Regulation of Thymocyte Development through CD3: Functional Dissociation between p56\textsuperscript{ck} and CD3\(\zeta\) in Early Thymic Selection

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Summary

We studied the extent of functional linkage between CD3\(\zeta\) and p56\textsuperscript{ck} in pre-TCR-dependent thymocyte development. Differentiation of DN to DP cells was examined by treatment of RAG2/CD3\(\zeta\) and RAG1/p56\textsuperscript{ck} double-deficient mice with anti-CD3\(\zeta\) antibodies. The results suggest that CD3\(\zeta\) has no specific role in this maturation step, but may be important for amplification of signaling through the pre-TCR. In contrast, p56\textsuperscript{ck} is the main protein tyrosine kinase associated with signaling through the pre-TCR-CD3 complex. In DP thymocytes, the Ca\textsuperscript{2+} response to anti-CD3\(\zeta\) was totally abolished in CD3\(\zeta\)-null but only reduced in p56\textsuperscript{ck}-null mice, and in vivo responses to anti-CD3\(\zeta\) differed from one another. Thus, CD3\(\zeta\) and p56\textsuperscript{ck} are functionally not tightly associated and their deficiencies cause distinct developmental defects.

Introduction

T cells develop in the thymus, where they undergo multiple steps of differential gene expression and gene rearrangements. Cells having successfully completed one step are selected to mature to the next. At an early stage, defined by the expression of the interleukin 2-R\(\alpha\) (IL-2R\(\alpha\)) chain, thymocytes attempt to produce a functional T cell receptor \(\beta\) (TCR\(\beta\)) chain gene by random rearrangement of the TCR\(\beta\) V, D, and J gene segments (Spits, 1994). If rearrangement was successful, the pre-TCR is expressed on the cell surface, consisting of the TCR\(\beta\) chain, dimerized with the pre-TCR\(\alpha\) chain, and members of the CD3 complex (Groettrup et al., 1993; Groettrup and von Boehmer, 1993; Saint-Ruf et al., 1994). The surface expression of the pre-TCR, alone or by interaction with a ligand on thymic stroma cells, selects thymocytes with a functional TCR\(\beta\) chain to continue maturation. This results in allelic exclusion by arrest of further rearrangement of the TCR\(\beta\) locus, a burst of cell divisions, down-regulation of the IL-2R\(\alpha\) chain, and the expression of the coreceptor CD4 and CD8 (Groettrup and von Boehmer, 1993; Levelt and Eichmann, 1993). During the CD4+CD8\textsuperscript{+} double positive (DP) stage, rearrangements in the TCR\(\alpha\) locus take place. Thymocytes with a functionally rearranged TCR\(\alpha\) chain gene are then selected according to the specificity of their mature \(\alpha\) TCR (Kisielow et al., 1993; Rothenberg, 1994). Thymocytes that are potentially self-reactive are negatively selected and clonally deleted by apoptosis. Nonelected thymocytes also die. Thymocytes that are self-restricted are positively selected and continue maturation into the CD4+ single-positive (SP) or CD8+ SP cells depending on the restriction of their TCR. The difference between positive and negative selection is most probably dictated by quantitative parameters of the interactions between the TCR and the selecting ligands (Hogquist et al., 1994; Ashton-Rickardt et al., 1994), resulting in distinct signal intensities (Eichmann, 1995).

Mice that carry mutations leading to the failure to produce a TCR\(\beta\) chain, such as scid mice (Schuler et al., 1986), mice deficient for RAG1 (Mombaerts et al., 1992), RAG2 (Shinkai et al., 1992), or mice with a null mutation in the TCR\(\beta\) chain genes (Mombaerts et al., 1992), show a nearly complete block in the maturation of CD4+CD8\textsuperscript{+} double-negative (DN) thymocytes to the DP stage. Less severe deficiencies are observed in mice deficient for molecules that are involved in signal transduction through the TCR. For example, mice that are deficient for CD3\(\zeta\) (Liu et al., 1993; Ohno et al., 1993; Malissen et al., 1993; Love et al., 1993) have a small thymus, with 5-15% of the normal number of DP thymocytes. Furthermore, positive selection is impaired, with virtually no SP cells in the thymus. A phenotypically similar incomplete developmental block is observed in mice deficient for p56\textsuperscript{ck} (Molina et al., 1992). In contrast, mice that overexpress a dominant-negative form of Ick show a complete block of thymocyte development at the DN stage (Levelt et al., 1993), presumably because of competition for additional PTK substrates. PTKs are likely also to play a role in the regulation of allelic exclusion, as a TCR\(\beta\) transgenic does not lead to allelic exclusion in mice overexpressing dominant-negative Ick (Anderson et al., 1993).

In previous work, we have shown that cross-linking of CD3\(\zeta\) on DN thymocytes accelerated their maturation to DP thymocytes, whereas cross-linking of CD3\(\zeta\) on DP thymocytes induced their deletion (Levelt et al., 1993a). Moreover, cross-linking of CD3\(\zeta\) on DN thymocytes of TCR\(\beta\)-deficient mice restored their maturation to the DP stage (Levelt et al., 1993b). These studies suggested that signaling events through the CD3 complex controlled these early as well as the late selection events. Here, we analyzed mice deficient for CD3\(\zeta\) and for Ick, asking the question as to what extent these components of CD-mediated signaling are functionally connected with one another during the early phases of thymic development.
Mice were bred to become double deficient for RAG1 (or RAG2) and either Ick or CD3e, and double-deficient newborn mice were treated with anti-CD3e, thus addressing the role of CD3e and Ick in the differentiation of DN to DP thymocytes. Furthermore, by studying CD3e and Ick single-deficient mice, we analyzed the responses of immature DP thymocytes upon CD3 cross-linking. The results suggest that CD3e and Ick are involved in partially independent signal transduction pathways with divergent functions in pre-TCR-dependent thymic selection.

Results

Signaling through CD3e Is Not Essential for Early Thymocyte Maturation

Newborn F2 generation offspring from intercrosses between CD3e-deficient and RAG2-deficient parental mice were injected with 10 μg/g bodyweight anti-CD3e monoclonal antibody (MAb), 1 day after birth. Thymocytes were isolated at day 8 after birth and the mice were typed by intracellular staining for CD3e (Levett et al., 1993c) and by reverse transcription polymerase chain reaction (RT-PCR) for RAG2. Remaining thymocytes were stained for CD4, CD8, and IL-2Ra. In Figure 1, the effects of anti-CD3e MAb treatment on thymic development in RAG2-deficient (E–H) and RAG2/CD3e double-deficient mice (A–D) are shown. As expected from our previous results on RAG1-deficient mice (Levett et al., 1993b), anti-CD3e MAb caused full induction of CD4/CD8, down-regulation of IL-2Ra, and a substantial increase in cell numbers in RAG2-deficient thymi. No significant differences in any of these parameters were detected between RAG2-deficient mice and CD3e⁻/⁻/RAG2⁻/⁻ double mutants. Absolute cell numbers of thymocytes in the anti-CD3e MAb-treated double-mutant mice were similar to those found in untreated wild-type newborn mice, and four to six times greater than those found in untreated CD3e single-deficient mice (see Table 1). CD3e single-deficient mice also showed full induction of DP cells (see below). These results suggest that the defect in thymocyte development observed in CD3e⁻/⁻ mice can be overcome by potent signalling through CD3γδ and alone. The signaling function of CD3e.
Table 1. Total Thymocytes in Wild-Type and Mutant Mice after Injection of Anti-CD3e MAb* (× 10^6)

<table>
<thead>
<tr>
<th>Mice</th>
<th>Day after injection</th>
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<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>WT</td>
<td>58.1 ± 4.6</td>
</tr>
<tr>
<td>CD3e-/-</td>
<td>10.7 ± 1.5</td>
</tr>
<tr>
<td>Lck+/-</td>
<td>13 ± 1.6</td>
</tr>
<tr>
<td>RAG2-/-</td>
<td>2.9 ± 0.3</td>
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*Results are means ± SEMs of 2–6 mice for each data point.

during early thymocyte differentiation is therefore either redundant or merely an amplification of the CD3γδε signal. In addition, CD3ε may increase the surface expression of the immature TCR.

**Lck** Is Important for Efficient Signal Transduction through CD3 during Early Thymocyte Differentiation

The results on CD3e-deficient mice suggested that CD3γδε is sufficient for the early signal that induces maturation to the DP stage. If this signal was mediated by PTLs other than lck, lck-deficient thymi should be fully inducible by anti-CD3e. This was tested by injecting RAG1-/-/lck-/- double-mutant mice with anti-CD3e MAbs at day 1 after birth. The newborn mice were typed by PCR of tail DNA, and flow cytometry of thymocytes was done on day 8 after birth. As for RAG2-deficient mice, expression of CD4 and CD8 was down-regulated of IL-2Ra, and proliferation is induced upon anti-CD3e MAb treatment in thymocytes of RAG1-deficient mice (Figures 2E–2H). In contrast, RAG1-/-/lck-/- double-mutant mice showed only a poor reconstitution of early thymocyte development upon anti-CD3e treatment. Down-regulation of IL-2Ra was incomplete (Figures 2B and D), and the absolute number of DP cells was less than 15% of that in thymi of anti-CD3e MAb-treated RAG1-deficient mice (Figures 2A and 2C). These results suggest that lck is an important element in signal transduction through both the CD3e and the CD3γδε modules during this stage of development.

We found that one of the earliest parameters of induction by anti-CD3e MAbs is the expression of CDB6, which coincides with the down-regulation of IL-2Ra in RAG2-deficient
thymi. (Figures 3A and 3B). CD69 is lost 1 day later, together with IL-2Ra (Figures 3C and 3D). It is likely that this occurs also in physiological thymocyte differentiation, regulated by the pre-TCR treatment; CD69 expression was detected on IL-2Ra knockout and wild-type thymi from day 16 wild-type embryos before expression of CD4 or CD8 (data not shown). These and additional early consequences of CD3 cross-linking were compared in RAG1-deficient and RAG1+/−/Ick−/− double-mutant mice. In the Ick-deficient thymi, fewer cells expressed high levels of CD69 and more cells were found with intermediate levels (Figures 4A and 4C); down-regulation of IL-2Ra was less effective in all thymocytes, including those that expressed CD69; and expansion of DN thymocytes and reduction in cell size were less pronounced (Figures 4B and 4D). This demonstrates that a number of early events in the maturation of DN thymocytes to the DP stage are impaired in the absence of Ick.

Responses of DP Thymocytes to Anti-CD3
Treatment in Mice with Defects in Signal
Transduction through TCR-CD3

The responses to CD3 cross-linking of thymocytes that spontaneously matured to the DP stage in CD3−/− or Ick−/− mice were studied. Newborn mice deficient for either Ick or CD3−/− were treated with anti-CD3e MAb 1–3 days before analysis. As controls, wild-type newborn mice, and RAG2−/− newborn mice were used. Table 1 shows the absolute numbers of thymocytes in these mice over time. In wild-type mice, the antibody treatment resulted in a rapid decrease of the absolute number of thymocytes, reflecting negative selection by deletion. In contrast, CD3−/− deficient mice and RAG2−/− mice responded with an increase in the absolute numbers of thymocytes. Ick−/− deficient mice showed no significant changes in the absolute thymocyte number.

Apoptosis upon anti-CD3 treatment, as analyzed by gel electrophoresis or intracellular DNA staining with propidium iodide, was marginal or undetectable in CD3−/− or Ick−/− deficient thymocytes (data not shown). Because a relative resistance to dexamethasone-induced apoptosis was also observed, it is likely that DP thymocytes in CD3−/− or Ick−/− deficient mice do not efficiently mature to an apoptosis-sensitive stage.

The kinetics of expansion of DN and DP thymocytes in CD3−/− deficient mice after cross-linking of CD3e were studied in more detail. Figure 5 shows the absolute numbers of DN and DP thymocytes with or without functionally rearranged TCRβ genes of newborn mice on days 1–3 after anti-CD3e MAb treatment. Beginning from day 1, the increase in cell numbers was largely restricted to DP thymocytes expressing intracellular TCRβ chain. Production of new DP thymocytes from DN thymocytes upon anti-CD3 treatment takes 3 days, and results in a block in rearrangement of the TCRβ locus (Levet et al., 1995), also in CD3−/− deficient animals (Wang et al., 1995). Therefore, these data indicate that CD3 cross-linking induces proliferation of preexisting DP TCRβ+ thymocytes in CD3−/− deficient mice.
Figure 5. Expansion of the DP Population in CD3ζ-Deficient Thymines upon anti-CD3ε MAb Treatment

CD3ζ−/− newborn mice were injected with anti-CD3ε MAb, 1-3 days before analysis, and absolute cell numbers of different thymocyte populations were determined, as indicated. The DP cells that proliferate in the CD3ζ-deficient mice during the first 2 days after treatment are labeled completely by thymocytes expressing intracellular TCRβ chain (DP TCRβ+). A few DP thymocytes not expressing TCRβ (DP TCRβ−) are detected 3 days after treatment. The results represent mean cell numbers of 2-5 mice for each data point; variations between individual mice were less than ± 20% on day 2, less than ± 15% on all other days.

Figure 6. Impaired Ca²⁺ Mobilization in lck-Deficient Thymocytes

Thymocytes were isolated from heterozygous littermates (left) or lck−/− (right) mice at day 8 after birth, stained for CD4 and CD8, and loaded with Indo-1. Analysis was performed on a FACScan Plus flow cytometer. CD4−CD8− DP thymocytes were 30% for lck−/− mice and 42% for lck+−/− mice, respectively. Density plots represent the course of the violet-blue ratio, which is linearly related to the molarity of intracellular Ca²⁺ over time. Ca²⁺ mobilization is detected in lck-deficient mice whose thymocytes are stimulated at 40 s with 3 μg/ml anti-CD3ε MAb, followed by 80 s by 30 μg/ml anti-hamster IgG (A). When 0.3 μg/ml anti-CD3ε was used (B), the response of lck-deficient thymocytes was abolished.

Discussion

Surprisingly, CD3ζ-deficient mice had up to normal numbers of peripheral T cells. In this respect lck−/− mice differ from CD3ζ-deficient mice, as the possess only 5%-10% of the normal number of peripheral T cells (Molina et al., 1992). The initial aim of the present study was to use these mice to test our hypothesis that the cross-linking of CD3 on thymocytes induces their differentiation to the DP stage by a process involving sign transduction (Levelt et al., 1993a, 1993b). In addition, the alleviation of terminal responses of thymocytes and anti-CD3ε MAb allowed us to delineate the different roles of CD3ζ and lck during early thymocyte differentiation in more detail than has been previously possible in a phenotypic analysis alone.

We first investigated whether CD3ζ was involved in signal transduction through the immature TCRβ–CD3 complex, and if it had a specific or only an amplifying role in the CD3ζ. Recent studies have shown that not only CD3ζ, but also CD3ζ can mediate TCR signaling (Letourneau and Klausner, 1992; Wegener et al., 1992). It was suggested that different PTKs are involved in signal transduction through the CD3ζ module and the CD3ζ module (Letourneau and Klausner, 1992). Recently, evidence accumulates that the same PTKs mediate signaling through both CD3ζ and CD3ζ (Weiss and Litman, 1994; Wange et al., 1994; Duplay et al., 1994), though with differential affinities. Signal transduction through CD3ζ may be more efficient th
through CD3ε because its cytoplasmic tail contains three ARAMs, signaling motifs containing two tyrosines that can be phosphorylated by specific PTKs, as compared with a single motif in the cytoplasmic tail of CD3ζ (Weiss and Littman, 1994). A second reason why CD3ζ may be important in regulating the strength of the signal through TCR–CD3 is that, in the absence of CD3ζ, the assembly and surface expression of TCR–CD3 is extremely inefficient (Weissman et al., 1989).

To address these questions, we made use of our previous finding that in FTOC of mice deficient in TCRβ gene rearrangement, cross-linking of CD3 with anti-CD3ε MAb fully restored early thymocyte differentiation. By injection of newborn mice with anti-CD3ε MAb (Jacobs et al., 1994; Shinkai and Alt, 1994), we found that RAG2-deficient and RAG2/CD3ζ double-deficient mice were equally competent in the generation of DP thymocytes upon anti-CD3ε MAb treatment. Induced DP thymocyte numbers were similar to those found in normal untreated newborn mice of the same age. We conclude that signaling through CD3ζ/ε is sufficient for the full induction of this maturation step.

Subsequently, we addressed the question whether signal transduction through the immature CD3ζ/ε module involved primarily PTKs other than lck, used by the CD3ζ module. In that case, induction of thymocyte development in lck/RAG1 (or RAG–) double-mutant mice by anti-CD3ε MAb treatment should be successful. By injecting anti-CD3ε MAb into lck/RAG1 double-deficient mice, we observed production of DP cells, but the absolute number did not exceed 15% of that obtained in anti-CD3ζ MAb-treated RAG1-deficient mice. Moreover, down-regulation of IL-2Rα on DN cells was incomplete. These results suggest that lck is the main PTK in signal transduction through both the CD3ζ and CD3ζ/ε modules at this stage of thymocyte development.

The developmental block in lck-deficient mice was not restricted to the proliferation of DP thymocytes, but also affected the proliferation and differentiation of DN thymocytes. We found that during early thymocyte selection, the early activation marker CD69 was transiently expressed, similar to its expression upon late positive selection of DP thymocytes (Bendelac et al., 1992; Yamashita et al., 1993; Swat et al., 1993). In lck/RAG1 double-mutant mice, expression of CD69 after 1 day of anti-CD3ε MAb treatment occurred on fewer thymocytes than in RAG1 mutant mice. Furthermore, down-regulation of IL-2Rα, proliferation of DN thymocytes, and reduction in cell size all were less effective in the absence of lck. Whether this represents a complete block in the development of a proportion of the cells, or a general slowdown for all cells, cannot be decided from our results. In either case, the data suggest that lck is important in signal transduction through the pre-TCR already before the acquisition of the DP phenotype. In this context, it is interesting that allelic exclusion of the TCRβ locus in TCRβ transgenic lck-deficient mice is almost complete (Wallace et al., 1995). It is possible that different responses to pre-TCR signaling are associated with different biochemical pathways or are restricted by different quantitative thresholds.

In lck- or CD3ζ-deficient mice, DP thymocytes differ phenotypically from normal DP thymocytes. It has been described before (Crompton et al., 1994) that in CD3ζ-deficient animals, IL-2Ra expression is detected on many DP thymocytes. This was also observed on lck-deficient DP thymocytes (C. N. L., unpublished data). In addition, we observed functional differences between DP thymocytes from wild-type mice and those from lck- or CD3ζ-deficient mice. lck- or CD3ζ-deficient DP thymocytes were highly resistant to deletion by in vivo treatment with anti-CD3ε MAb. This could partially be due to inefficient signal transduction through the TCR–CD3 complex in the absence of either CD3ζ or lck. Indeed, Ca2+ mobilization was absent in DP thymocytes of CD3ζ-deficient mice, and reduced in lck-deficient DP thymocytes. However, impaired signaling may not be the only explanation for the resistance to deletion, as reduced sensitivity to dexamethasone-induced apoptosis was also observed. It is therefore likely that, in addition to maturation of DP thymocytes is incomplete in CD3ζ- or lck-deficient mice and does not proceed efficiently to an apoptosis-sensitive stage. In line with this idea is our finding that in CD3ζ-deficient mice, DP thymocytes remained sensitive to anti-CD3ε-induced proliferation, a response typical of immature thymocytes. The lack of this immature response in lck-deficient DP thymocytes may highlight the importance of lck in the proliferative response of immature thymocytes, as well as observed in anti-CD3ζ-treated RAG1/lck double-deficient animals. Our observation that lck-deficient DP cells can mobilize Ca2+ does not argue against this: the signal inducing early thymocyte proliferation is not likely to depend on Ca2+ mobilization, as suggested by previous results (Leveille et al., 1995b), and by the proliferation induced in CD3ζ-deficient DP thymocytes.

Is the cross-linking of CD3 complexes on TCRβ thymocytes indeed comparable to signaling through the pre-TCR? In this context, it is interesting that the cytoplasmic tail of the pre-TCRα chain contains phosphorylation sites consistent with an involvement in signal transduction (Saint-Ruf et al., 1994). While CD3 complexes have been detected on TCRβ thymocytes with biochemical means (Wiest et al., 1994), it is not known whether the pre-TCRα chain can be expressed on the cell surface without TCRβ as part of these incomplete CD3 complexes. Alternatively, CD3 cross-linking by MAb may generate a strong signal that overrides the requirement for pre-TCRα, or this molecule may not be involved in signal transduction. Taken together, the results presented in this report show that maturation of DN thymocytes into the DP stage, including proliferation of immature DP thymocytes, depends on signaling through the CD3 complex. For the signals dictating pre-TCR-dependent thymocyte maturation, a cooperation between lck and CD3ζ/ε seems essential. CD3ζ does not play a specific role in this process, but may be of importance in the augmentation of signaling through the pre-TCR–CD3 complex.

Experimental Procedures

Mice

Newborn RAG1+−, RAG1+−, CD3ζ−−, and lck−− mice or crosses of these strains, were obtained from the specific pathogen-free breeding.
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facility at the Beth Israel Hospital, Harvard Medical School, Boston, Massachusetts, and at the Max Planck Institut für Immunologie, Freiburg, Federal Republic of Germany. RAG1-deficient and Ick-deficient mice were typed by PCR analysis of tail DNA. The following primers were used: RAG1 sense, 5'-TACCCTGAGTCAAGTCTGC-3'; RAG1 antisense, 5'-CAAGAGTCGCTTCAAGTGC-3'; Nemoxycin sense, 5'-TATCCAGGAGTGCTTGGATCCC-3' (Molina et al. 1997); Ick antisense, 5'-CTTAAAGTCAAGCCTTGCTGAAAGTAA-3' (Molina et al. 1997); Ick sense, 5'-GAACGTTCAACTGCTCCGAAAGC-3'.

RAG2-deficient mice were typed by staining thymocytes for intracellular TCRβ chain expression and FACS analysis. If results were not evident because of the anti-CD3e MAb treatment, the mice were tested for RAG2 expression in the thymus by RT-PCR, using the following primers: RAG2 sense, 5'-CAACATGAGAAGGAGAGGAGGAC-3' and RAG2 antisense, 5'-GCTAGGAGAAGCAACAGTCTGCTTAA-3'.

CD3ε-deficient mice were typed by intracellular staining of thymocytes for CD3ε.

Mabs

Anti-CD3ε antibody 500A2 (Havran et al. 1987) was isolated from culture supernatants by affinity chromatography over protein A columns (Pharmacia, Freiburg, Federal Republic of Germany). Flow cytometry employed labeled anti-Ly-42 (53-67, anti-LT4 (8D5-4D4), anti-TCRβ (5H7-597), anti-CD3ε (500A2), anti-CD69 (H1.2F3), anti-IL-2Rα (7D4) (all purchased from Pharmingen), and biotin-labeled anti-CD3ε antibody Hy146-958 (Punett et al., 1991), using a FACScan flow cytometer (Becton Dickinson, San Jose, California).

Flow Cytometry

Two- and triple-color stainings were performed using fluorescein isothiocyanate-phycoerythrin-, and biotin-labeled antibodies. As a third color, Red 670-conjugated streptavidin (GIBCO, Gaithersburg, Maryland) was used. Intracellular stainings were performed as described (Levet et al., 1993a, 1993b). Events were collected using a FACScan flow cytometer (Becton Dickinson, San Jose, California).

Antibody Treatment of Newborn Mice

Newborn mice were injected intraperitoneally with 10 μg bodyweight anti-CD3ε MAb. To avoid variations in thymocyte numbers by differences in the age of the newborn mice, analyses were always performed at day 8 after birth. Injections were given at varying days before analysis, as indicated with each experiment. Because no effects of intraperitoneal injections of normal hamster IgG were detected, some control newborn mice were not treated.

Mobilization of Intracellular Free Calcium

Calcium mobilization studies were performed as described (Rabinovitch et al., 1988). Cells were isolated from thymi derived from 8-day-old newborn mice. Thymocytes (5 x 10⁶/ml) were loaded with Indo-1 by incubation with its acetoxy-methyl ester (Molecular Probes, Eugene, Oregon) (53 M) and 0.02% sucrose (Molecular Probes) for 45 min at 37°C. Cells were washed and resuspended in Iscoves modified Dulbecco medium supplemented with 1% fetal calf serum and 1% glutamine. Analysis followed immediately and was performed on a FACStar flow cytometer (Becton Dickinson), in combination with staining for CD4 and CD8.

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References


