

## Special Feature

# T cell activation: *in vivo* veritas

BARBARA FAZEKAS DE ST. GROTH, ADRIAN L SMITH and CAROLINE A HIGGINS

*Centenary Institute of Cancer Medicine and Cell Biology, Newtown, NSW 2042, Australia*

**Summary** Phenotypic changes in CD4<sup>+</sup> T cells undergoing antigen-dependent activation were compared *in vivo* and *in vitro*. The most obvious difference was in expression of CD25, the alpha chain of the high affinity receptor for IL-2. High level expression of CD25 *in vivo* is restricted to a small fraction of the cells at the leading edge of the cell division profile, whereas all activated cells express high levels of CD25 in cultures responding to antigen. Because IL-2 is known to upregulate expression of CD25 in preactivated T cells, this suggests a difference in IL-2 exposure in the two responses. A number of other markers, including CD54, show a similar difference in the pattern of expression *in vivo* and *in vitro*. Using 6-colour flow cytometry, it was demonstrated that the small percentage of cells expressing CD25 *in vivo* coexpresses a very high level of a number of other activation markers, including CD38, CD44 and Ly-6A/E, suggesting that these may also be upregulated by autocrine IL-2.

**Key words:** activation, T cells, thymus.

### Introduction

T cells undergo a wide range of phenotypic and functional changes upon activation. The advent of genomic and proteomic analysis has generated an increasingly complex and detailed data set relating to activation-induced changes in gene transcription and protein expression within lymphocytes, and this has stimulated a renewed effort to rigorously define the process of T cell activation at a cellular and biochemical level.<sup>1</sup> Such efforts will inevitably increase the level of detail in our knowledge of possible T cell activation states. Our ability to understand what *does* happen, rather than what *can* happen, during immune responses will assume greater importance with the unrelenting increase in available information. In particular, in order to reap the benefits of our new knowledge to design effective new treatments for immunological conditions, it will be essential to fully understand immune responses *in vivo*, and to avoid the pitfall of generalization on the basis of *in vitro* data.

### The basic program of activation-induced changes

Activation-induced changes affect many classes of molecules within CD4<sup>+</sup> T cells including, but not restricted to, those involved in survival, cell cycle control, adhesion and migration, and effector function. While the expression of some molecules is irreversibly altered by T-cell activation, others undergo repetitive up- and downregulation. In mature T cells, these changes are linked to the receipt of a TCR-dependent signal, and can be ordered in a hierarchy that reflects the

strength of the TCR-dependent signal required to induce their expression (Smith and Fazekas de St. Groth, unpubl.). Thus, upregulation of CD69, one of the earliest and most sensitive measures of antigen recognition in the periphery, can be achieved with a lower antigen exposure than upregulation of CD44 or CD38. CD25 lies intermediate between these two extremes.

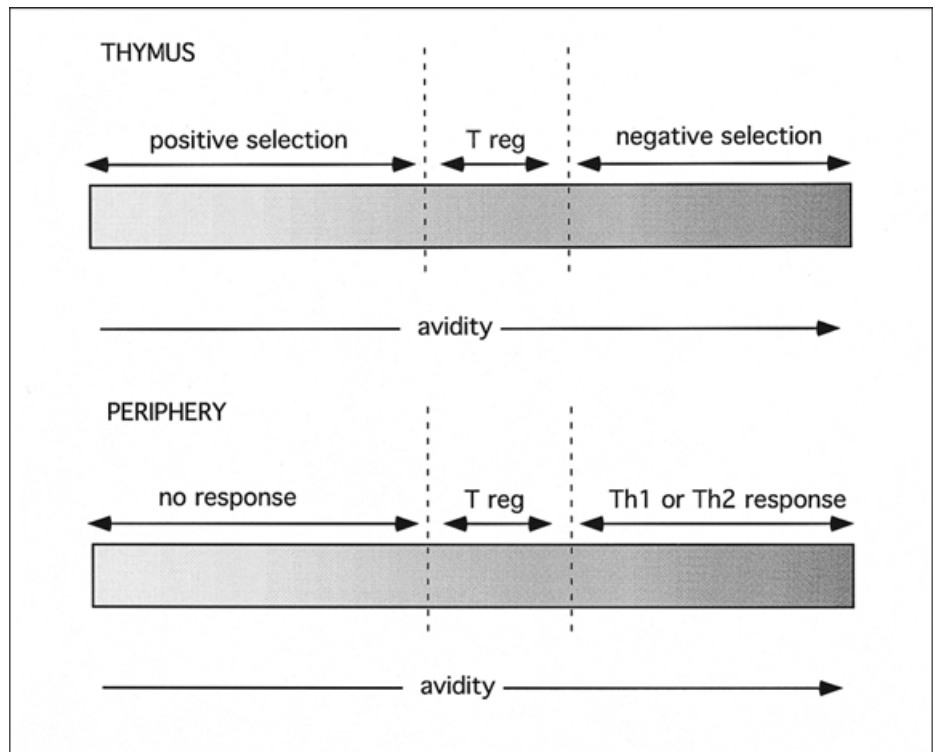
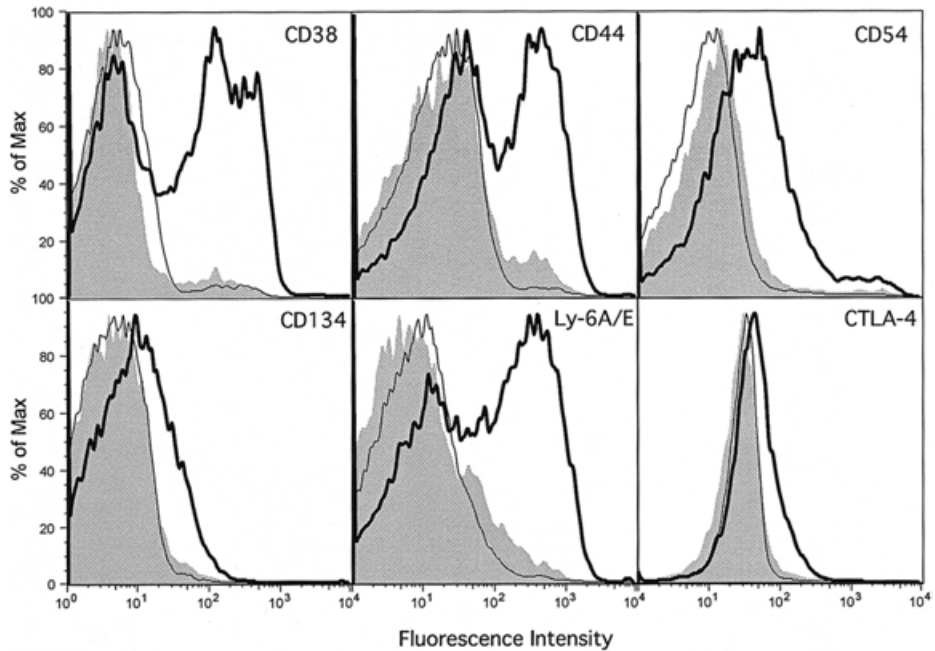
In the thymus, molecules such as CD44, CD25, CD38, CD54 and Ly-6A/E are transiently transcribed as part of the pro- and pre-T-cell development program.<sup>2</sup> At this stage, they are clearly independent of TCR signalling, although dependent on at least some of the same transcription factors, such as members of the NF- $\kappa$ B family. Once TCR rearrangement has been achieved, thymic selection on the basis of specificity is also linked to expression of activation markers. Interestingly, the hierarchy of marker expression as a function of strength of TCR signal, mentioned above, is also seen in the thymus. Thus, transient expression of CD69 is associated with positive selection in the thymus, believed to require only a very low level of TCR-dependent signalling.<sup>3</sup> The phenotypic accompaniments of *in vivo* negative selection, which requires a higher TCR-dependent signal,<sup>4,5</sup> are not as clearly established, principally because the majority of such cells die rapidly. However, the study of thymic selection in double transgenic models, in which cells can survive a negative selection signal by losing expression of their autoreactive transgenic TCR, indicates that negative selection appears to turn on a number of classical 'activation markers' that are associated with TCR signals of higher intensity. These markers include CD38, CD44, CD54 and Ly-6A/E (Fig. 1). Recently it has been demonstrated that differentiation to a regulatory phenotype in the thymus is also associated with acquisition of activation markers, in particular CD25.<sup>6–9</sup> While there is still some controversy about the avidity of signals mediating selection into the CD25<sup>+</sup> compartment in the thymus, the majority of reports are consistent with the view that the signal lies between those that result in positive and negative selection, respectively (Fig. 2).

Correspondence: Dr Barbara Fazekas de St. Groth, Locked Bag No.6, Newtown, NSW 2042, Australia.

Email: B.Fazekas@centenary.usyd.edu.au

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**Figure 1** Expression of activation markers by CD4<sup>+</sup> thymocytes in double transgenic mice expressing both a transgenic TCR and its ligand as a neoself antigen. 6-colour flow cytometry data were collected using a FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA, USA) and analysed using FlowJo software (Treestar, San Carlos, CA, USA). Data were gated for forward/side scatter, expression of CD4 and lack of expression of CD8, and exclusion of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Histogram overlays show marker expression by CD4<sup>+</sup> cells from double transgenic (bold line), TCR transgenic (fine line) and antigen transgenic mice (filled profiles).



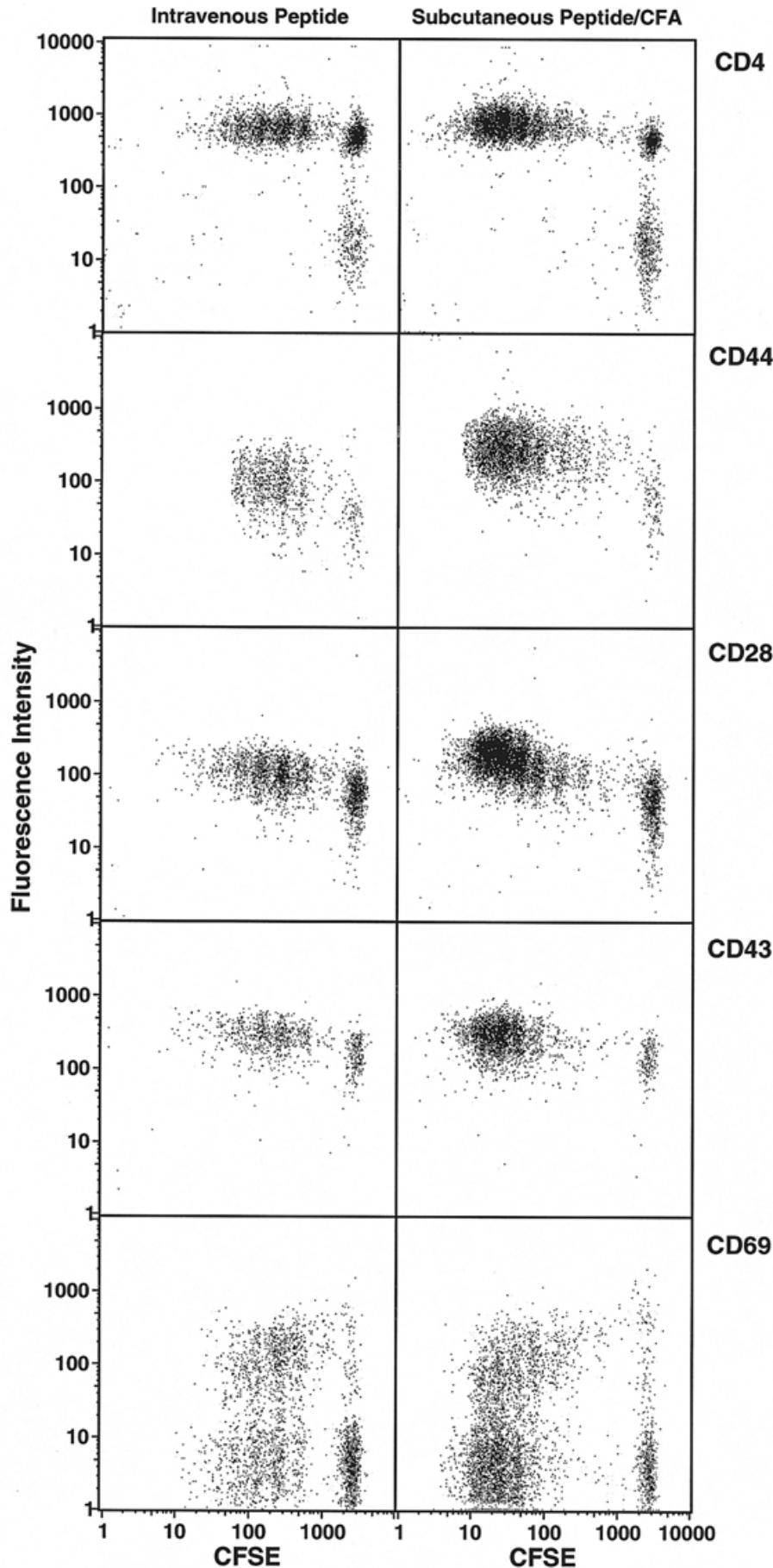
**Figure 2** Postulated avidity constraints for generation of antigen-specific CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells in the thymus vs. the peripheral lymphoid tissues.

**Peripheral T-cell activation**

While the basic pattern of activation-induced changes is probably common to all T-cell activation in the periphery, the subtleties remain to be defined. It is often assumed that this basic pattern will accord with that seen *in vitro*. To examine this hypothesis, we have made a detailed study of CD4<sup>+</sup> T-cell activation *in vivo* and *in vitro*, measuring the phenotypic changes detected by flow cytometric analysis using monoclonal

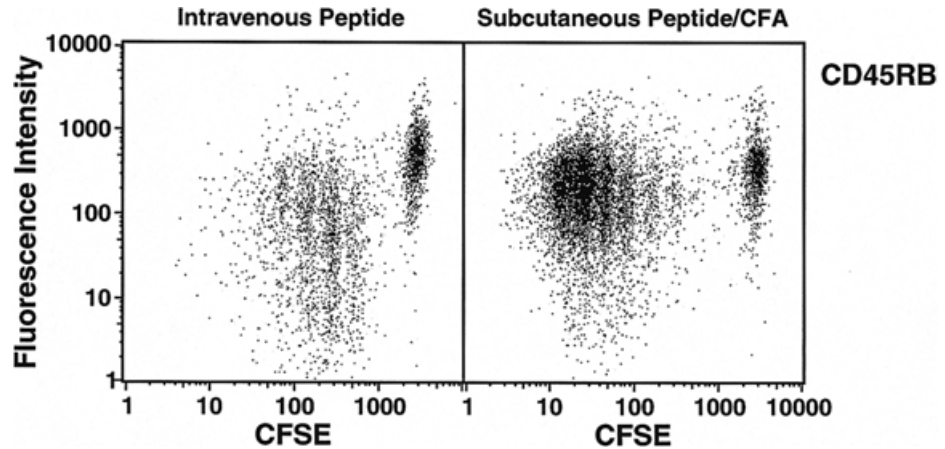
antibodies in combination with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE).

An additional aim of this study was to define phenotypic correlates of T-cell fate *in vivo*, using the 5C.C7 TCR transgenic model specific for moth cytochrome C peptide (MCC) residues 87–103 in association with IE<sup>k</sup>.<sup>10</sup> By comparing the response of antigen-specific CD4<sup>+</sup> TCR transgenic T cells to immunogenic versus tolerogenic stimuli, we had hoped to uncover phenotypic differences between T cells destined for



**Figure 3** Surface molecules showing similar regulation in both intravenous and subcutaneous responses.  $8 \times 10^6$  CFSE-labelled 5C.C7 TCR transgenic lymph node cells were adoptively transferred to CD45.1 congenic non-transgenic recipients. Recipients were immunized either intravenously with 10  $\mu$ g moth cytochrome C peptide (MCC) peptide (residues 87–103) or subcutaneously with 10  $\mu$ g MCC peptide emulsified in CFA. Lymph nodes (or draining lymph nodes, in the case of subcutaneous immunization) were collected 1, 2, 3, 4 or 17 days after immunization, 5-colour flow cytometry was performed using a FacstarPlus flow cytometer and data analysed using FlowJo software. Data were gated for forward/side scatter, expression of CD4, lack of expression of CD45.1 and exclusion of propidium iodide. Day 3 profiles are shown as dot-plots of CFSE vs. surface marker.

**Figure 4** Surface molecules down-regulated during or after the first cell division *in vivo*. Experiment as described in the legend to Fig. 3.



deletion (in response to intravenous peptide) and those destined to acquire effector/memory function (in response to subcutaneous peptide emulsified in CFA).<sup>11</sup> Such differences would allow us to predict the fate of activated T cells by analysing their phenotype, saving considerable time and resources in our attempt to define the *in vivo* consequences of encounter with different types of dendritic cells.<sup>12–14</sup> Although contact with intravenous versus subcutaneous antigen leads to fundamentally different outcomes, no phenotypic differences predictive of fate have been convincingly documented in the literature to date. To test whether the *in vivo* phenotypes corresponded with those seen *in vitro*, CFSE-labelled CD4<sup>+</sup> TCR transgenic T cells were also stimulated with specific peptide *in vitro*.

#### Phenotypic differences between effector/memory cells and those destined for deletion

Antigen-specific cells responding to intravenous versus subcutaneous peptide share many phenotypic and functional features. One of the most fundamental is entry into cell division. The division profiles of CFSE-labelled CD4<sup>+</sup> T cells are remarkably similar in the two responses during the early period of rapid proliferation (days 1–3).<sup>11,15</sup> At later times, cells responding to intravenous peptide stop dividing, while those responding to subcutaneous peptide continue to divide.

Virtually every surface marker for which an antibody is available can be demonstrated to undergo changes in level of expression as a result of *in vivo* activation. In the experiments described here, we studied the expression of a panel of 15 markers at days 1, 2, 3, 4 and 17 after immunization with soluble intravenous peptide or subcutaneous peptide emulsified in CFA. For the purposes of this review, only the profiles from day 3 will be shown. They are grouped according to their pattern of expression over the course of cell division.

#### Surface molecules with similar regulation in both intravenous and subcutaneous responses

Five of the 15 markers show very similar expression patterns in the two responses (Fig. 3). A small but consistent increase in expression of CD4 is a hallmark of all acute antigen responses *in vivo*. This phenotype has been used to isolate activated antigen-specific cells directly *ex vivo*.<sup>16</sup> In the

lymphoid tissues, the level returns to normal as activated cells rest down to become memory cells.

Undivided cells express increased levels of CD44 within 24 h of stimulation (not shown), and the degree of upregulation increases over the course of the first 2–3 cell divisions. Because expression remains elevated on all antigen-experienced cells, CD44 is often used to differentiate naive from activated/memory cells.<sup>17</sup> Unfortunately, naive CD4<sup>+</sup> T cells already express intermediate levels of CD44, so that the degree of activation-dependent upregulation for CD4<sup>+</sup> T cells is smaller than for CD8<sup>+</sup> T cells. Hence, it is not always possible to make a clean separation between naive and activated/memory cells on the basis of CD44 expression.

Like CD44, the level of CD28 increases over the course of cell division and remains elevated on memory/effector T cells. CD43 shows a similar pattern.

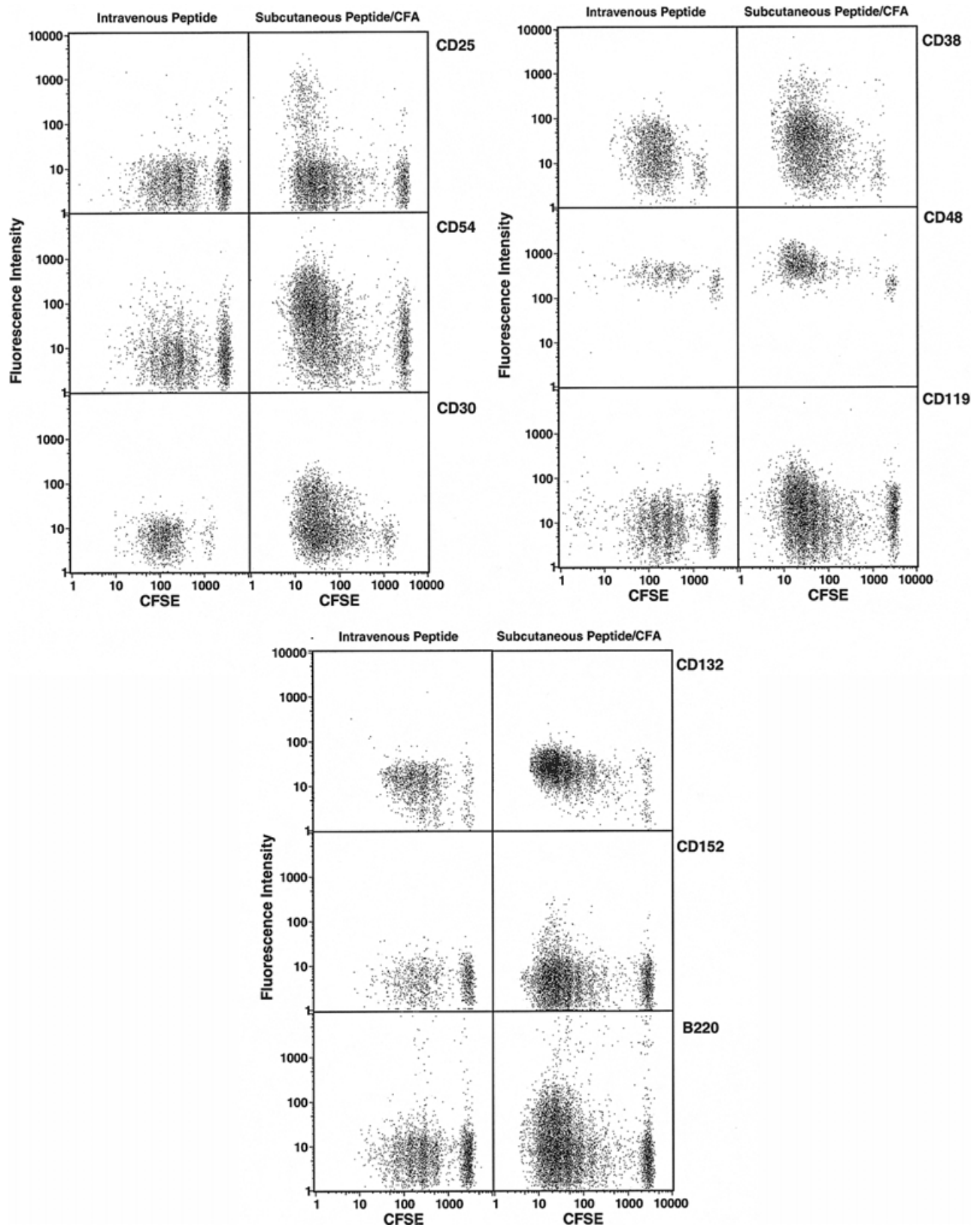
CD69 is expressed only transiently after activation and CD4<sup>+</sup> T cells migrating into the bloodstream are uniformly CD69 negative.<sup>18</sup> Our data indicate that every CD4<sup>+</sup> T cell destined to divide will express CD69, whereas the inverse is not true in that, at low antigen doses, a significant number of T cells express CD69 but never undergo cell division (not shown).

#### Surface molecules downregulated during or after the first cell division

CD45RB shows a complex pattern of regulation during *in vivo* responses (Fig. 4). Although the downregulation in response to intravenous peptide is consistent with the published data (derived mainly from *in vitro* studies), the pattern in response to subcutaneous peptide is more complex. An early decrease on days 1–2 after immunization (not shown) is followed by an increase. The average level within the divided population several weeks after immunization is reduced, although divided cells can still be found within the CD45RB<sup>hi</sup> gate (not shown).

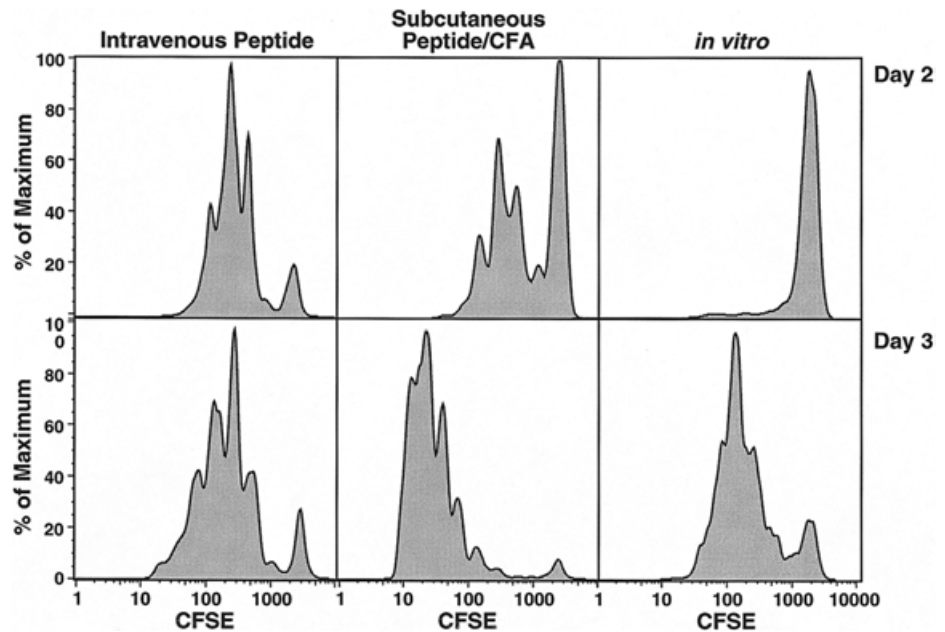
#### Surface molecules showing differential regulation in intravenous versus subcutaneous responses

CD25 is expressed early (day 1–2) by most cells responding to either intravenous or subcutaneous peptide (not shown), but expression is rapidly lost so that by day 3 there is



**Figure 5** Surface molecules showing differential regulation in intravenous vs. subcutaneous responses. Experiment as described in the legend to Fig. 3.

**Figure 6** Comparison of cell division profiles *in vivo* and *in vitro*. *In vivo* experiment as described in the legend to Fig. 3. *In vitro* cultures set up at  $10^6$  CFSE-labelled lymph node cells per ml in RPMI supplemented with 10% FCS,  $5 \times 10^{-6}$  M 2-ME, 2 mmol/L l-glutamine and 10  $\mu$ mol/L MCC peptide. Cultures were harvested on days 2 or 3, and 4-colour flow cytometry was performed using a FacstarPlus flow cytometer and data analysed using FlowJo software. Data were gated for forward/side scatter, expression of CD4 and the transgenic TCR and exclusion of propidium iodide.



surprisingly little expression in either response (Fig. 5). Expression of CD25 is known to be controlled by a combination of cell intrinsic and extrinsic factors (reviewed in <sup>19</sup>). Of the latter, exogenous IL-2, acting via the IL-2 receptor and STAT-5a, is one of the most potent.<sup>20</sup> Exposure to exogenous IL-2 is required to upregulate expression of CD25 to a level that is easily detected by antibody staining. In the subcutaneous response, such high level expression of CD25 is restricted to a fraction of the cells at the leading edge of the cell division profile, and the pattern of CD25 expression is very similar to that of cells that synthesize IL-2.<sup>15</sup> This suggests that the same cells both make and respond to IL-2 *in vivo*, via an autocrine loop, and that paracrine access to IL-2 is more limited. Because CD4<sup>+</sup> T cells are clustered around dendritic cells during this time, the data suggest that IL-2 is effectively sequestered within the immunological synapse, limiting its potential to mediate bystander effects.

The pattern of CD54 upregulation is similar to that of CD25, although it involves a larger proportion of cells, both in terms of the number of positive cells within each cell division peak, and the number of peaks manifesting the 'leading edge' pattern. A similar pattern is seen for CD30, CD38, CD48, CD119 (the IFN- $\gamma$  receptor  $\gamma$  chain), CD132 (the common cytokine receptor  $\gamma$  chain), CD152 (CTLA-4, whose expression is known to reflect the strength of TCR-mediated signalling),<sup>21</sup> and B220. Taken together, these data and the IL-2 synthesis data mentioned in the preceding paragraph suggest that strength of signal *in vivo* is greatest at the leading edge of the cell division profile and that the level of expression of all these markers reflects signal strength, either directly or indirectly.

### *In vivo* versus *in vitro*

#### *Proliferation*

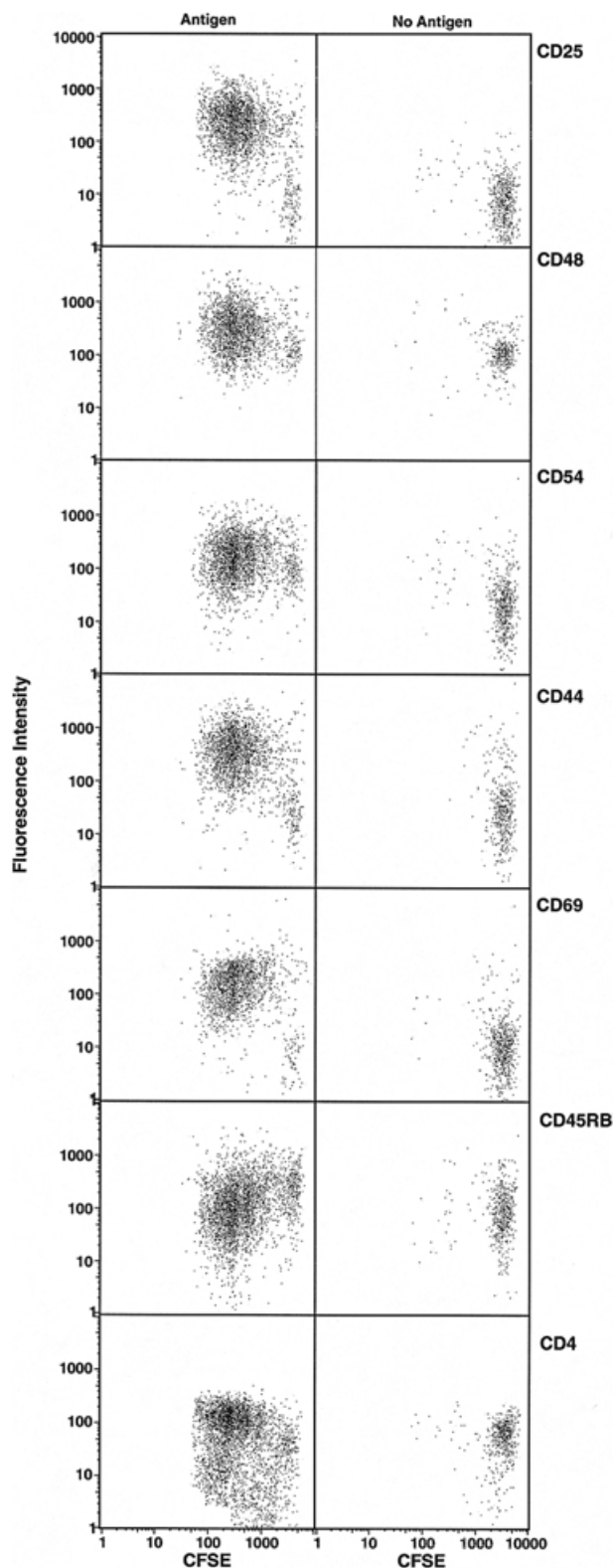
*In vitro* antigen-specific responses display different kinetics to those of *in vivo* responses. The time to the first cell division

is at least 24 h longer, and the cell cycle time is also considerably longer (Fig. 6). Even in the presence of saturating concentrations of antigen and IL-2, it is not possible to mimic the speed of the *in vivo* proliferative burst. Another crucial difference is the tendency for T cells to die *in vitro*, regardless of whether they have recognized antigen or not.<sup>22</sup> In contrast, calculation of the CFSE content of early *in vivo* responses (in response to either intravenous or subcutaneous antigen) indicates that very few cells die during the early proliferative burst.<sup>11,15</sup>

#### *Expression of activation markers*

*In vitro* expression of a subset of the markers described above is shown in Fig. 7. One of the most obvious differences between the phenotype of CD4<sup>+</sup> T cells undergoing activation *in vivo* and *in vitro* is in expression of CD25. The high level of expression on the majority of dividing T cells *in vitro* is in stark contrast to the situation *in vivo*, and provides indirect evidence that far more T cells are exposed to high IL-2 levels *in vitro* than *in vivo*, even when no exogenous IL-2 has been added to the cultures. This observation is consistent with the prominence of bystander or paracrine activation *in vitro*, and underlines the need for caution in the interpretation of *in vitro* data, particularly in relation to IL-2 dependence and the balance between the autocrine and paracrine pathways. Why IL-2 should reach unphysiological levels *in vitro* is not clear, although the absence of the normal degradative pathways for extracellular material may provide at least a partial explanation. Another possibility is that T cells synthesize supra normal amounts of IL-2 *in vitro*, due to the unphysiologically high availability of antigen.

A number of other markers whose expression displays the 'leading edge' pattern *in vivo* are also expressed at a uniformly high level *in vitro*. They include CD48 and CD54 (Fig. 7). Whether this difference reflects higher exposure to IL-2 *in vitro* is not clear. Other differences are the tendency



**Figure 7** Antigen-dependent expression of surface molecules by transgenic T cells stimulated *in vitro*. Cultures were set up as described in the legend to Fig. 6. MCC peptide was omitted from control cultures.

to lose expression of CD4 on some activated cells, and less prominent downregulation of CD69 (Fig. 7).

### Correlations with expression of CD25 *in vivo*

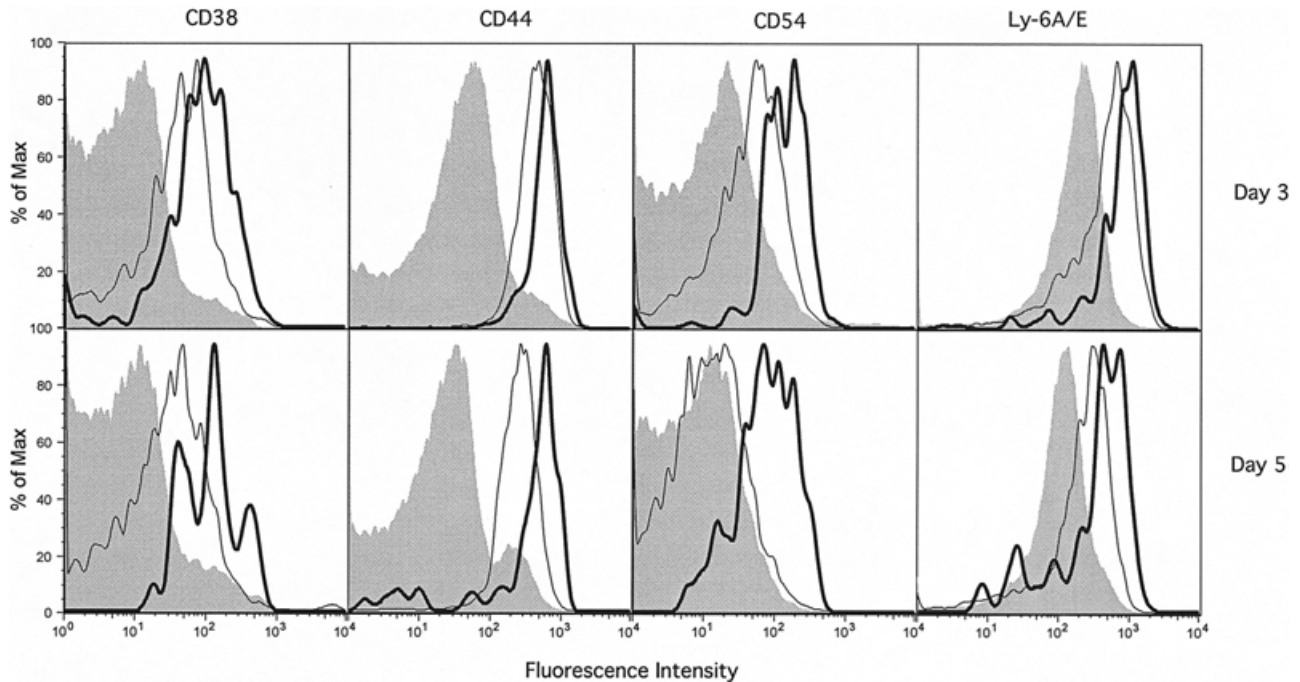
The uniformly high level of expression of CD25, CD48 and CD54 *in vitro* suggests that expression of all the 'leading edge' activation markers (Fig. 5) might be augmented by IL-2/Stat5a-dependent signals. The data presented in Figure 5 suggests a correlation between expression of CD25 and the other markers, but a direct demonstration that they are coexpressed by individual cells responding to specific antigen requires the use of six colour flow cytometry (Fig. 8). By gating on proliferating antigen-specific T cells that do or do not express CD25, it is possible to test whether *in vivo* expression of CD25 is predictive of increased expression of other activation markers. Figure 8 indicates a strong correlation between expression of CD25 and that of CD38, CD44, CD54 and Ly6A/E on days 3 and 5 of an acute response to subcutaneous antigen/CFA *in vivo*. This effect was particularly marked on day 5 of the response, when fewer than 1% of responding cells expressed CD25. Whether the expression of all these markers is linked to the autocrine IL-2 loop, or whether expression of some or all of them is linked directly to the same stimuli that drive IL-2 synthesis, remains to be established. In contrast, expression of CD4, CD45RB and CD62L showed no correlation with expression of CD25 in the acute antigen-specific response (not shown).

### Conclusions

This analysis of marker expression during antigen-specific *in vivo* and *in vitro* responses indicates significant differences, particularly with regard to expression of CD25 and a number of markers whose upregulation *in vivo* correlates with CD25 expression. Significant numbers of antigen-experienced, dividing CD4<sup>+</sup> T cells that do not express high levels of CD25 fall outside the 'activated' phenotype defined by *in vitro* analysis. Our data indicate that even expression of CD44, one of the most useful indicators of previous exposure to antigen, is modulated significantly by recent antigen exposure. These findings are consistent with previous reports underlining the complexity of transcriptional regulation during the transition from activation to memory within the CD8<sup>+</sup> T-cell compartment and indicate that many of the complexities of T-cell behaviour *in vivo* remain to be elucidated.

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**Figure 8** Comparison of surface phenotype of CD25<sup>+</sup> and CD25<sup>-</sup> T cells during the *in vivo* response of naive CD4<sup>+</sup> T cells to subcutaneous antigen.  $8 \times 10^6$  CFSE-labelled 5C.C7 TCR transgenic lymph node cells on a recombinase activating gene (RAG)-1<sup>-/-</sup> background were adoptively transferred to CD45.1 congenic non-transgenic recipients. Recipients were immunized subcutaneously with 10  $\mu$ g MCC peptide emulsified in CFA. Draining lymph nodes were collected 1, 3, 5 or 8 days after immunization, 6-colour flow cytometry was performed using a FACS Vantage flow cytometer and data analysed using FlowJo software. Data were gated for forward/side scatter, expression of CD4 and exclusion of DAPI. Histogram overlays show donor (CD45.1<sup>-</sup>) CD25<sup>+</sup> cells (bold line), donor (CD45.1<sup>-</sup>) CD25<sup>-</sup> cells (fine line) and host (CD45.1<sup>+</sup>) CD25<sup>-</sup> cells (filled profiles).

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