile, retaining full activity after boiling for 5 minutes. The activity of this supernatant fluid is not restricted to bovine cells and causes a similar, selecive migration of some human lymphocyte subsets. the basic functional and biochemical nature of this supernatant fluid distinguishes the active factor(s) from any known lymphocyte migration altering cytokine, including all known chemokines. Funded by NRI 96-02218.


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**Introduction:**
INTRODUCTION

gd T cells are commonly found in extralymphoid, epithelial cell associated tissues, such as the gut mucosa, tongue, and skin; whereas, few cells are found in organized lymphoid tissues. The unique mechanisms accounting for the selective tissue accumulation of gd T cells are not yet completely understood. Selective migration of leukocytes into different tissues can be controlled, in part, by adhesive interactions between the leukocyte and endothelial cells lining the vessels in a given tissue and/or by the release of leukocyte-specific migratory factors (chemotactic factors). We have found that certain adhesive interactions may account for the predilection of gd T cells to migrate into some inflammatory lesions. It is, however, unlikely that adhesion alone can explain all of the trafficking patterns on these cells in the animal. For example, immunohistology of some tissues reveals large numbers of gd T cells bound to vessel walls, but very few of the cells having extravasated into the tissue. Therefore we have tested several "activating" factors for their ability to alter the capacity of gd T cells to migrate through endothelial layers. Here we show that PHA- or PMA-stimulated lymphocyte conditioned medium causes a dramatic lymphocyte-specific increase in the transendothelial migratory capacity of bovine gd T cells.

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**Materials and Methods:**

Methods

LBSF was generated by culturing bovine PBMCs for three days in the presence of 5 ug/ml PHA or PMA and collecting the supernatant fluids.

Migration assays were performed in a transwell migration assay. PBMCs were added to the top chamber with or with out activating factors. Migration assays were typically run for 3 hours. In some assays, LBSF was added to the

bottom well of the chamber to test for chemotactic properties.

Activating factors which were used included: LBSF (1%), Con A (5ug/ml), PHA (5ug/ml), PMA (5ug/ml), LPS (2ug/ml). All assays were done using RPMI media supplemented with 10% FBS.

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**Results:**

Results and Summary

Here we describe the active component of an activated lymphocyte conditioned media (LBSF) that causes the selective migration of gd T cells through endothelial monolayers. The activity described here is unique from that described for any previously described cytokine. The major group of cytokines previously shown to be involved in migration, chemokines, are chemotactic factors, whereas LBSF is chemokinetic. Furthermore, chemokines in general bind heparin, whereas the initial characterization of the active factor in LBSF shows it does not bind heparin (data not shown). We have not shown that this factor is produced in vivo. Therefore, we can not rule out that it is an artifact of in vitro culturing. However, even if this is the case we have demonstrated that this supernatant fluid has profound activity on the migratory capacity of gd T cells both in vivo and in vitro.

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**Discussion and Conclusions:**

Conclusions

* LBSF causes a dramatic increase in the number of lymphocytes migrating through 3 um pore size filters, and through endothelial monolayers
* LBSF selectively acts on bovine gd T cells.

* LBSF is chemokinetic, not chemotactic.

* LBSF is active both in vitro and in vivo.

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**References:**

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**Comments:**

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Address questions and comments about this abstract to Eric Wilson ( wilson@trex2.oscs.montana.edu).

Previously presented at AAAI/AAI/CIS Joint meeting on Feb. 21-26 1997.

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# Questions and Answers

**Q:** I'm having trouble visualizing the physical setup of the Transwell migration chambers. If there is contact between the fluid in the upper and lower chambers, then it shouldn't matter where the LBSF is placed: it will diffuse equally. Therefore it isn't clear to me how to interpret the results seen in Figure 4. Please explain.

**A:**The top and bottom of the well of the transwell chamber are in physical contact,and it would appear that any soluble molecule would diffuse and reach equilibrium very quickly. However, this does not appear to be the case and the actual diffusion across the filters is quite slow, I believe studies by others have shown in chemotaxis assays that it takes several hours to reach equilibrium.

It may also be that when the LBSF is added to the top chamber with the cells in it that the cells have a high binding affinity for the molecule(s) active in LBSF, thus binding much of it before it diffuses into the lower chamber. When added to the lower chamber no LBSF would be available in the upper chamber until it had diffused up, and it would never reach as high a concentration as it did when added to the top well.

**A:**I was really interested by your poster on selective transmigation of bovine lymphocytes through endothelial monolayer. Could you indicate me some details about your experimental conditions of endothelial cells: at which step of culture do you add the leucocytes ? Are your endothelial cells activated (experimentally, or due to growing on membrane) ? What is the status of these endothelial cells in relation with leucocytes (MHC) ?

**Q:** In those assays where endothelial monolayers were used the cells were originally obtained from calf fetuses. All experiments were done with cells from passage 4, cells from 3 different fetuses were used in the experiments. Endothelial cells were cultured to confluence in EGM media (usually 48-72 hours).

Transwell chambers were then transferred to 6 well tissue culture plates containing prewarmed cRPMI media. Approximately 1 hour later, leukocytes were added to the top well and LBSF added immediately. The endothelial cells are not activated experimentally in the assays shown. Some minimal activation may take place on the transwell chambers due to the nature of the membrane itself, or due to the stress of changing to cRPMI media. However, I feel this effect is minimal and is taken into account in control wells that receive no LBSF. All endothelial cells used are allogenic, normally from another breed of cow. We have also performed these experiments on human endothelial cells and obtained the same results.

**Q:** Can you inject your LBSF intradermally and see an increase in accumulation of gamma/delta T cells histologically? If so what affect would stress have on gamma/delta T cell accumulation in this same tissue?

**A:** We have not injected LBSF under or into the skin. However, the wiffle ball experiments described are essentially the same as a subcutaneous injection. I am unsure of the effect of stress on the accumulation of gd T cells in the bovine system. I believe it may in fact increase the number of cells migrating into our wiffle ball tissue. However, in control wiffle balls injected with PBS we see far fewer numbers of gd T cells than in those balls injected with LBSF.

**Q:** I found your virtual poster presentation very fascinating and look forward to the full publication of your interesting discovery and findings. Have you made any allowances to exclude the possibility that your LBSF contains any residual PHA or PMA? (Its the "old pain in the behind" question of mitogen carry-over in the activity-containing supernatant. I realize you tested PHA as an activating factor, and it had very low activity but activity nonetheless. So, have you made any other provision to exclude this as a confounding factor?)

**A:** I am sure that LBSF does in fact contain residual PMA or PHA. As you mentioned, I have "controlled out" this effect with the addition of PMA or PHA alone. However, I am not realy satisfied with this, if you have any suggestion on how to get rid of this residual PMA or PHA please let me know.

**Q:** Could you explain to me why the "magnitude" of response to LBSF was different between Fig1 (>20%) and Fig4 (<5%)? In both experiments I believe you used the same concentration of LBSF (that is, 1%), and the assay was conducted over the same time period (that is, 3 hours), and I couldn't find any other conditions that may have been different (this could be my oversight). Could there have been some differences in the experimental setup or are there differences in the LBSF "potency" from batch to batch?

**A:** The LBSF used in the different experiments shown was from the same batch. However, the experiments were performed on differnet days, I do see some variation from day to day. I am not sure where this variation comes from, whether it is variation between cows from day to day or if it is something subtle I am doing and not aware of, I am not sure.

There is in fact variation from batch to batch in LBSF generation. In fact the generation of LBSF is very highly variable at this time, just one more problem I am trying to work out.

**Q:**Your in vivo experiments were particularly interesting for me. Would infusion of PBS, PHA or PMA, or even ConA for that matter, in your subcutaneously placed balls also induce a comparable accumulation of gamma/deltas? I realize you may not have conducted these experiments, but I feel they represent important controls in understanding the specificity of gamma/delta extravasation and accumulation in vivo. For instance, could other non-specific irritants also cause selective gamma/delta localizations? (just being rhetorical here)

**A:** I have injected PBS and PMA in the in vivo experiments and saw significantly fewer gd T cells than in the LBSF injection. The PMA experiment was done in a different wiffle ball in the same calf on the same day. I agree that Con A and PHA should also be tested. I need to do this as well as do some repeats of the experiments mentioned above. If you have any other ideas please let me know I appreciate your help.