Lymphocyte immune thresholds in bovine mastitis

LYMPHOCYTE NON-PROTECTIVE AND PROTECTIVE RESPONSES IN INFECTIOUS BOVINE MASTITIS

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SUMMARY

The presence of lymphocyte-related cut-off points that distinguish protective from non-protective anti-bacterial responses was investigated in bovine mastitis. Interactions among lymphocyte CD3, CD2, CD4, CD8 CD11b and CD45r surface density, and the percent of lymphocytes expressing these markers, were investigated by flow cytometry in bovine blood and milk cells before and times after experimental intra-mammary infusion with *Staphylococcus aureus*. The somatic cell count (SCC) and bacterial counts in milk cultures were also recorded. The surface density of these markers per cell (measured as median fluorescent intensity or MFI) provided confidence intervals that differentiated non-mastitic from mastitic animals and, among inflammed cows, identified very early (1 day post-infusion) from later cases (1-2 week[s] post-infusion). Critical values for immune markers were observed ("immune thresholds"). Above them, no bacterial counts and SCC <500,000 cells/ml were found. Regardless of time from infection onset, protective thresholds were also observed when milk CD2+ and CD45r+ lymphocytes exceeded 73 % and 21%, respectively. All 6 markers showed identical MFI thresholds for both outcome indicators (SCC and CFU). When individual time points and multi-factor interactions were considered, the value of the immune threshold increased over time for the percentage of milk CD3+ cells, and decreased for the percents of CD2+, CD11b+ and CD45r+ cells, and for the marker surface density per cell of all markers. Findings indicated that determination of time of infection (early versus late inflammation) is necessary to make inferences on the effect of the immune response, information provided by immune data. Because measurement of immune thresholds also facilitates predictions on outcomes to future bacterial invasions, it may be applicable for animal selection against bovine mastitis.

There are, conceptually, at least three levels of scale for the measurement of anti-bacterial immune responses. For bovine mastitis, they are: i) the somatic cell count (SCC) or mixed leukocyte level, in which

no individual leukocyte is accounted for; ii) the leukocyte differential level, in which lymphocytes, monocytes/macrophages and polymorphonuclear cells (PMN) are individually evaluated; and iii) the sub-cellular level in which cell functions are considered, which can be characterized by, at least, two dimensions. These dimensions are the percentage of cells of a given type showing specific surface molecules relevant in immune responses (antigen differentiation markers), and (for cells expressing a given immune marker), the marker surface density per cell. While research on bovine mastitis has emphasized the first level, relatively few studies have considered the remaining levels.

A major objective of immunological research is to describe the events that determine the outcome of host-pathogen interactions (i.e., whether a bacterial invasion will lead to bacterial multiplication and, eventually, infection, or, alternatively, to a response that will prevent bacteria from colonizing the host's tissues). The central event of the immune response is the process by which the T cell receptor (TCR) is stimulated in numbers and speed such that a cascade of responses will be triggered promptly and efficaciously. In humans, approximately 8000 TCR molecules per lymphocyte must be activated before T cells reach certain critical activation status such that the immune response begins (Viola & Lanzavecchia, 1996). However, the threshold for TCR triggering also appears to be influenced by non-CD3 molecules (Grossman et al., 2001; Tanchot et al., 2001). While previous studies have assessed immune thresholds, (i.e., variables representing early phenomena, like cell proliferation and cytokine synthesis), only a few studies have included outcome indicators, such as infections (Hesse et al., 2001). Neither immune thresholds nor the effect of non-CD3 lymphocyte molecules on CD3 triggering have been determined in dairy cattle.

While multiple immune markers have been measured simultaneously in other species (Falcioni et al., 1996), interactions among multiple immune markers have not been conducted in the bovine species. Although individual lymphocyte phenotype percentages have been determined in relation to bovine mastitis (Taylor et al. 1994; Rivas et al. [2000, 2001a,b, 2002]), previous studies did not assess relationships

between multiple immune markers (predictors) and the outcome to bacterial invasion (infection vs. protection). Measurement of cell percentage showing specific immune markers and/or marker density per cell may identify healthy from non-healthy individuals (Bikoue et al., 1996; Resino et al., 2000).

The objectives of this study were: 1) to evaluate whether assessment of surface density in conjunction with percentage of lymphocytes expressing immune markers could identify cut-off points that differentiated mastitic from non-mastitic animals and, if so, to determine whether immune markers could identify (early vs. late) inflammatory phases; 2) to identify cut-off points that characterize the outcome of the host-pathogen interaction (infection vs. protection), 3) to explore whether cut-off points vary as a function of immune marker interactions and/or time and, 4) to assess whether immune markers may predict the outcome of future bacterial invasions.

MATERIALS and METHODS

Animals

Five first-lactation, non-periparturient Holstein heifers were investigated. They had no history of mastitis. At least three consecutive microbiological tests yielded no bacterial growth of specific pathogens from milk samples, and no individual mammary gland quarter showed somatic cell counts (SCC) > 200,000 cells/ml.

Intra-mammary infusions

Staphylococcus aureus ribotype 116-232-S3 (Rivas et al., 1997), a strain isolated from a New York commercial herd, was cultured in sterile Todd-Hewitt broth at 37 C until the exponential growth phase was reached. The number of colony-forming units (CFU) was determined, and cultures were diluted to 200 CFU/ml in sterile Todd-Hewitt broth and kept at 4 C until infused. After the morning milking on day 0, 1 ml of inoculum (200 CFU in Todd-Hewitt broth) was infused into each of the right front and left hind

mammary gland quarters.

Bacteriological analysis and SCC

Decimal dilutions (100 µl each) of milk were cultured in triplicate onto blood agar plates and incubated at 37 C for 24 hours. The mean CFU in each replicate was determined as described (Rivas et al., 2001b). All *S. aureus* isolates recovered from milk of inoculated cows were the same ribotype as the inoculating strain (ribotype 116-232-S3). Duplicate samples of somatic cell counts were determined with a cell counter at the North East Dairy Herd Improvement Association (Ithaca, New York, USA).

Sample collection

At least one liter of milk from each tested mammary quarter was collected before bacterial infusion (day 0), at 1 day post-infusion (1 dpi), and at 1 and 2 weeks post-infusion (wpi). Milk samples were transferred to sterile 1-liter bottles containing 10 ml (100 X) of an antibiotic-antimycotic (penicillin, streptomycin, and fungizone) solution (# 15240-039, Gibco, Grand Island, NY, USA) and 12.5 µg/ml of gentamicin (Gibco). Blood samples were collected from the tail vein. Milk and blood were transported at 4 C.

Isolation of blood and milk leukocytes

Blood leukocytes were isolated using gradient centrifugation (Ficoll-Hypaque, Amersham-Pharmacia Biotech, Inc., Piscataway, NJ, USA), and washed 3 times. Milk leukocytes were isolated as described previously (Rivas et al., 2001b). Milk was diluted in an equal volume of pH 7.2 PAE buffer (phosphate buffer saline [PBS] solution with 10% acid citrate dextrose, 20m*M* EDTA and 0.1 sodium azide) and centrifuged (350 X g) for 40 minutes at 15 C. The supernatant and fat layer were poured off, and the cell pellet was washed 3 times in PAE buffer. The washed cell pellet was resuspended in 30 ml of Hank's

116 balanced salt solution (HBSS, Gibco), layered on a density gradient, and centrifuged (800 X g) for 30
117 minutes at 15 C. Leukocytes were collected, washed 3 times in HBSS containing 10% fetal bovine serum
118 (FBS, Hy-Clone, Logan, Utah, USA), and diluted into 5 ml of complete media (RPMI 1640 [Gibco]
119 containing 10% FBS and 5% of a tissue culture cocktail [0.1 m*M* nonessential amino acids, 2 m*M* L-
120 glutamine, 1 m*M* sodium pyruvate, 10 m*M* HEPES buffer, and 1X antibiotic-antimycotic mix, Gibco]).
121
122 *Immunophenotyping of blood and milk leukocytes*
123 Seven primary antibodies were used for phenotyping (isotype control, CD3, CD2, CD4, CD8, CD45r, and
124 CD11b). Seven million blood or milk leukocytes were resuspended in PAE buffer containing 2% rabbit
125 serum (first wash buffer) and centrifuged at 350 X g for 10 minutes. One million cells were then transferred
126 to each of seven 12 × 75 mm polypropylene tubes and resuspended in 50 µl of 10% rabbit serum in PAE
127 buffer (second wash buffer). After 10 minutes on ice, 50 µl of the appropriate primary antibody was added
128 to each tube, and tubes were incubated for 30 minutes on ice. A negative isotype control antibody (mouse
129 IgG1[catalog # 08-6599, Zymed, South San Francisco, CA, USA] diluted 1:25 in first wash buffer) was also
130 used as a primary antibody. Mouse monoclonal antibodies against either bovine CD3, CD2, CD4, CD8,
131 CD45r or CD11b (all IgG1 isotypes, VMRD Inc., Pullman, Washington, USA) were diluted 1:25 in the
132 same buffer. After incubation with primary antibodies, cells were washed in first wash buffer and incubated
133 for 30 minutes at 4 C with 100 µl of FITC-conjugated rabbit anti-mouse IgG diluted 1:50 in second wash
134 buffer (heavy and light chains, Zymed). Cells were then washed with second wash buffer (4X), fixed in 500
135 µl of 2% paraformaldehyde in PBS solution containing 0.1% sodium azide, and kept at 4 C until analyzed
136 with a cytometer (FACSCalibur, Becton-Dickinson, San Jose, California, USA). Cells were processed and
137 fixed within 12 hours of collection.
138

Flow cytometry analysis

Cell types were identified on the basis of forward and side scatter, as described earlier by Rivas et al. (2001a). Backgating was conducted on T cells (CD3+) or non-T lymphocytes ($CD3^-$), and the median fluorescence intensity (MFI) per cell was determined. At least 40,000 cells were acquired per test to obtain enough cells of the type least represented in each sample. Data were acquired and analyzed, using commercial flow cytometry software (Becton-Dickinson).

Statistical analysis

Three-factor interactions were assessed with lowess-generated curves fitting the data. Analysis of interactions involving lymphocyte and phagocyte phenotypes is reported elsewhere (associated manuscript). Medians, correlation and regression coefficients, confidence intervals and lowess-based curves were determined with a commercial software package (Minitab 12.2, Minitab, State College, PA, USA). To test the null hypothesis of a common model for the relationship between the responses (SCC, CFU) and each of the 6 predictor variables at the 3 post-challenge time points (1 dpi, 1 and 2 wpi) against the alternative hypothesis of differences among the models at the 3 time points, a likelihood test was performed (Mood et al., 1974). This test was based on the difference between fitting a common gamma function and fitting 3 separate gamma functions to the data from the 3 time points. The gamma function was chosen as the appropriate model because of its shape, rising from the origin to its maximum value and then decreasing to response of 0, as the level of the prediuctor varianle increases without bound. For all tests, $P < 0.05$ was considered significant.

RESULTS

Longitudinal lymphocyte phenotype (percentages)

Table 1 shows the phenotype of blood and milk lymphocytes expressed as percentage of cells displaying six markers. A critical percent value of some phenotypes was associated with protection (Fig. 1). Cows with >63% blood lymphocytes displaying CD2, or > 73% CD2+ milk lymphocytes, showed a median bacterial count of 0, while a median of 1405 CFU/ml was found in animals showing < 73% CD2+ milk T cells or < 63% CD2+ blood lymphocytes. Animals showing values above those critical thresholds showed low SCC (< 500,000 cells/ml). The percentages of blood vs. milk CD2+ cells were positively correlated (r= .71, $P<0.03$). Similarly, animals with > 21% of milk CD45r+ lymphocytes managed to prevent bacterial multiplication and did not show SCC above 500,000/ml. The median bacterial counts of animals showing > 21% milk CD45r+ lymphocytes was 0, while a median of 2134 CFU/ml was found in animals showing < 21% milk CD45r+ T cells ($P<0.02$). While the percent of blood CD45+ lymphocytes was not predictive of the milk CD45r+ cell percent, pre-infusion milk CD45+ lymphocyte percentage was positively correlated with the 1-day post-infusion percent, relationship that approached statistical significance (r=0.82, $P=0.09$). Interactions among immune markers were suggested by regression analysis. While the level of infection (expressed as bacterial counts) was not explained or marginally explained by individual markers (CD3, CD2, CD11b), the percentages of three markers explained 75% of all bacterial counts (Table 2).

Longitudinal lymphocyte phenotype (immune marker density per cell)

The surface density per cell of each of the six markers under study is shown in Table 3. It is expressed as median fluorescence intensity (MFI). Increased values were suggested after infusion (especially between 1 day and 1 week post-infusion), although they did not reach statistical significance (Fig 2 A-L). While post-challenge immune marker surface density data were partially overlapping, 95% confidence intervals provided estimates that facilitated the identification of non-mastitic from mastitic animals, and differentiated early (<l week pi) from late (>1 wpi) inflammatory phases (Table 4).

185

186 *Differentiation antigen cut-off points associated with immunity*

187 A critical cut-off point separated the data range characterizing non-protective responses from the
188 range of protective responses ("immune thresholds"). A threshold-like response was observed in all 6
189 investigated immune factors, both in blood and milk post-infusion comparisons (vertical broken line, Fig. 3
190 A-X). Neither bacterial counts nor SCC>500,000/ml were observed above that threshold. For each marker
191 and cell compartment, the same threshold was found in relation to both outcome variables (bacterial
192 [CFU/ml] and leukocyte counts [SCC/ml]).

193

194 *Longitudinal thresholds*

195 Two-factor analysis of immune thresholds indicated significant changes over time. Seventeen of the
196 24 assessments showed that all investigated markers in blood cells and 4 of the 6 milk thresholds showed
197 lower values at later observations (when the outcome variable was CFU), and 7 of the 14 thresholds
198 expressed in relation to SCC also diminished over time (Table 5).

199

200 *Interactions involving immune markers expressed as percentages*

201 The presence of immune thresholds was further explored by analysis of individual time points at
202 which interactions between pairs of immune factors were considered. Three-factor plots expressed bacterial
203 counts (infection) and SCC (inflammation) as peaks above the plot surface, while immunity (lack of
204 infection, lack of excessive inflammatory response) was visualized as a flat plot surface or as a depression
205 below that surface (Fig. 4). For instance, a non-protective response range was observed (at 1 day post-
206 infusion) approximately below 50 % for CD4+ cells and below 82% for CD2r+ cells. In contrast, the effect
207 was protective (no bacterial counts were observed) above those critical values.

208 Immune thresholds were characterized by two points: i) the "early inflexion point" (where a pro-
209 infective effect begins to become protective or *vice versa*), and ii) the "final inflexion point" (where the full
210 effect is seen, as when no bacterium is found [point where the plot surface is intersected, Fig. 4]). For
211 example, the interaction involving CD4 and CD2 showed an early inflexion point (where protection began)
212 at, approximately, 38 % (for CD4+ cells). However, the "final inflexion point" (i.e., total protection) was
213 achieved by CD4+ cells only at about 52%. Thus, the "distance" (percentage points) between these inflexion
214 points provided an additional characterization of immune thresholds.
215 The 3-factor analysis revealed that thresholds varied over time (both increasing and decreasing),
216 both in effects and cut-off values. All interactions involving milk CD3+ cell percents showed higher
217 thresholds of non-protective effects at 2 wpi than at 1 dpi, as indicated in the example shown in Fig. 5. An
218 inflexion point (where bacterial counts began to increase) was noticed for 2-wpi CD3 milk cells at around
219 89% (Fig. 5 C). In contrast, interactions involving the percent of CD45r+ and CD2+ cells and those of
220 CD45r+ and CD11b+ cells showed lower (and protective) thresholds at 1 or 2 wpi than at 1 dpi (not shown).
221
222 *Interactions involving immune marker surface density expression*
223 Three-factor analysis of marker density data suggested four major findings: 1) significant lower
224 thresholds occurred in milk than in blood cells at any given time, 2) lower thresholds were displayed at later
225 times that at 1 dpi by all markers, both in blood and milk cells, 3) interactions including CD8 and CD45r
226 revealed bi-modal patterns (showing protective effects at lower MFI values and pro-infective effects at
227 higher MFI values); and 4) different thresholds could result (at any given time) when the same marker
228 interacted with other markers.
229 Significant lower thresholds were observed in milk than in blood cells at 1 dpi. For instance, the
230 interaction between blood CD2 vs. blood CD4 lymphocyte surface densities showed no immune threshold

before 170 MFI units (Fig. 6 A). In contrast, the interaction between 1 dpi milk lymphocyte CD2 MFI vs. that of CD4 showed a lower threshold (approximately 70 MFI units for each marker), above which no bacterial counts were observed (Fig. 6 B).

Each cell compartment (blood or milk) showed decreased thresholds over time. For example, at 2 wpi, thresholds involving CD2 and CD4 markers were lower than those observed at 1 dpi (Fig. 6 D). Major threshold reductions were observed at 1wpi in blood cells for: CD4 x CD2, CD8 x CD11b, and CD11b x CD45r (not shown). At 2 wpi, milk CD3, CD2, CD4 and CD45r MFI showed lower thresholds than at 1 wpi (not shown).

Eight bi-modal interactions were observed in blood and milk cells, characterized by protective effects at low MFI values and non-protective (pro-infective) effects at high MFI values. CD45r participated in 5 of those bi-modal interactions: blood CD3 vs. CD45r, CD2 x CD8, and CD11b x CD45r interactions; milk (1dpi) CD8-CD4, CD8-CD3 and CD45r vs. CD8; and 1 wpi CD45r-CD11b and CD45r-CD4 (i.e., Fig. 7 D). However, no bi-modal interactions were shown at either 1 dpi or 2 wpi by milk CD4 and CD45r (Fig. 7 B, 7 E). At 1 dpi, the milk CD4-CD45r MFI interaction showed a protective effect throughout its entire range.

Significant differences among markers were suggested by their early vs. final inflexion points. For instance, blood CD4 resulted in a lower inflexion (protective) point when interacted with CD3, than when interacted with CD2, CD8, CD11b and CD45r (not shown).

Longitudinal comparisons between bacterial and leukocyte counts

Opposite CFU-SCC patterns were suggested by milk cells at 1 dpi both in relation to phenotype percents (which expressed opposite slopes, Fig. 4) and MFI (Fig. 8 A, D, G, J). Except CD2, all markers showed negative relationships between CFU and SCC. The number of bacteria decreased at the same immune

254 marker point where the SCC increased.

255 In contrast, identical patterns were found at 1 wpi between bacterial and leukocyte counts in relation
256 to both phenotype percents and surface density (Fig. 8 B, E, H, K). At 2 wpi, relationships between CFU
257 and SCC were not statistically significant. Some interactions including lymphocyte percentages showed
258 mixed SCC-CFU relationships. For instance, the interaction including percentages of milk CD4+ and
259 CD11b+ lymphocytes showed a bi-modal profile (protective at lower percentages for CD4 and infective at
260 higher percentages [not shown]).

261

262 *Comparisons across variables and time*

263 Five major findings were noticed in comparisons across immune variables and time: 1) phenotype
264 percents and marker density were, in most cases, not related; 2) pre-infusion marker density values could
265 predict later outcomes; 3) non-CD3 markers showed no protective effects by themselves, but resulted in
266 protective immunity when interacted with CD3 (even lower than those seen when CD3 was assessed
267 alone); 4) CD2 induced lower thresholds than other markers; and 5) linear relationships were observed
268 among longitudinal thresholds such that early (1 dpi) thresholds predicted later ones.

269 Only at 1 dpi a positive linear relationship was noted between phenotype percentage and MFI and
270 only for 3 of the six markers (CD3, CD2 and CD45r, Fig. 9). Of those, only CD45r showed a positive linear
271 relationship between phenotype percentage and MFI at later times (Fig. 9 R, S, T). Non-linear relationships
272 were observed for CD4 at all times.

273 These findings prompted us to explore whether pre-infusion milk MFI values could predict post-
274 infusion outcomes. Negative correlations were found in all interactions involving pre-/post-infusion milk
275 CD2, CD3, CD4 and CD8, and at least some involving CD11b and CD45r (Fig. 10). This indicated that
276 animals showing the lowest pre-challenge milk MFI values were likely to show the highest post-challenge

277 MFI values (event of protective effects), whereas those showing high MFI before experimental challenge
278 (an indication of ongoing immune responses of probably anergic consequences) were likely to fail in
279 eliciting adequate activation once challenged (event of non-protective or pro-infective consequences).
280 Except CD8, all other non-CD3 markers were protective only when associated to CD3. While interactions
281 involving a single marker indicated pro-infective effects (Fig. 10 C, E, G, I), a lower (and protective)
282 threshold was induced when each non-CD3 marker interacted with CD3 (Fig. 10 B, D, F, H).

283 Among non-CD3 markers, CD2 appeared to induce the most stimulatory effect. Pre-infusion blood
284 CD2 predicted immunity at, approximately, 95 CD3 MFI units (Fig. 11 A). In contrast, pre-infusion CD4,
285 CD8 or CD11b in interaction with CD3 were associated with infection even at higher CD3 MFI values (i.e.,
286 Fig. 11 E). Milk CD2 also indicated the lowest protective threshold that interacted with CD3 (even lower
287 than the one showed by CD3 when assessed alone, Fig. 10 B).

288

289 *Concordance between raw data and lowess-based interactions*

290 The hypothesis that longitudinal immune thresholds followed a linear relationship was further
291 explored by regression analysis, using lowess-fitted data. A positive and linear positive relationship was
292 observed (Fig. 12). This suggested that determination of early thresholds could predict later ones. It also
293 provided support to the hypothesis that 3-factor assessments based on lowess-fitted curves, are valid
294 procedures.

295

296 DISCUSSION

297 Critical values for the percentages of CD2+ and CD45r+ milk lymphocytes (around 73% and 21%,
298 respectively) indicated the threshold above which immune responses were protective. These findings are
299 consistent with published studies. In mice, depletion of CD2+ cells is associated with development of

300 opportunistic infections (ie, *Pneumocystis carinii* pneumonia), in spite of normal T cell numbers (Beck et
301 al., 2003). Similarly, infected calves (with bovine respiratory syncytial virus) show a CD45r$^-$ phenotype,
302 while uninfected calves are predominantly CD45r+ (McInnes et al., 1999).

303 Higher thresholds of pro-infective effects were suggested over time by the percent of milk CD3+
304 cells. When CD3+ exceeded 89% and two weeks had elapsed from the time of challenge, greater bacterial
305 counts were found. Immune responses ongoing for over a week may result in anergic lymphocytes
306 (Lanzavecchia & Sallusto, 2001; Knudsen et al., 2002; Schwartz, 2003). This indicates that phenotype
307 percentages alone cannot be informative unless the time from challenge (inflammatory phase) is determined,
308 observation that also applies to other phenotype percentages (later CD2+ and CD45r+ percents showed
309 lower protective thresholds).

310 Reduced protective thresholds at later times after initiation of immune responses (displayed by
311 marker density data), are consistent with the immune memory function. That is, the function by which naïve
312 (immuno-incompetent) cells become competent (memory) cells. In the early response (1 dpi-1 wpi),
313 CD45r+cells are characterized by two sub-sets: i) one with low density per cell, and ii) one characterized by
314 high CD45r MFI. However, two weeks after the bacterial encounter, naïve T cells may become immuno-
315 competent (Bell et al., 1998). Consequently, it may be expected that during the first phase (1 dpi-1 wpi)
316 either memory only or memory (low MFI) and naïve (high MFI) cells may coexist (which would be
317 expressed as bi-modal curves). Here, CD45r+ cells showed very protective effects at 1 dpi and bi-modal
318 curves were observed at 1 wpi. As expected, the 2 wpi CD45r-CD4 interaction was protective in its entire
319 range (uni-modal pattern). In agreement with this, five of the 8 bi-modal curves observed between 1 dpi and
320 1wpi involved CD45r. Although other bi-modal patterns were also observed at 1 dpi (i.e., the CD4-CD8
321 interaction), they were likely due to the suppressive or non-protective role of CD8, since no bi-modal but a
322 very low (and protective) threshold was seen at 1 dpi by the CD4-CD2, and CD4-CD45r interactions. CD4+

323 and CD45r+ cells showed bi-modal patterns at 1 wpi. In other species, such profile has been reported both *in*
324 *vitro* and *in vivo* (Bell et al. 2001; Bitmansour et al., 2002).

325 The interaction including CD2 percentage and surface density showed the lowest threshold at 1 dpi.
326 CD2 also seemed to induce lower thresholds than either other non-CD3 markers or CD3 itself (Figs. 11 &
327 12). Immune thresholds may be reduced when CD2 is present (Sasada et al., 2002).

328 At 1 dpi, the percent and marker density of CD11b+ lymphocytes resulted in the lowest "early
329 inflexion pont" with protective effects (i.e., Fig. 8A). While additional analysis of lymphocyte-phagocyte
330 interactions (involving CD11b density) are presented in the accompanying article, these findings alone
331 indicate that CD11b may induce lower thresholds, as reported by Randriamampita et al.
332 (2003). In contrast, CD11b did not appear to influence later immune thresholds (Fig. 8 A-C). Again, this
333 suggests that assessment of immune markers is inconclusive if time is not assessed.

334 CD8+ cells did not display anti-bacterial effects at early phases. The percentage of CD8+ cells
335 displayed a negative linear relationship with the percent of milk CD11b+ cells at 1 dpi, which resulted in
336 pro-infective effects. Suppressive consequences were associated with greater CD8 MFI (bi-modal patterns
337 with pro-infective effects in relation to CD3, CD4 and CD45r at 1 dpi, [i.e.,Fig. 7 A]). In other species,
338 CD8+ cells require much higher antigen concentration than CD4+ cells in order to express protective
339 immune responsiveness (Pettersson & Gronvik, 2003). At 1-2 weeks post-challenge, a similar percentage of
340 interacting CD8+ and CD11b+ cells, was protective. The fact that the same percentage or antigen density
341 value varied in effects over time highlights the importance of determining time of infection.

342 Bacterial count profiles were symmetrically opposed to leukocyte counts (SCC) only at the very
343 early phase (1 dpi). Later, they were either identical or not related. This means that assessment of SCC, if
344 time is not accounted for, prevents from making inferences in regard to its effects. This implies a limitation
345 of current diagnostic approaches (SCC), since they do not account for time, cell type or cell function. In

346 contrast, the immune factors assessed in this study estimated all three of these variables.

347 To the best of our knowledge, these findings provide the first report on lymphocyte thresholds in the
348 bovine species. In addition, they document that immune thresholds vary over time and that, when expressed
349 as antigen differentiation density per milk lymphocyte, early immune thresholds can predict later ones.
350 These findings may apply to animal selection practices. Because both blood and milk pre-challenge marker
351 density per cell predicted, at least for some markers, thresholds that were protective after challenge (Figs. 10
352 & 11), it is suggested that outcomes to future bacterial challenges can be predicted from non-infected
353 animals, information applicable to animal selection purposes. If such testing were conducted in a way such
354 that pre-challenge and post-challenge immune factors were measured (i.e., a challenge with a sterile
355 bacterial protein), anergy/immune responsiveness, as well as the other factors here enumerated, could be
356 determined. Because early post-challenge responses appear to predict later thresholds in a linear fashion
357 (Fig. 12), such testing could generate not only qualitative information (i.e., whether an individual would
358 become infected after challenge) but also, how much.

359 The median fluorescence intensity values reported here are indirect indicators of the number of
360 immune molecules per cell. While such number was not determined, this protocol could be adapted to
361 directly quantify the number of molecules per cell (Bikoue et al., 1996). Three-factor relationships were
362 based on data estimated by locally weighted robust analysis (lowess). Lowess-adjusted data displayed the
363 same final inflexion point for both bacterial and leukocyte count data (measures independent from each
364 other) in each of the 60 comparisons conducted. While lowess is regarded to be robust to many types of
365 measurement error (Borkowf et al., 2003), it does not preclude alternative curve-fitting models. Additional
366 studies considering other statistical analyses in which the immune marker density per cell is directly
367 measured and cytokine synthesis are assessed, are recommended.

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Table 1. Percentage of lymphocytes expressing differentiation antigens (predictors) and outcome indicators

	BLOOD							MILK							SCC	CFU
	B Ct	b CD 3	b CD 2	b CD 4	b CD 8	b CD 45r	b CD 11b	m Ct	m CD 3	m CD 2	m CD 4	m CD 8	m CD 45r	m CD 11b		
A,0	0.8	66.7	61.1	39.3	15.5	64.7	11.8	0.4	56.1	77.9	12.5	16.7	34.2	0.6	21	0
1 d	0.7	86.1	79.9	49.3	15.7	49.9	9.1	7.6	90.3	89.4	28.2	30.3	50.3	13.3	403	0
1 w	1.1	76.1	74.9	46.4	16.4	53.8	14.6	5.6	75.7	75.7	17.3	29.0	45.8	15.3	420	0
2 w	0.4	77.9	66.8	45.4	16.7	42.0	6.4	0.9	85.4	85.9	13.5	21.1	34.0	3.4	65	0
B,0	0.3	73.4	68.0	40.7	17.9	58.0	11.5	1.0	70.6	73.0	9.1	18.0	25.7	1.2	42	0
1 d	0.2	76.2	73.3	46.3	18.6	52.6	10.5	1.5	90.4	89.2	61.6	26.7	13.8	3.3	370	0
1 w	0.3	69.8	70.2	38.5	18.7	48.5	15.8	0.5	90.9	91.6	63.3	21.6	9.9	1.6	135	0
2 w	0.9	58.3	66.2	35.2	17.5	61.7	*	1.9	*	82.6	48.3	32.7	14.4	2.9	55	0
C,0	0.5	64.9	55.2	33.4	15.7	56.4	12.9	0.7	71.8	71.9	15.7	32.1	27.0	0.9	47	0
1 d	0.5	62.5	53.7	40.6	15.3	50.0	13.8	0.3	72.6	77.7	29.3	28.1	20.5	1.2	23	1575
1 w	0.2	67.2	59.0	41.8	14.8	61.3	11.2	0.2	73.1	65.4	43.3	15.7	8.9	9.6	6479	16675
2 w	0.7	65.9	58.3	38.5	13.6	37.8	10.7	0.2	91.9	67.7	38.3	31.6	10.9	3.5	1453	50430
D,0	0.6	74.0	71.3	47.8	16.3	57.0	12.6	0.1	95.0	94.5	25.2	43.7	18.0	1.2	15	0
1 d	0.4	72.5	69.7	44.5	16.4	56.6	7.5	1.1	84.4	79.9	48.4	28.9	16.1	4.7	36	1.5
1 w	0.6	79.0	77.9	48.2	19.2	67.4	6.8	0.3	88.9	86.7	18.9	39.5	17.8	4.6	90	2685
2 w	2.5	69.6	68.5	42.1	17.8	64.7	10.1	0.9	91.5	87.1	32.6	35.9	15.1	2.5	2267	1405
E,0	1.1	64.2	54.4	34.7	13.1	62.2	13.0	3.0	83.6	85.6	19.6	20.0	26.5	0.9	94	0
1 d	0.5	54.0	48.7	32.3	10.3	73.1	18.9	3.5	67.7	70.0	30.6	18.8	22.9	13.8	174	165
1 w	0.5	61.1	54.4	35.8	10.9	71.6	12.0	0.7	66.5	59.8	44.8	8.9	7.6	30.8	3266	3135
2 w	0.8	54.4	42.1	29.6	10.5	65.4	25.8	0.8	88.1	72.0	39.1	24.4	15.1	15.8	5104	5435

Ct: isotype (negative control) antibody. b: blood. m: milk. SCC: somatic cell counts (1×10^3 cells/ml). CFU: bacterial counts in milk cultures (colony forming units/ ml). Results show the longitudinal values of individual animals (A-E) recorded before challenge (0), and 1 day, 1 week or 2 weeks after challenge (1 d, 1 w, 2 w, respectively).

Table 2. Regression analysis of bacterial counts (CFU/ml) on immune phenotype (percentage of milk lymphocytes expressing differentiation antigens CD3, CD2 and CD11b)

Outcome variable	Predictive variable(s)	Regression equation	R^2 (adjusted)	P
CFU	CD3	CFU= –15326 + 256 (CD3)	0.0 %	0.54
CFU	CD11b	CFU= 6625 – 139 (CD11b)	0.0 %	0.74
CFU	CD2	CFU= 53521 – 611 (CD2)	15.1 %	0.08
CFU	CD2 , CD11b	CFU=106770 – 1179 (CD2) – 998 (CD11b)	38.2%	0.02
CFU	CD2, CD3	CFU= 8411 +1369 (CD3) – 1476 (CD2)	61.4%	0.002
CFU	CD3, CD2, CD11b	CFU=54801+1175 (CD3) –1175 (CD2) –761 (CD11b)	75.5%	0.001

447
448 Table 3. Differentiation antigen density per lymphocyte

	Blood MFI							Milk MFI						
	Ct	b CD 3	b CD 2	b CD 4	b CD 8	b CD 45r	b CD 11b	Ct	m CD 3	m CD 2	m CD 4	m CD 8	m CD 45r	m CD 11b
A,0	18.7	92.3	54.0	52.0	46.5	201.8	43.1	33.7	18.1	26.0	18.3	19.5	94.7	17.5
1 d	32.6	319.5	177.9	175.0	132.6	625.9	120.7	72.0	209.0	184.0	162.0	101.0	451.0	167.0
1 w	55.0	280.0	174.0	187.0	131.0	492.0	119.0	42.0	97.0	123.0	61.0	75.0	312.0	62.0
2 w	18.0	115.0	60.0	71.0	58.0	181.0	52.0	19.0	62.0	60.0	50.0	34.0	160.0	34.0
B,0	23.0	120.0	64.0	90.0	65.0	245.0	52.0	22.5	34.0	41.0	40.0	34.0	72.5	31.4
1 d	24.0	113.0	26.0	103.5	65.4	230.0	53.3	15.5	55.6	62.7	48.3	40.0	91.0	23.3
1 w	35.0	83.0	57.8	75.0	56.0	142.0	52.6	44.0	42.0	57.0	38.0	35.0	61.0	32.0
2 w	18.0	25.0	63.0	89.0	59.0	198.0	51.0	25.6	55.0	64.0	48.0	44.0	91.0	32.0
C,0	25.9	80.0	49.6	64.5	48.9	203.5	46.7	43.8	49.7	52.1	59.4	42.2	142.1	44.2
1 d	19.7	93.9	55.1	80.0	54.1	199.4	48.3	24.0	47.8	51.4	52.3	39.6	125.2	42.9
1 w	40.0	93.9	50.9	66.7	57.2	259.5	48.7	22.7	37.2	46.1	43.3	27.8	51.9	39.2
2 w	32.2	103.7	59.9	80.0	50.0	228.8	43.3	23.9	41.2	40.3	45.3	22.5	45.1	37.5
D,0	12.4	99.1	47.0	42.9	50.5	162.5	30.8	31.9	78.4	62.6	56.2	55.2	129.8	36.2
1 d	18.1	103.7	52.8	43.7	58.8	145.5	34.0	23.3	71.7	62.1	48.3	48.7	87.8	37.5
1 w	20.0	123.0	51.4	62.6	60.4	200.0	41.4	35.4	82.0	62.1	44.1	49.6	137.0	50.9
2 w	71.4	93.1	46.6	*	*	153.3	36.5	35.2	75.0	70.4	61.5	54.2	117.6	42.7
E,0	19.7	112.4	58.8	76.3	52.3	199.9	54.5	27.8	54.7	63.8	47.0	36.2	101.8	96.9
1 d	25.5	110.4	43.7	67.3	50.9	162.4	53.8	28.0	70.0	73.0	63.2	47.0	139.5	55.5
1 w	25.5	95.6	43.3	54.7	47.2	140.7	44.1	26.7	57.8	51.4	52.8	39.4	51.6	68.5
2 w	19.5	106.5	49.6	59.3	45.1	93.9	57.2	11.1	52.6	42.5	42.5	14.4	33.2	36.7

449
450 MFI: median fluorescence intensity. Ct: isotype (negative control) antibody. b: blood. m: milk. Results show
451 the longitudinal values of individual animals (A-E) recorded before challenge (0), and 1 day, 1 week or 2
452 weeks after challenge (1 d, 1 w, 2 w, respectively).
453
454
455
456

Table 4. Lymphocyte surface marker density confidence intervals

Blood cell MFI	Mean	95% C.I.	Milk cell MFI	Mean	95% C.I.
Pre-infusion					
CD3	100.7	(81.0, 120.5)	CD3	47.4	(20.1, 74.7)
CD2	54.6	(46.1, 63.2)	CD2	49.2	(29.8, 68.6)
CD4	65.1	(41.8, 88.4)	CD4	44.4	(24.9, 64.1)
CD8	52.6	(43.6, 61.6)	CD8	41.9	(26.7, 57.0)
CD45r	202.5	(166.3, 238.8)	CD45r	107.2	(71.7, 142.7)
CD11b	45.4	(33.8, 56.9)	CD11b	47.3	(12.1, 82.6)
One day p.i.#					
CD3	148.1	(28.8, 267.4)	CD3	90.8	(7.9, 173.8)
CD2	71.1	(-4.4, 146.6)	CD2	86.6	(18.4, 154.9)
CD4	93.9	(31.5, 156.3)	CD4	74.8	(13.8, 135.8)
CD8	72.4	(30.0, 114.7)	CD8	55.3	(23.1, 87.4)
CD45r	272.6	(24.1, 521.2)	CD45r	178.9	(-12.0, 369.8)
CD11b	62.0	(20.1, 103.9)	CD11b	65.2	(-6.8, 137.3)
One week p.i.#					
CD3	135.1	(32.9, 237.3)	CD3	63.2	(31.2, 95.2)
CD2	75.5	(6.8, 144.2)	CD2	67.9	(29.0, 106.9)
CD4	89.2	(20.7, 157.7)	CD4	47.84	(36.58, 59.10)
CD8	70.4	(27.8, 112.9)	CD8	45.36	(22.57, 68.15)
CD45r	246.8	(66.1, 427.5)	CD45r	122.7	(-16.0, 261.4)
CD11b	61.2	(20.7, 101.7)	CD11b	50.52	(31.62, 69.42)
Two weeks p.i.#					
CD3	88.7	(43.4, 133.9)	CD3	57.16	(41.68, 72.64)
CD2	55.82	(46.84, 64.80)	CD2	55.44	(38.84, 72.04)
CD4	74.83	(54.63, 95.02)	CD4	49.46	(40.40, 58.52)
CD8	53.03	(42.45, 63.60)	CD8	33.82	(13.95, 53.69)
CD45r	171.0	(107.6, 234.4)	CD45r	89.4	(24.6, 154.2)
CD11b	48.00	(37.91, 58.09)	CD11b	36.58	(31.54, 41.62)

Lower and upper limits (95% confidence intervals) are reported within parentheses. # Post-infusion.

If milk cell MFI for: CD3> 74.7, CD2> 68.6, CD4>65, CD8>57.0, CD11b> 82.6, and/ or CD45r>142.7 =mastitic. If milk MFI for: CD3 > 95.2, CD2 > 106.9, CD11b 69.4, and/or CD45r > 261.4= very early (1 dpi) mastitis.

Table 5.

Predictor variable	Response variables	
	CFU	SCC
Blood lymphocytes		
CD3 MFI	10.734	12.540
CD2 MFI	3.758	8.188
CD4 MFI	4.051	8.572
CD8 MFI	8.018	12.335
CD45r MFI	7.980	7.029
CD11b MFI	5.109	8.892
Overall *P* value:	<0.001	>0.1
Milk lymphocytes		
CD3 MFI	5.899	6.082
CD2 MFI	8.054	21.378**
CD4 MFI	19.396**	15.659*
CD8 MFI	2.348	9.118
CD45r MFI	6.589	3.626
CD11b MFI	8.513	12.187
Overall *P* value:	<0.01	<0.05

Hypotheses and critical values: i) Ho: there is a common cubic polynomial model for all three post-infusion (pi) times (1 day pi, 7 days pi, and 14 days pi); ii) Ha: there are differences among the three times in the cubic polynomial model coefficients; and iii)critical values are chi-square (6,.95) = 12.592, and chi-square (6,.99) = 16.812.

* Significant at p=0.05

**: Significant at p=0.001

LEGENDS

Fig. 1. Relationships involving post-infusion lymphocyte percentages in blood and milk, and bacterial (CFU/ml) or leukocyte counts (SCC/ml, expressed in 1 x 10^3 cells)(n=15). **A, C**: CD45r. **B, D**: CD2. A vertical broken line indicates the threshold above which no bacterial counts (with one exception, CD2) and low leukocyte counts (SCC<500,000) are observed. **E**: Blood vs. milk percentage of CD2 + cells. Two clusters are suggested by the data. The upper right quadrant includes observations above 60% (blood) or 73% (milk), values associated with protective effects, whereas the lower left quadrant displays values related to infection.

Fig. 2. Longitudinal expression of differentiation antigen density per lymphocyte in blood and milk. **A, B**: CD3. **C, D**: CD2. **E, F**: CD4. **G, H**: CD8. **I, J**: CD11b. **K, L**: CD45r. Boxplots indicate the median, 25 and 75 percentiles ([lower, upper] external horizontal lines) and 5 and 95 percentiles (lower, upper vertical lines) obtained at pre-infusion, 1 day, 1 week and 2 weeks post-infusion (d/wpi) (n=5).

Fig. 3. Post-infusion differentiation antigen density per lymphocyte in blood and milk, and bacterial (CFU/ml) or leukocyte counts (SCC/ml)(n=15). **A-D**: CD3. **E-H**: CD2. **I-L**: CD4. **M-P**: CD8. **Q-T**: CD11b. **U-X**: CD45r. A threshold (vertical broken line) is suggested in each of the 24 plots, above which no bacterial counts and low leukocyte counts (SCC<500,000) are observed. SCC are expressed as 1 x 10^3 cells.

Fig. 4. Three-factor host-pathogen relationships. The graph displays interactions involving pairs of predictor factors (immune variables, as percentage of lymphocytes expressing surface molecules CD4

498 and CD2) and outcome factors (bacterial counts [CFU], leukocyte counts [SCC]) at a specific post-
499 challenge time (1 day post-infusion or dpi). Predictor and response variables are estimated by lowess.
500 The graph shows an example of 1 dpi responses measured on 5 cows, which are expressed as
501 bacterial counts (CFU, **A**) or leukocyte counts (SCC, **B**). They display: i) a 2-factor relationship
502 involving the percentage of CD4+ and CD2+ lymphocytes, and ii) the net outcome resulting from the
503 overall predictor-response interaction. In this example, the relationship between the 2 immune
504 markers is positive and linear (increases in one factor [i.e., the percentage of CD4+ cells] result in
505 increases in the other factor [i.e., the perentage of CD2+ lymphocytes]). The relationship between
506 immune factors and the outcome factor (i.e., CFU) shows a threshold (vertical line) above which a
507 protective effect (no bacterial counts) is noticed. The same threshold (with opposite effects) is
508 observed in relation to both outcome variables (CFU, SCC). The "immune threshold" can be
509 characterized by 2 inflexion points: i) the point above which the effect reverses (i.e., the early
510 inflexion point where the non-protective effect begins to become protective), and ii) the point at
511 which the final effect is achieved (i.e., when protection [0 bacterial counts] is complete).
512
513 **Fig. 5.** Longitudinal thresholds involving percentage of lymphocytes expressing specific surface
514 markers (CD3, CD2) and bacterial counts (CFU). **A, B, C**: one day post- infusion, one week and two
515 weeks post-infusion, respectively. A linear and positive relationship is observed between immune
516 markers at 1 dpi and 1 wpi, while a rather negative and non-linear relationship is suggested at 2 wpi.
517 While the percentage of CD3+ lymphocytes above, approximately, 88% is associated with protective
518 effects at 1 dpi-1 wpi, the same range displays pro-infective effects at 2 wpi. Sample size per
519 comparison: n=5.
520

521 **Fig. 6.** Cross-sectional and longitudinal comparisons between blood and milk lymphocyte marker
522 density in relation to bacterial counts. **A**: blood, **B-D**: milk cells.
523
524 **Fig. 7.** Examples of longitudinal bi-modal relationships (protective at lower marker density values,
525 pro-infective at higher marker density values) in milk lymphocytes. The graph shows that the marker
526 density interactions between CD4 and CD45r at 1 week post-infusion results in a bi-modal pattern
527 (**D**), which is nor observed at 1 dpi (**B**) and at 2 wpi (**F**). CD8 MFI displays bi-modal patterns at 1 dpi
528 (**A**) and 1 wpi (**C**), but not at 2 wpi (**E**).
529
530 **Fig. 8.** Longitudinal profiles of response variables. The graph shows examples of CD3-CD11b and
531 CD8-CD45r marker density longitudinal expression in relation to bacterial counts (CFU, **A-C**, **G-I**)
532 and leukocyte counts (SCC, **D-F**, **J-L**).
533
534 **Fig. 9.** Longitudinal relationships between percentage of lymphocytes expressing specific surface
535 markers and density per cell of the same marker, in relation to bacterial counts. Left column: 1 dpi;
536 central column: 1 wpi; right column: 2 wpi.
537
538 **Fig. 10.** Effects on 1 dpi bacterial counts induced by non-CD3 and CD3-nonCD3 milk lymphocyte
539 marker density interactions. **A, C, E, G, I**: pre-infusion vs. 1 dpi MFI for the same marker (CD3, 2, 4,
540 8, 45r, respectively). **B, D, F, H**: 2-marker comparisons between the pre-infusion MFI of [CD2, CD4,
541 CD8, CD45r, respectively) vs 1 dpi CD3 MFI.
542
543 **Fig. 11.** Effects on 1 dpi bacterial counts induced by pre-infusion blood lymphocyte marker density

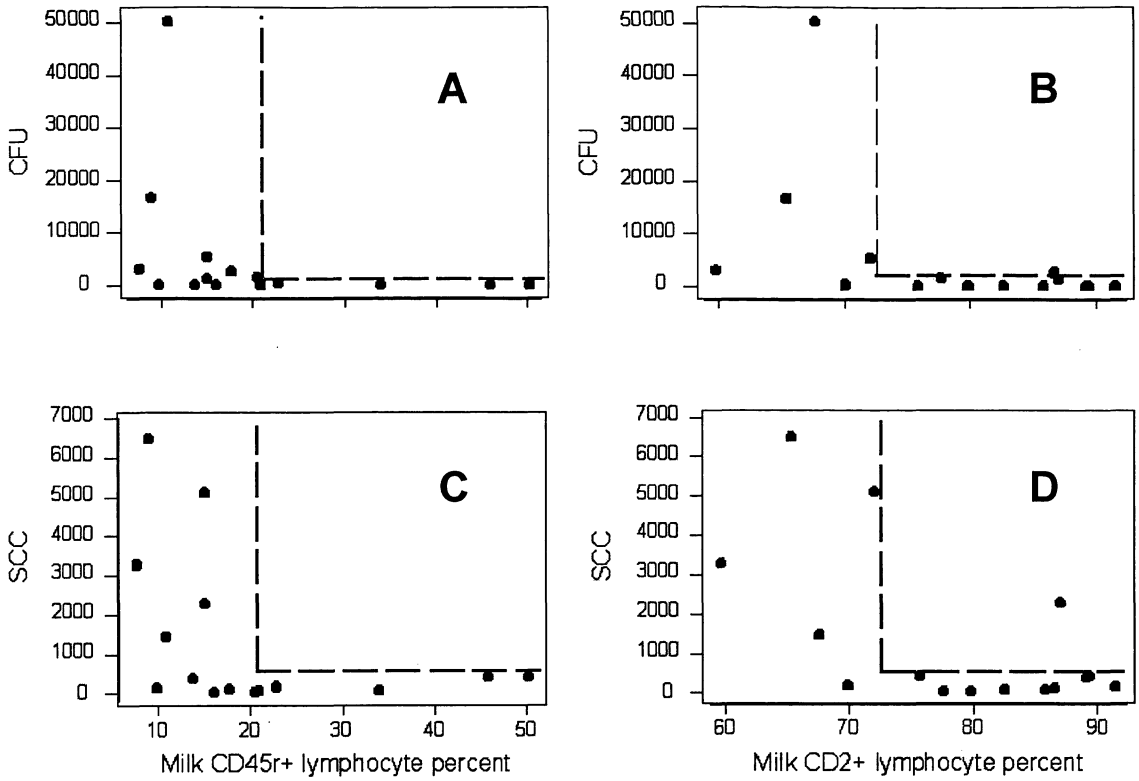
544 interactions. The graph shows interactions between non-CD3 and CD3 density per cell.

545

546 **Fig. 12.** Regression line (broken line) and confidence interval (95% confidence, dotted lines) of 1 dpi
547 MFI milk lymphocyte thresholds regressed on 2 wpi thresholds (adjusted R^2= 0.929, P<0.001), as
548 indicated by the inflexion point of each of the six differentiation antigen density per cell (MFI) above
549 which bacterial counts are ~ 0 (plots [not shown] generated from data reported in Table 2).

550

Fig 1



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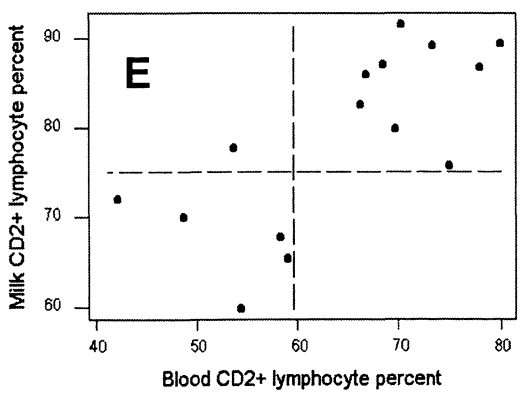

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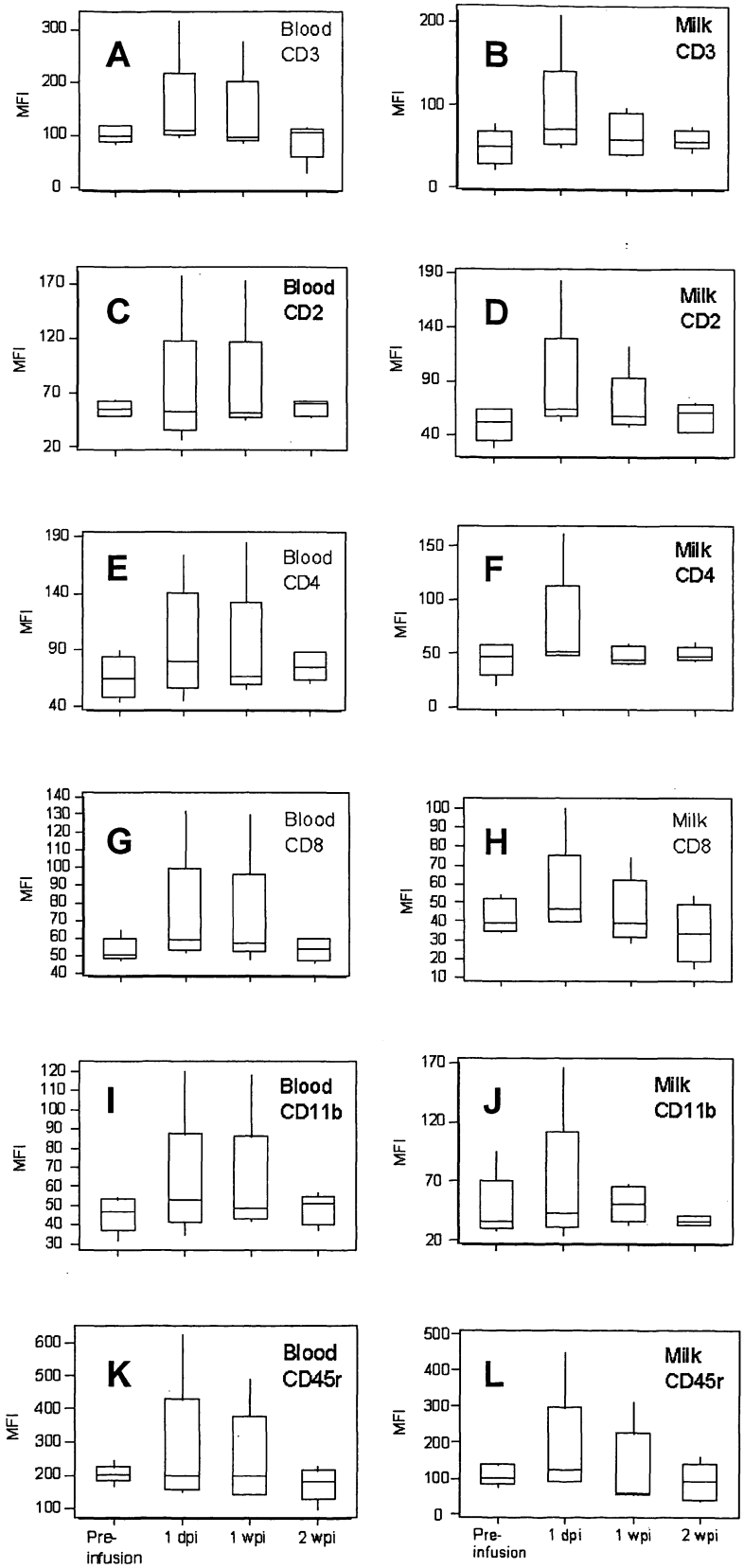
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CD4
G
Blood
CD8
H
Milk
CD8
I
Blood
CD11b
J
Milk
CD11b
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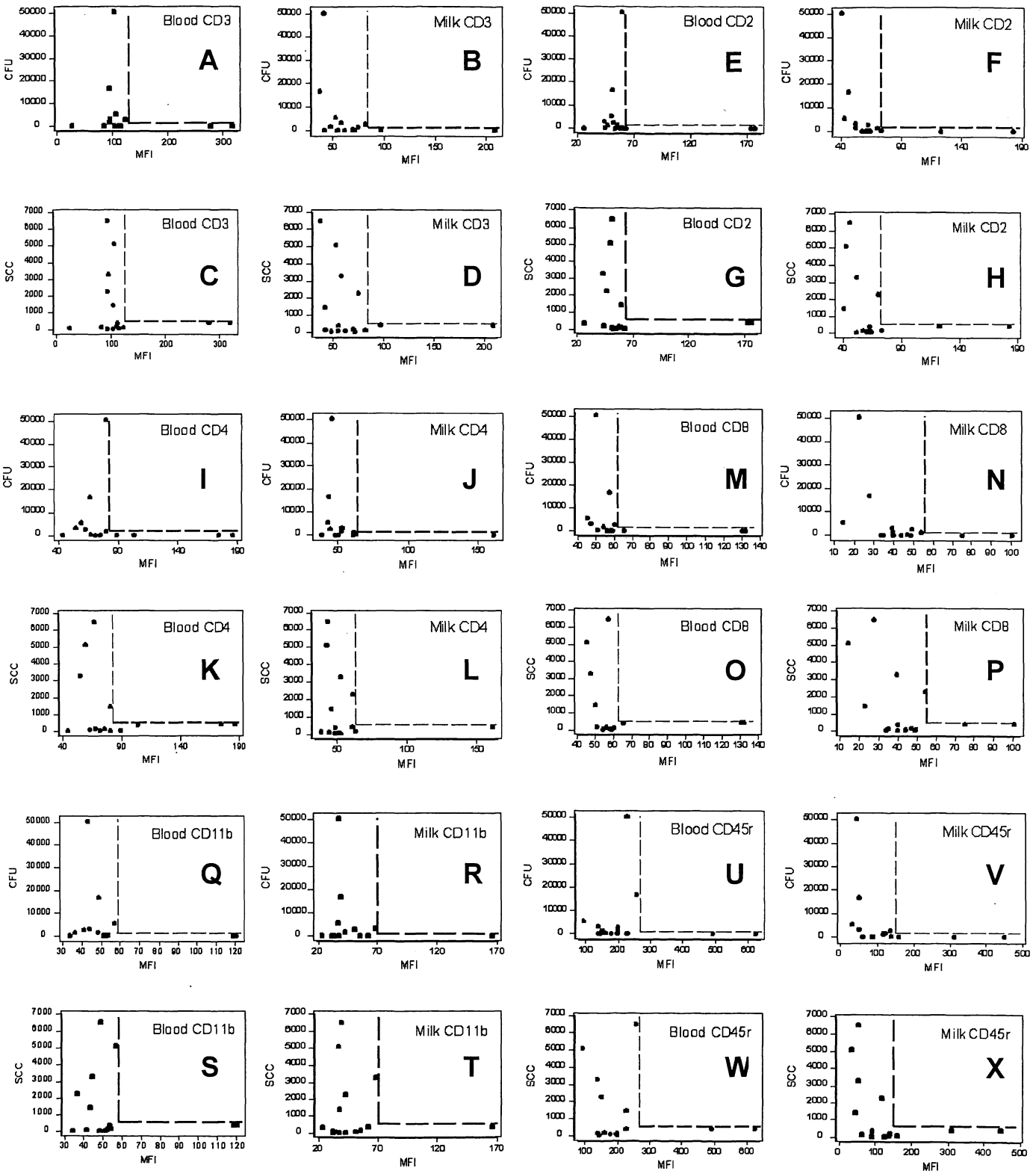
Pre-infusion
1 dpi
1 wpi
2 wpi

Fig 2

Fig 3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P
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Q
Milk CD11b
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Blood CD45r
U
Milk CD45r
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SCC
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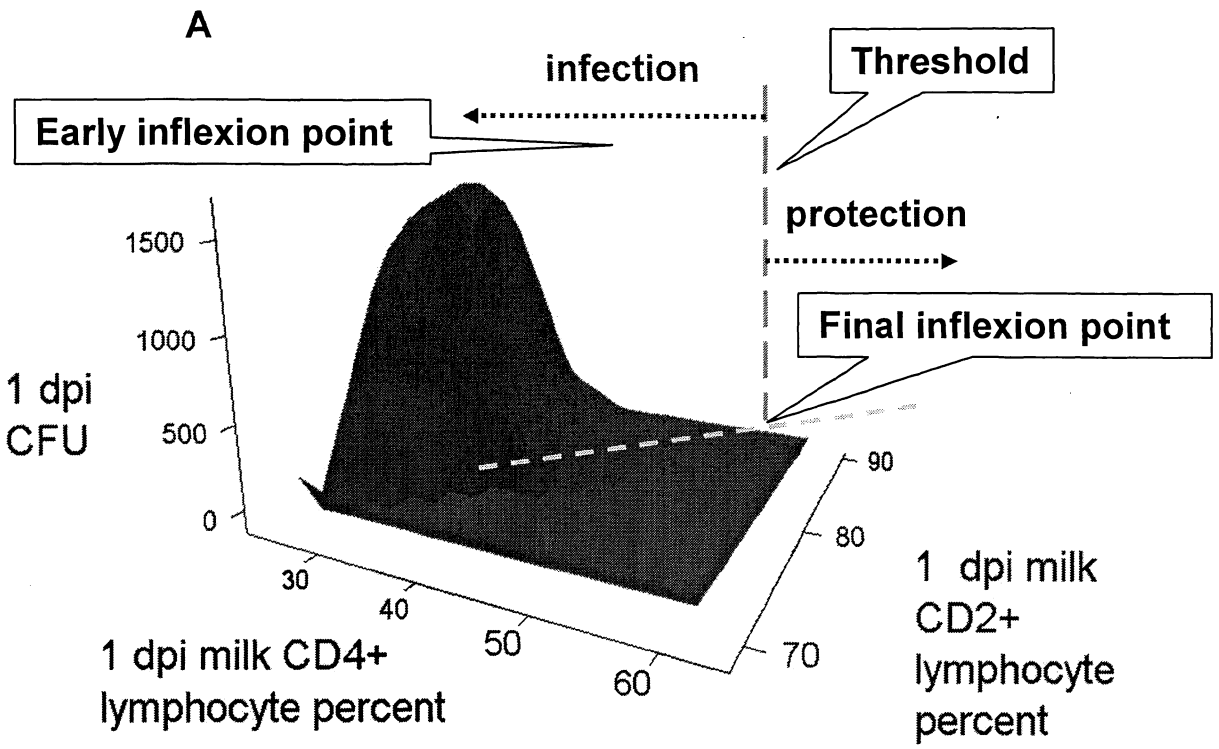
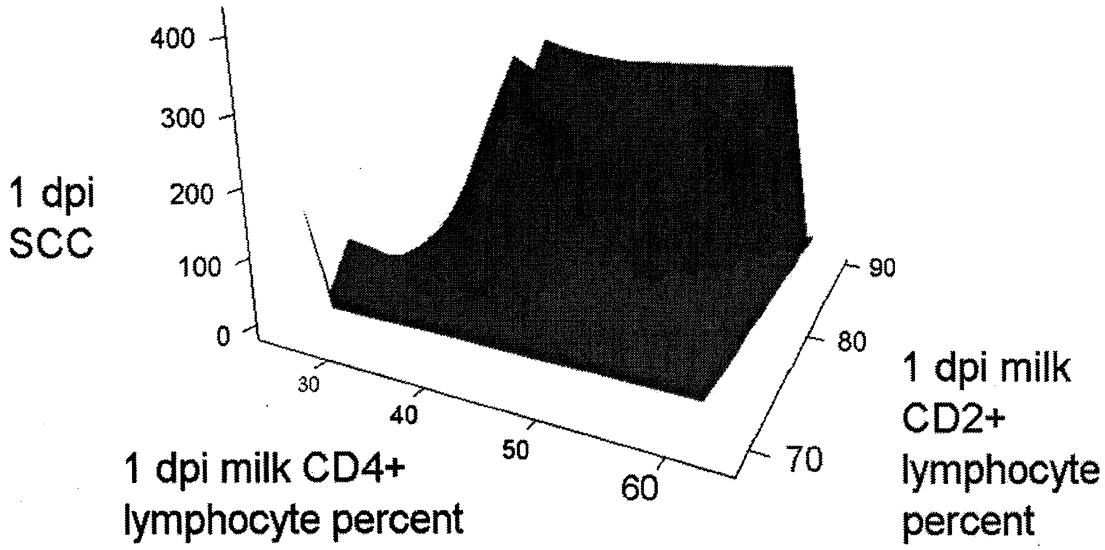
Fig 4



562
563

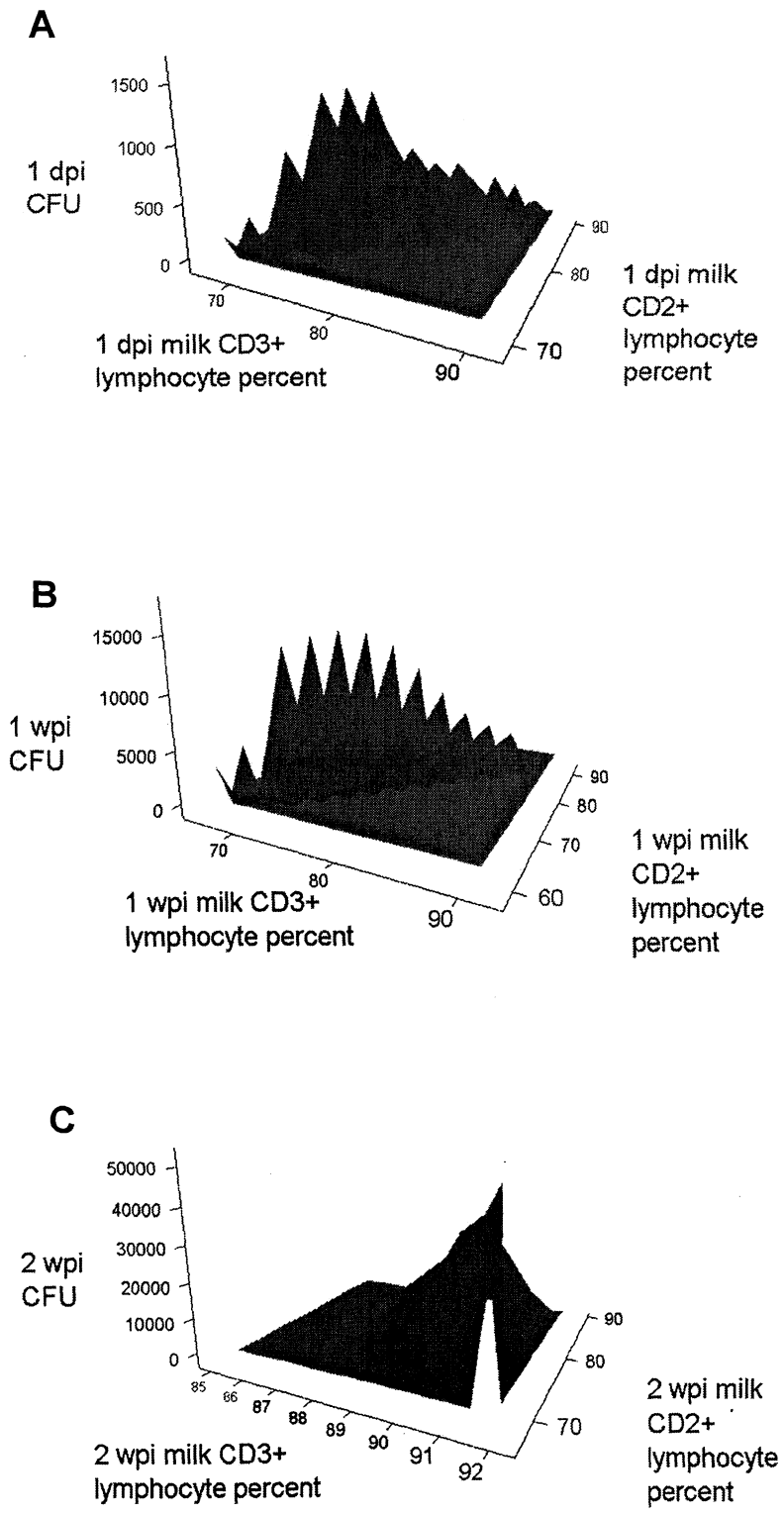
B


1 dpi
SCC
400
300
200
100
0
30
40
50
60
1 dpi milk CD4+
lymphocyte percent
90
80
70
1 dpi milk
CD2+
lymphocyte
percent

564

565
566

Fig 5

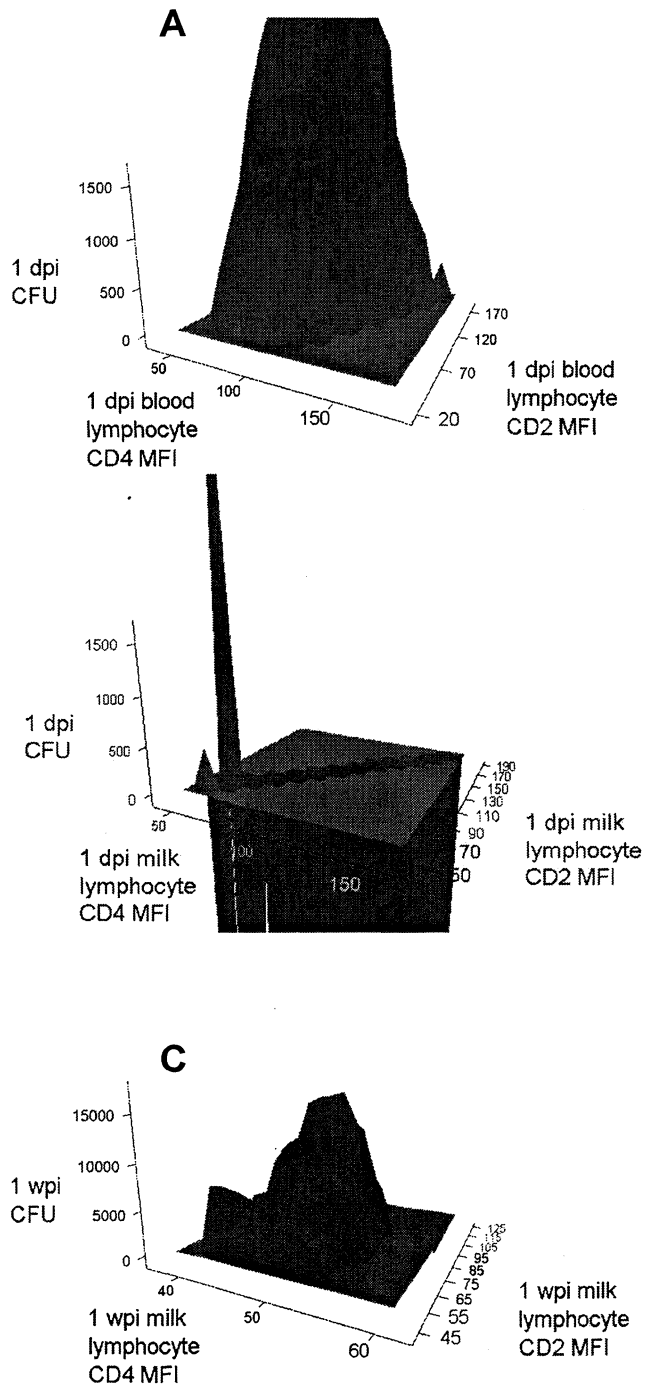


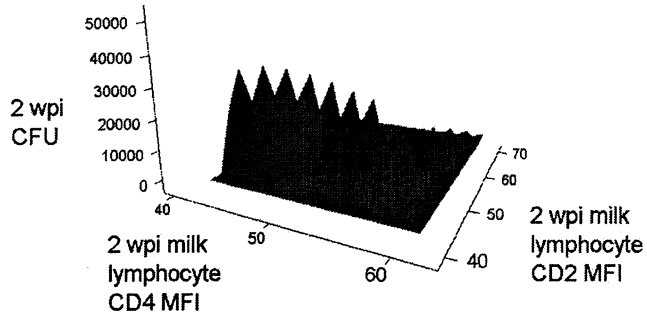
567

568

569

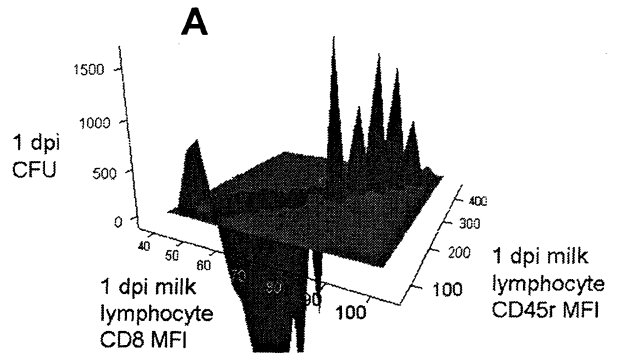
Fig 6

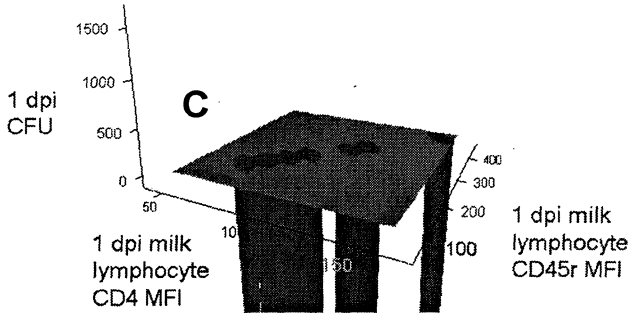

A
B
1500
1000
500
0
1 dpi
CFU
50
100
150
1 dpi blood
lymphocyte
CD4 MFI
170
120
70
20
1 dpi blood
lymphocyte
CD2 MFI
1500
1000
500
0
1 dpi
CFU
50
100
150
1 dpi milk
lymphocyte
CD4 MFI
190
170
150
130
110
90
70
50
1 dpi milk
lymphocyte
CD2 MFI
C
D
15000
10000
5000
0
1 wpi
CFU
40
50
60
1 wpi milk
lymphocyte
CD4 MFI
125
115
105
95
85
75
65
55
45
1 wpi milk
lymphocyte
CD2 MFI

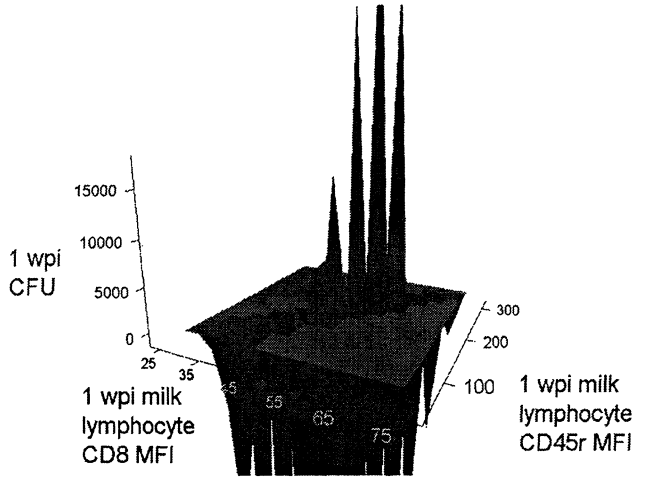

50000
40000
30000
20000
10000
0
2 wpi
CFU
40
50
60
2 wpi milk
lymphocyte
CD4 MFI
70
60
50
40
2 wpi milk
lymphocyte
CD2 MFI

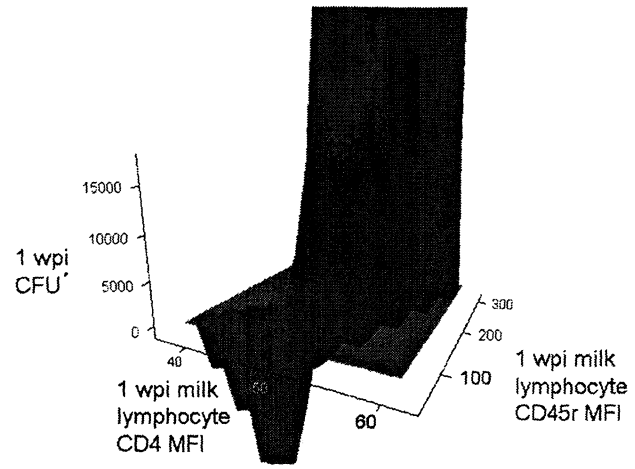
580

Fig 7


A
1500
1000
500
0
1 dpi
CFU
40
50
60
1 dpi milk
lymphocyte
CD8 MFI
100
200
300
400
1 dpi milk
lymphocyte
CD45r MFI


B
1500
1000
500
0
1 dpi
CFU
C
50
1 dpi milk
lymphocyte
CD4 MFI
100
150
200
300
400
1 dpi milk
lymphocyte
CD45r MFI

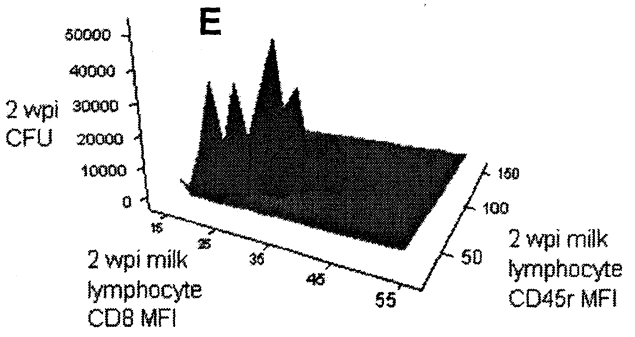

D
15000
10000
5000
0
1 wpi
CFU
25
35
55
65
75
1 wpi milk
lymphocyte
CD8 MFI
100
200
300
1 wpi milk
lymphocyte
CD45r MFI



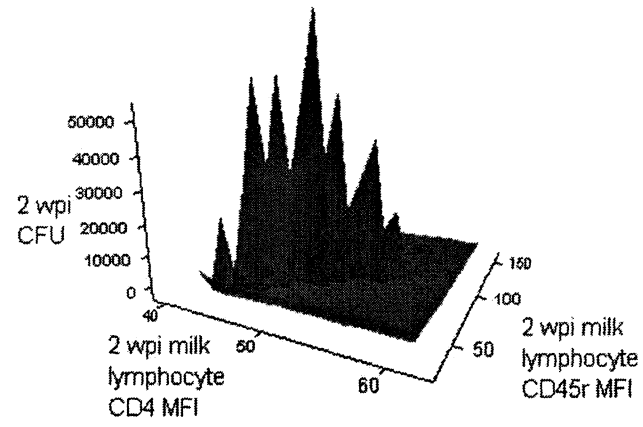
588

E

F

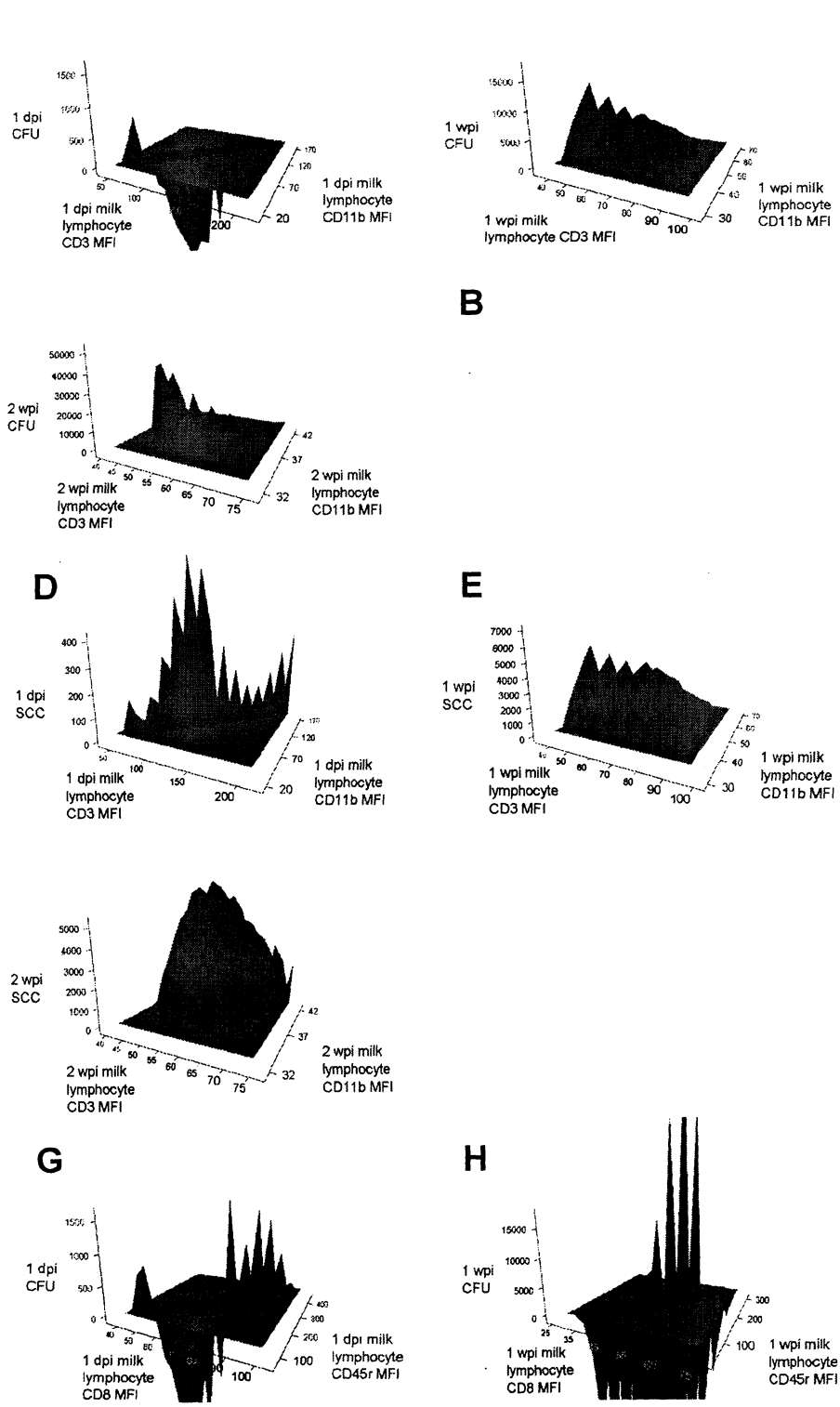


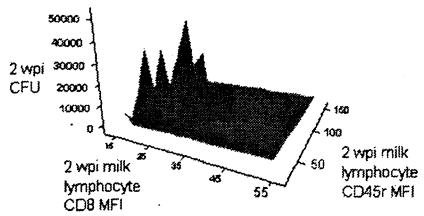
589



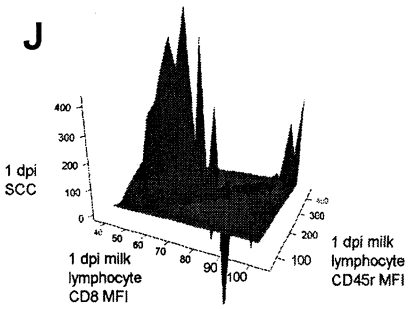
590

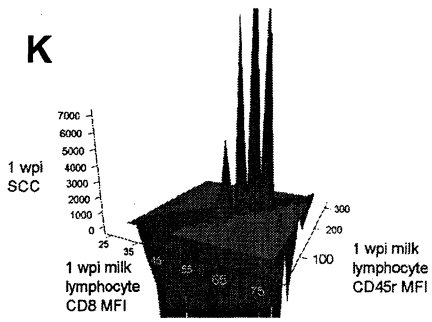
Fig 8


A
1 dpi CFU
1 dpi milk lymphocyte CD3 MFI
1 dpi milk lymphocyte CD11b MFI
B
1 wpi CFU
1 wpi milk lymphocyte CD3 MFI
1 wpi milk lymphocyte CD11b MFI
C
2 wpi CFU
2 wpi milk lymphocyte CD3 MFI
2 wpi milk lymphocyte CD11b MFI
D
1 dpi SCC
1 dpi milk lymphocyte CD3 MFI
1 dpi milk lymphocyte CD11b MFI
E
1 wpi SCC
1 wpi milk lymphocyte CD3 MFI
1 wpi milk lymphocyte CD11b MFI
F
2 wpi SCC
2 wpi milk lymphocyte CD3 MFI
2 wpi milk lymphocyte CD11b MFI
G
1 dpi CFU
1 dpi milk lymphocyte CD8 MFI
1 dpi milk lymphocyte CD45r MFI
H
1 wpi CFU
1 wpi milk lymphocyte CD8 MFI
1 wpi milk lymphocyte CD45r MFI
I

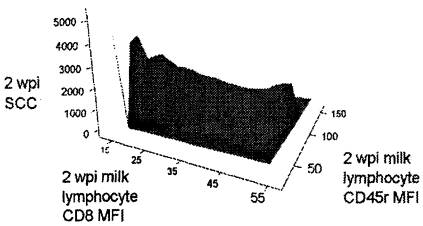

2 wpi
CFU
2 wpi milk
lymphocyte
CD8 MFI
2 wpi milk
lymphocyte
CD45r MFI

600


J
1 dpi
SCC
1 dpi milk
lymphocyte
CD8 MFI
1 dpi milk
lymphocyte
CD45r MFI

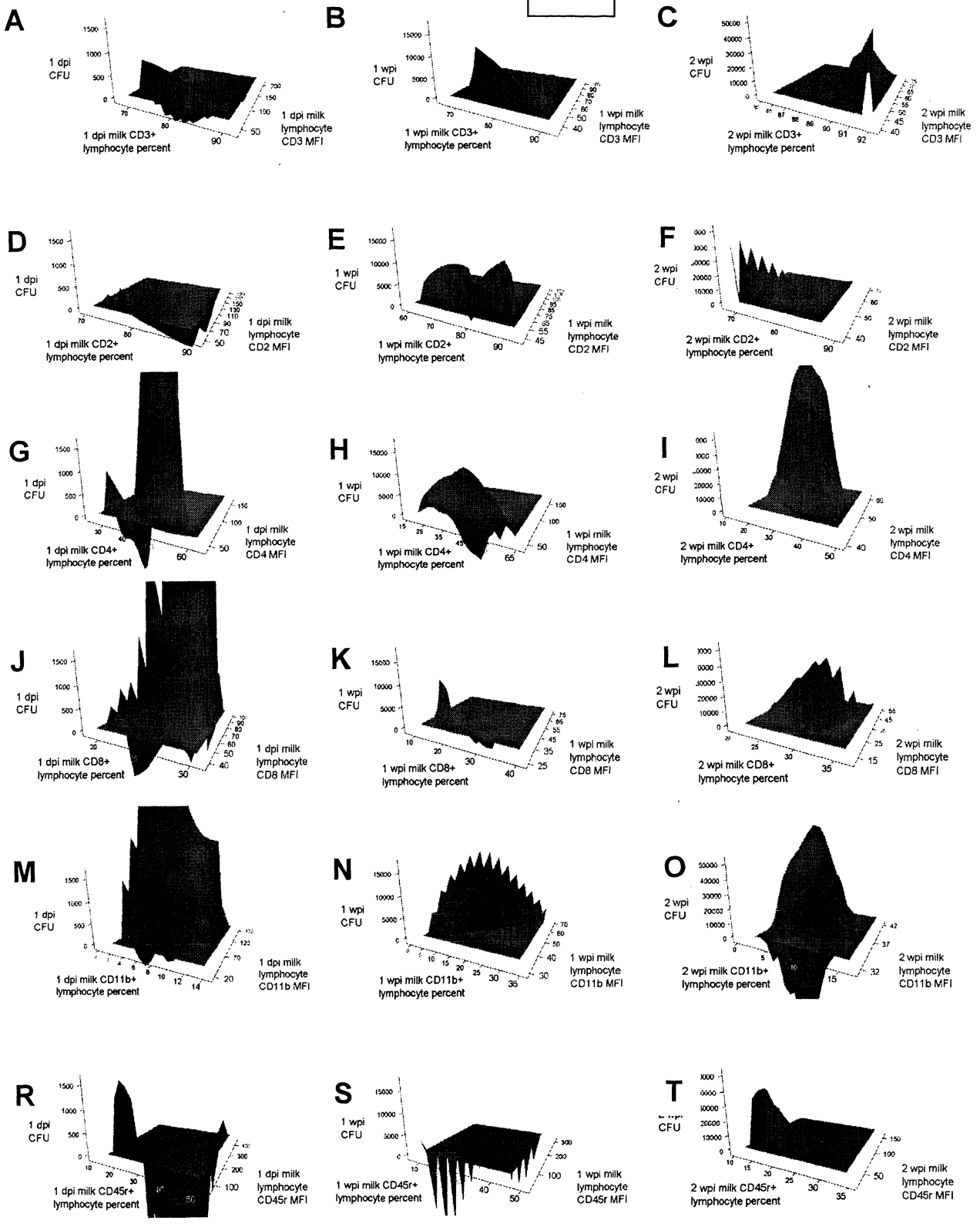

K
L
1 wpi
SCC
1 wpi milk
lymphocyte
CD8 MFI
1 wpi milk
lymphocyte
CD45r MFI

601


2 wpi
SCC
2 wpi milk
lymphocyte
CD8 MFI
2 wpi milk
lymphocyte
CD45r MFI

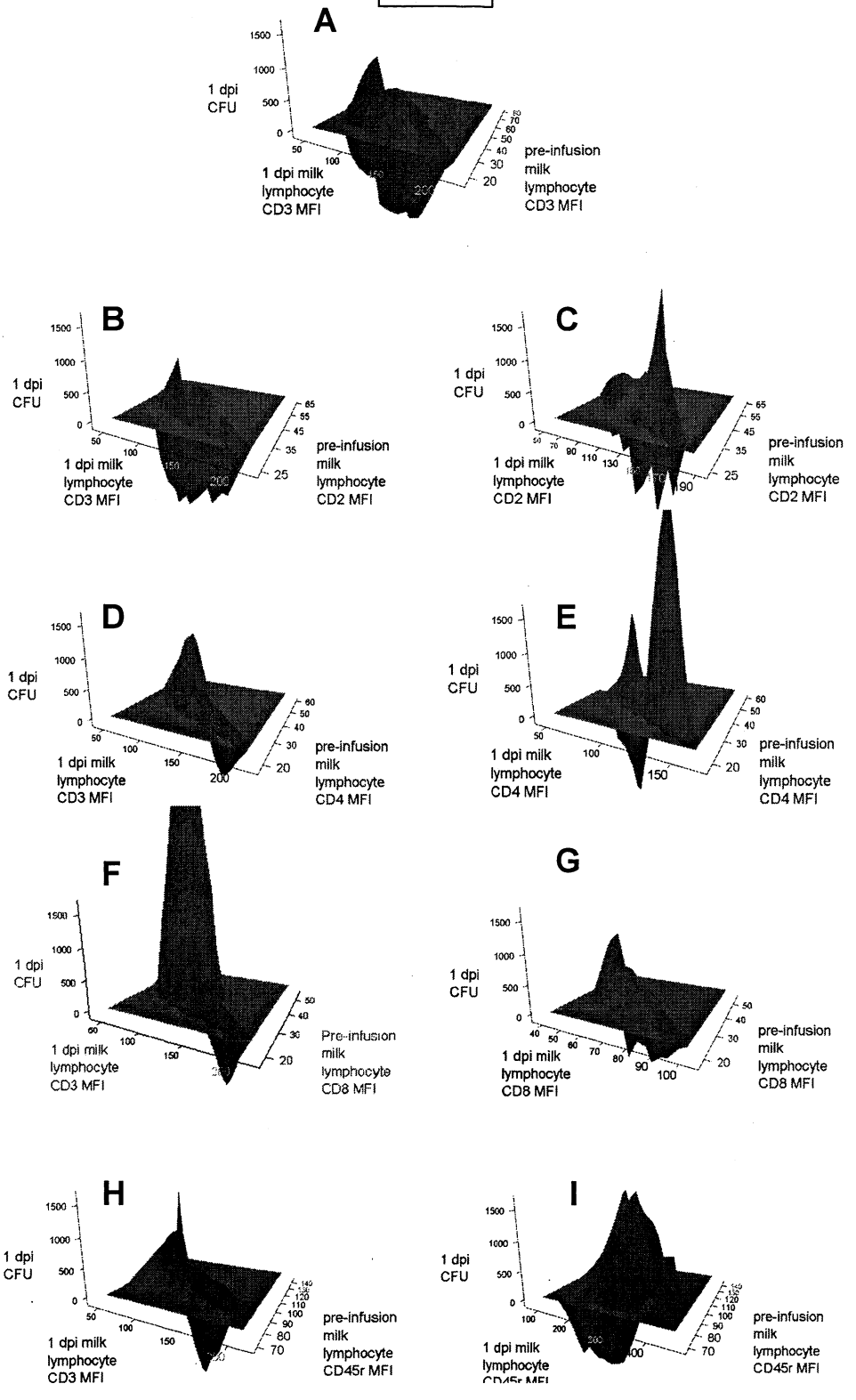
602

Fig 9


A
1 dpi CFU
1 dpi milk CD3+ lymphocyte percent
1 dpi milk lymphocyte CD3 MFI
B
1 wpi CFU
1 wpi milk CD3+ lymphocyte percent
1 wpi milk lymphocyte CD3 MFI
C
2 wpi CFU
2 wpi milk CD3+ lymphocyte percent
2 wpi milk lymphocyte CD3 MFI
D
1 dpi CFU
1 dpi milk CD2+ lymphocyte percent
1 dpi milk lymphocyte CD2 MFI
E
1 wpi CFU
1 wpi milk CD2+ lymphocyte percent
1 wpi milk lymphocyte CD2 MFI
F
2 wpi CFU
2 wpi milk CD2+ lymphocyte percent
2 wpi milk lymphocyte CD2 MFI
G
1 dpi CFU
1 dpi milk CD4+ lymphocyte percent
1 dpi milk lymphocyte CD4 MFI
H
1 wpi CFU
1 wpi milk CD4+ lymphocyte percent
1 wpi milk lymphocyte CD4 MFI
I
2 wpi CFU
2 wpi milk CD4+ lymphocyte percent
2 wpi milk lymphocyte CD4 MFI
J
1 dpi CFU
1 dpi milk CD8+ lymphocyte percent
1 dpi milk lymphocyte CD8 MFI
K
1 wpi CFU
1 wpi milk CD8+ lymphocyte percent
1 wpi milk lymphocyte CD8 MFI
L
2 wpi CFU
2 wpi milk CD8+ lymphocyte percent
2 wpi milk lymphocyte CD8 MFI
M
1 dpi CFU
1 dpi milk CD11b+ lymphocyte percent
1 dpi milk lymphocyte CD11b MFI
N
1 wpi CFU
1 wpi milk CD11b+ lymphocyte percent
1 wpi milk lymphocyte CD11b MFI
O
2 wpi CFU
2 wpi milk CD11b+ lymphocyte percent
2 wpi milk lymphocyte CD11b MFI
R
1 dpi CFU
1 dpi milk CD45r+ lymphocyte percent
1 dpi milk lymphocyte CD45r MFI
S
1 wpi CFU
1 wpi milk CD45r+ lymphocyte percent
1 wpi milk lymphocyte CD45r MFI
T
CFU
2 wpi milk CD45r+ lymphocyte percent
2 wpi milk lymphocyte CD45r MFI

609

Fig 10



610

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612

613

614

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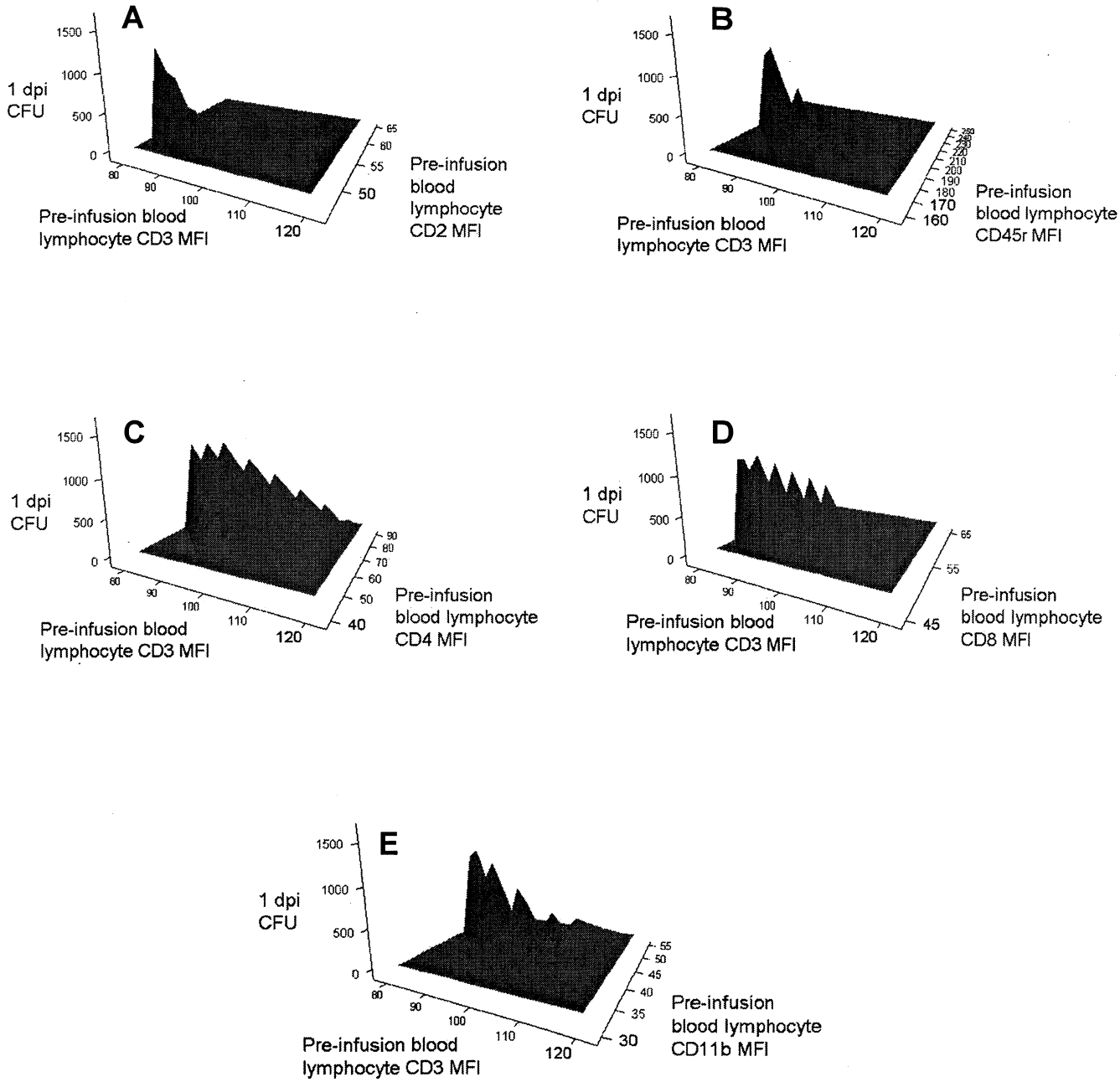
Fig 11



616

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619

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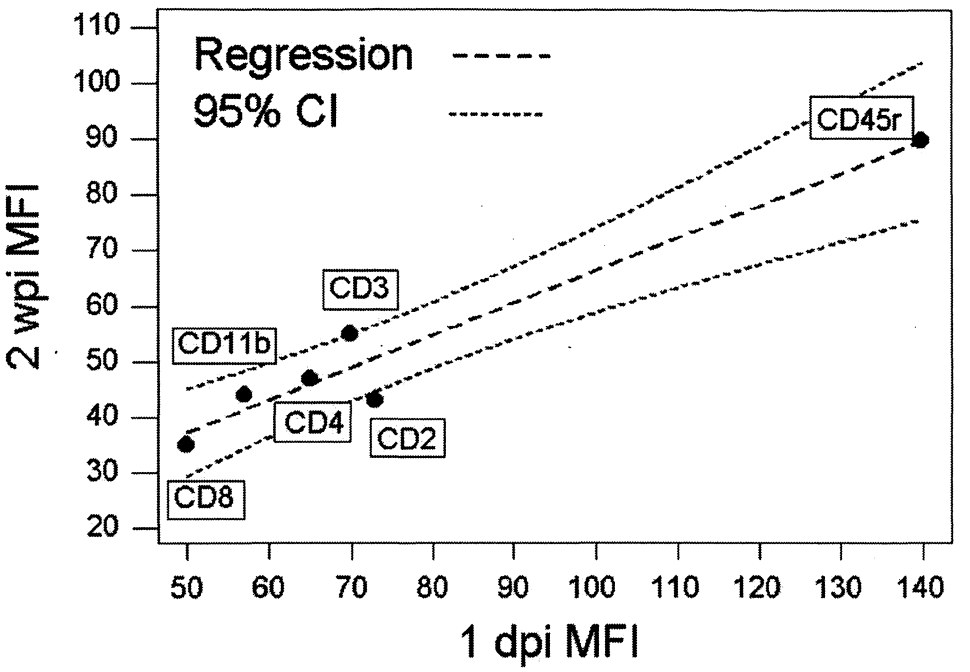
621
622

Fig 12



623