Chromatin condensation via the condensin II complex is required for peripheral T-cell quiescence

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Naive T cells encountering their cognate antigen become activated and acquire the ability to proliferate in response to cytokines. Stat5 is an essential component in this response. We demonstrate that Stat5 cannot access DNA in naive T cells and acquires this ability only after T-cell receptor (TCR) engagement. The transition is not associated with changes in DNA methylation or global histone modification but rather chromatin decondensation. Condensation occurs during thymocyte development and proper condensation is dependent on kleisin- β of the condensin II complex. Our findings suggest that this unique chromatin condensation, which can affect interpretations of chromatin accessibility assays, is required for proper T-cell development and maintenance of the quiescent state. This mechanism ensures that cytokine driven proliferation can only occur in the context of TCR stimulation.

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Introduction

Quiescence, marked by the absence of cell division, defines the majority of the lifespan of a peripheral T lymphocyte. During this time, which can be considered indefinite (Sprent, 1993), the cell has very low-energy output, hindered ability to produce macromolecules, and many of its organelles (if present) are rudimentary (Jaehning *et al*, 1975; Morley *et al*, 1993; Paul, 2003; Frauwirth and Thompson, 2004). This long-lived quiescence is bookended by periods of intense proliferative activity, first as developing precursor cells in the bone marrow and thymus and finally as activated T cells participating in an immune response in the periphery.

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Naive peripheral T cells remain in this quiescent, G₀ phase, until they encounter their T-cell receptor (TCR)-specific antigen. Following activation via the TCR, these relatively small T cells will undergo massive transformation into large lymphoblasts with the capacity to proliferate and ultimately clear the foreign antigen.

Establishment and maintenance of the quiescent state is marked by drastically reduced global levels of transcription (Jaehning *et al*, 1975). Epigenetic phenomena have been linked to transcriptional activity and silencing. Methylation of CpG dinucleotides has been shown to silence genes, including those involved in T-lymphocyte development and function (Fitzpatrick *et al*, 1998; Lee *et al*, 2002). Modification of the amino-terminal tails of histones has also been associated with transcriptional competence (Li *et al*, 2007). Histone modifications have also been studied on a global scale in CD4⁺ and CD8⁺ T lymphocytes (Barski *et al*, 2007; Wang *et al*, 2008; Araki *et al*, 2009; Wei *et al*, 2009). Recently, a report has shown that global assessment of histone 'marks' could be used as an indicator of quiescence in B lymphocytes (Baxter *et al*, 2004).

The two highly related Stat5 proteins (Stat5a and Stat5b; hereafter referred to as Stat5) are essential for peripheral T-cell proliferation, as evidenced by the inability of Stat5-deficient T cells to proliferate in response to growth factors (Moriggl *et al*, 1999b). Therefore, proliferation depends on the transcriptional activity of Stat5. In the context of a proper immune response, TCR activation leads to the production of IL-2, which in turn activates Stat5 via the canonical Jak/Stat cascade (Ihle *et al*, 1995; Ihle, 1996; Rawlings *et al*, 2004), resulting in the clonal expansion of only those T cells that are able to recognize the foreign antigen. To avoid improper proliferation during an immune response, naive T cells, for which TCR engagement has not occurred, must have a mechanism to ignore the effects of this cytokine. One level of regulation exists at the level of receptor presentation. Naive T cells possess the intermediate affinity IL-2 receptor consisting of β and γ_c chains. The IL-2 receptor present on activated T cells possesses an additional chain (α) whose function is to increase ligand-receptor affinity (Lin and Leonard, 1997). However, even the addition of exogenous IL-2 at concentrations that would engage the low affinity receptor fails to elicit T-cell proliferation in the absence of TCR stimulation, indicating that there must be additional mechanisms, downstream of the IL-2 receptor, which regulate signalling and ultimately escape from quiescence.

We examined activation of T cells using IL-2/Stat5-target gene transcription as a model system for studying mechanisms of overcoming quiescence. As anticipated, IL-2 could induce the activation and nuclear translocation of Stat5 in naive T cells; however, Stat5 failed to access its target promoters. Changes in the global state of histone modification or altered DNA methylation at a Stat5-target gene were

not detected. However, we discovered that T-cell activation results in higher-order structural changes in chromatin that correlate with TCR induction of competence to respond to IL-2. We propose a model whereby T-cell activation reconfigures higher-order chromatin in naive T cells, which permits the engagement of Stat5 with its target promoters resulting in proliferation. Finally, we show that a mutation in a subunit of the condensin II complex results in defective chromatin condensation during T-cell development and failure to silence IL-2-target genes in naive peripheral T cells. Our findings suggest that in T cells, the mechanism for establishment of quiescence in the thymus and maintenance in the periphery even in the presence of growth factors lies in the ability to regulate higher-order chromatin structure via the condensin II complex. In the context of an immune response, this mechanism ensures that only those T cells that have been activated via the TCR will proliferate.

Results

IL-2 induces disparate transcriptional programs in naive versus activated peripheral T cells

We previously demonstrated that there is little overlap in the genes that IL-2 induces in naive T cells relative to activated T cells (Gatzka *et al*, 2006). Among these, known Stat5-target genes were induced in activated T cells, whereas none of the genes were induced in naive T cells (Figure 1A). The results for several genes in each set were validated by quantitative RT-PCR (Figure 1B–D) and confirmed that IL-2 induces distinct transcriptional programs in naive versus activated T cells and that known Stat5-target genes, such as *Cis*, are only induced in activated T cells. To explore possible differences in T-cell subpopulations, we used sorted peripheral T cells to assess the ability of IL-2 to induce *Cis*. *Cis* induction was readily detected in purified CD4⁺ or CD8⁺-activated T cells (Supplementary Figure S1). However, no induction of *Cis* was observed in purified, unstimulated CD8⁺ T cells. A weak induction of *Cis* was detected in purified, unstimulated CD4⁺ cells; however, this induction was eliminated by depleting the CD25⁺ subpopulation of CD4⁺ cells. As CD25 is a marker of T-cell activation (Minami *et al*, 1993), our findings suggest that the expression was associated with a subpopulation of CD4⁺ cells that were already activated (Supplementary Figure S1).

Peripheral T cells rapidly acquire the ability to induce expression of Stat5-target genes following TCR stimulation

The above results suggested that, with activation, T cells acquire the ability to respond to IL-2 and induce expression of Stat5-regulated genes. We therefore determined when, following TCR engagement, T cells acquire the ability to respond to IL-2 and induce expression of Stat5-regulated genes. Purified CD25[−] peripheral T cells were first primed with anti-CD3 antibodies for 0, 1, 3 or 6 h after which IL-2 was added for 1 h. We observed a significant increase in the ability of IL-2 to induce *Cis* gene expression after just 3 h of anti-CD3 stimulation, increasing following 6 h (Figure 2A). The induction of *Cis* gene expression was specifically dependent on IL-2 stimulation, as anti-CD3 treatment alone failed to activate Stat5 (Figure 2B) or elicit any significant changes in *Cis* expression (Figure 2A), consistent with previous

findings (Moriggl *et al*, 1999a). Similar results were seen with another Stat5-target gene, *Socs1*. However, TCR stimulation failed to provide any level of competence at the *Ifn γ* locus. This was expected, because it takes at least one cell division before *Ifn γ* can be expressed, presumably due to epigenetic silencing mediated by DNA methylation (Bird *et al*, 1998; Gett and Hodgkin, 1998). Conversely, *Bcl-X* proved promiscuous in our studies, consistent with the fact that it can be induced by other transcription factors such as Stat1 (Fujio *et al*, 1997), Stat3 (Rubin Grandis *et al*, 2000), NF κ B (Tsukahara *et al*, 1999; Chen *et al*, 2000) and Ets (Sevilla *et al*, 1999).

Stat5 fails to engage DNA in naive peripheral T cells

To explore the mechanisms for the lack of induction of Stat5-target genes, we initially assessed the ability of Stat5 to function properly in naive T cells. Stat5 transcriptional activity is activated via tyrosine phosphorylation resulting from its recruitment to the IL-2 receptor complex. This modification permits Stat5 dimerization, translocation to the nucleus and binding to target gene promoters (Rawlings *et al*, 2004). IL-2-induced tyrosine phosphorylation of Stat5 was readily detectable in both CD4⁺ and CD8⁺ naive or activated T cells (Figure 3A) with similar kinetics and comparable IL-2 concentration requirements (Figure 3B). Furthermore, we observed Stat5 nuclear translocation following IL-2 stimulation in naive peripheral T cells (Figure 3C). Lastly, previous studies demonstrated that naive peripheral T cells have the capacity for Stat5–DNA binding (Gatzka *et al*, 2006). Taken together, these findings suggest that although Stat5-target genes are not transcribed, naive peripheral T cells possess all the machinery needed for Stat5 activation and function.

The above results demonstrated that the inability of Stat5 to activate its target genes in naive T cells was either due to an inability to access its target promoters or the lack of assembly of a functional transcriptional complex. To address this, chromatin immunoprecipitation (ChIP) assays were used to assess the status of Stat5 at the *Cis* promoter. With IL-2 stimulation, or in combination with anti-CD3 antibodies, we readily observed an increase in the enrichment of Stat5 at the *Cis* promoter in activated T cells using either Stat5 antibodies or antibodies specific for phosphorylated (Y⁶⁹⁴) Stat5 (Figure 3D). Consistent with previous studies demonstrating the inability of TCR signalling to activate Stat5 (Figure 2B; Moriggl *et al*, 1999a), we did not observe any changes in enrichment of Stat5 at the *Cis* promoter of activated T cells stimulated with anti-CD3 alone. In contrast, stimulation of naive T cells under any of the conditions did not result in any detectable accumulation of Stat5 on the *Cis* promoter. These results demonstrate that the lack of induction of *Cis* expression is the result of the inability of Stat5 to access the promoter in naive T cells.

T-cell activation results in ultrastructural changes in nuclear architecture correlating with Stat5 competence

A number of possibilities existed to explain the activation-induced changes in the accessibility of the *Cis* promoter to activated Stat5. Initially, we examined T-cell nuclear morphology by transmission electron microscopy as they gained competence for activated Stat5 to induce gene expression (Figure 4A). As illustrated, there is a rapid, dramatic change in nuclear morphology following anti-CD3 treatment.

Figure 1 IL-2 induces disparate transcriptional programs in naive versus activated T cells. (A) Venn diagram illustrating the disparate transcriptional programs induced by IL-2 in naive versus activated peripheral T cells as revealed by microarray analysis (Gatzka *et al*, 2006). The number of genes induced more than three-fold in each class is indicated inside the diagram and representative genes of each class are indicated outside the diagram. (B–D) Purified naive T naive cells and those activated in culture (see Materials and methods) were stimulated with 1000 U/ml IL-2 for the times indicated. Levels of gene expression were determined by quantitative RT–PCR relative to HPRT and all values were calibrated to naive unstimulated cells. Genes are sorted into those that are induced in activated cells (B), those induced in naive cells (C) or induced in both cell types (D) according to the microarray.

In particular, nuclear material is highly condensed in naive T cells but becomes less condensed within hours after stimulation with anti-CD3 antibodies. The decondensation is not

seen with IL-2 treatment alone (data not shown) and precedes the expansion of the cytoplasm that characterizes ‘blasting’ T cells. Anti-CD3-mediated decondensation also

Figure 2 TCR stimulation rapidly primes naive T cells to permit IL-2/Stat5 signalling. **(A)** Purified naive T cells were primed with anti-CD3 antibodies for the times indicated followed by stimulation with IL-2. Stat5-target gene expression was assessed by quantitative RT-PCR. For comparison, activated T cells were starved overnight (S) and restimulated with 1000 U/ml IL-2 for 1 h. Gene expression was normalized to HPRT and calibrated to naive unstimulated cells. * $P < 0.05$, ** $P < 0.005$. **(B)** Purified naive T cells were stimulated with 1 μ g/ml anti-CD3 antibodies followed by 1000 U/ml IL-2 where indicated and assessed for activation of Stat5.

occurs with Stat5-deficient T cells (Teglund *et al*, 1998), indicating that decondensation is not Stat5 dependent (Figure 4B).

During the course of our experiments, we noted that the DNA of naive T cells is highly resistant to sonication-induced breakage and that this resistance is lost following stimulation with anti-CD3 antibodies (Figure 4C). Under the conditions described in the Materials and methods section, DNA from unstimulated naive T cells was completely resistant to breakage as indicated by the lack of significant DNA at lower molecular weights. Activation caused the DNA to become progressively more sensitive, such that we began to see lower molecular weight fragments beginning just 1 h following anti-CD3 treatment. Importantly, the kinetics of DNA fragmentation were comparable to those of the change in nuclear condensation seen by electron microscopy and both phenomena correlate with acquisition of Stat5 competence.

Nuclear condensation is not a function of CpG methylation

The similar kinetics of nuclear decondensation and acquisition of Stat5 competence would suggest that the inability of Stat5 to access its target promoters in naive peripheral T cells could be the result of DNA condensation and that a critical step in T-cell activation is to induce decondensation. CpG

methylation has been associated with transcriptional competence (Smale, 2003) and could be hypothesized to contribute to DNA condensation (Matarazzo *et al*, 2007). Recently, DNA methyltransferase activity has been linked to TCR engagement (Gamper *et al*, 2009) and has been shown to control expression of the Th1 (Fitzpatrick *et al*, 1998) and Th2 loci (Lee *et al*, 2002), implicating a role for DNA methylation in the control of gene expression during lymphocyte development. The *Cis* promoter contains a large CpG island that encompasses the Stat5-binding sequences (Li and Dahiya, 2002). Bisulfite sequencing of this region in both naive and activated T cells revealed that there were no major differences in the CpG methylation status between these cell types (Supplementary Figure S2), indicating that DNA methylation is not the mechanism for chromatin condensation in naive T cells.

Histones are not grossly modified as a consequence of TCR activation, rather they become more accessible

Several studies have implicated a role for histone modifications in chromatin structure and changes in gene expression (Kouzarides, 2007; Li *et al*, 2007), including during lymphocyte development (Baxter *et al*, 2004; Chang and Aune, 2007; Krangel, 2007). Therefore, the changes in nuclear condensation observed could be a response to changes in histone modifications. We therefore tested the status of histone H3

Figure 3 Stat5 cannot engage DNA in naive peripheral T cells. (A) IL-2 stimulation results in activation of Stat5 in naive CD4+ and CD8+ cells. Cells were stimulated with 1000 U/ml IL-2 for the times indicated. (B) IL-2 dose-response analysis of Stat5 activation in naive and activated T cells. Cells were stimulated for 30 min with the dose of IL-2 indicated. Ctrl lanes are DA3 cells expressing the Epo receptor stimulated with recombinant human Epo for 15 min (Pelletier *et al*, 2006) serving as a positive control. (C) Immunofluorescent localization of endogenous Stat5 in purified naive T cells and those stimulated with 1000 U/ml IL-2 for 30 min. (D) ChIP analysis on the *Cis* promoter of naive and activated T cells either unstimulated (control) or stimulated with anti-CD3 antibodies, IL-2 or combination of anti-CD3 antibodies and IL-2 for 1 h. Antibodies recognizing either activated (pY Stat5) or total Stat5 were used. (E) Schematic of the *Cis* locus showing the relative positions of the Stat5-binding elements and PCR assay used in the ChIP. An arrowhead marks the transcription start site.

using a variety of antibodies specific for modifications associated with both transcriptional activity and silencing by immunofluorescence microscopy. We reasoned that the magnitude of the changes in chromatin condensation we observed would require relatively global changes in histone modification. Remarkably, our ability to detect nuclear histones was severely impaired in naive T cells using any of the reagents we tested. However, all of the reagents were able to readily access nuclear histones following 20 h of TCR stimulation or in activated T cells proliferating in culture. The results were comparable for antibodies that detected unmodified histone H3, as well as a variety of modification-specific antibodies (Figure 5A). These findings were substantiated by flow cytometric analysis of intracellular stained peripheral T cells (Figure 5B; Supplementary Figure S3).

The above studies suggest that histone epitopes are masked in naive T cells; therefore, we used western blot analysis to determine potential changes in histone modification. Under standard whole-cell lysis conditions, it was evident that the ability to solubilize histones also dra-

matically changes during activation of T cells (Figure 6A). The ability to detect histone H3 is severely impaired in cell extracts from naive T cells but becomes readily detectable following TCR stimulation. As with the immunofluorescence, this was observed with antibodies that recognize unmodified H3 as well as antibodies that recognize various modified forms of histone H3. Importantly, the extraction kinetics are virtually identical to the nuclear decondensation observed by electron microscopy, the sensitivity of the DNA to sonication, the ability to stain histones for immunofluorescence and TCR-mediated Stat5 transcriptional competence.

Due to these differences in solubility, we used the more stringent method of acid extraction to assess the global status of histone modification during T-cell activation (Figure 6A). The importance of this is illustrated in Figure 6B in which naive or activated T cells were first lysed under normal conditions and the soluble fraction separated from the pellet and the pellet further solubilized by acid extraction. In naive T cells, the majority of histone H3 resided in the acid soluble

Figure 4 T-cell activation induces rapid ultrastructural changes to chromatin that allow access to DNA. **(A)** Transmission electron microscopy (TEM) was used to visualize purified naive T cells or those stimulated with anti-CD3 antibodies for the times indicated. **(B)** TEM of purified naive T cells from Stat5-deficient animals or those stimulated for 20 h with anti-CD3 antibodies. **(C)** Purified naive T cells were left untreated or stimulated with anti-CD3 antibodies for the times indicated and subjected to a sonication assay (see Materials and methods). Arrow indicates sonication resistant chromatin.

fraction (pellet), whereas in activated cells, histone H3 was readily detected in both fractions. We therefore used acid extraction to explore the potential changes in histone modifications following activation. Surprisingly, there were no detectable changes in the extent of any of the modifications tested (Figure 6A). These findings were also extended to the rest of the nucleosome, as we obtained similar results when using antibodies that recognize histone H1, H2A, H2B and H4 (Figure 6C). Our data indicate that there is no major net change in global histone modification as a function of activation, rather a change in accessibility.

While unlikely, it is possible that T-cell activation results in rapid increased synthesis of 'off chromatin' histones that are solubilized via the whole-cell lysis technique rather than a change in solubility of 'on chromatin' histones. To explore this possibility, naive T cells were activated in the presence of cycloheximide and histone accessibility was measured via western blot (Supplementary Figure S4). Cycloheximide had no effect on the kinetics of detection of histones following activation, indicating that these are changes in 'on chromatin' accessibility.

Chromatin condenses rapidly during thymocyte development

Immature CD4[−]CD8[−] double negative (DN) thymocyte precursors enter the thymus as relatively large, highly proliferative

cells. During development in the thymus, these cells become smaller and ultimately become quiescent prior to exit into the periphery as naive T cells, suggesting that chromatin condensation occurs during thymocyte development. As in the periphery, it has been suggested that epigenetic modification contributes to the silencing of loci important during thymocyte maturation *ex vivo* (Su *et al*, 2004). Therefore, we wanted to determine when chromatin condensation occurs during development in the thymus. Similar to naive peripheral T cells, we had difficulty in detecting histones in the smaller, more mature CD4⁺CD8⁺ double-positive (DP) and single-positive (SP) thymocytes, whereas histones were readily detectable in the larger, immature (DN) thymocytes (Figure 7A). Flow cytometric analysis using cell surface markers to delineate thymocyte subpopulations confirmed that chromatin condensation occurs as cells transition from the DN to the DP stage (Figure 7B).

Chromatin condensation is required for proper development of peripheral T cells and depends on the function of the condensin II complex

Given that chromatin condenses as thymocytes transition from the DN to DP stage, one could hypothesize that a failure to condense might have profound effects on T-cell selection and consequently proper differentiation. We searched the literature for mutations that resulted in thymocyte maturation

Figure 5 Standard immunofluorescence techniques fail to detect histones in naive peripheral T cells. **(A)** Naive T cells were left untreated, stimulated for 20 h with anti-CD3 antibodies or growing in culture for 5 days. DAPI was used to mark the nucleus. Antibodies to mono-, di- and tri-methylated histone H3K4, tri-methylated histone H3K9/27, and acetylated histone H3 were used to detect modification of histone H3. Antibodies to N-terminal and C-terminal epitopes of unmodified histone H3 are also shown. **(B)** Detection of intracellular H3K4me1 as a function of TCR β surface expression as shown by flow cytometry in unstimulated Thy1.2 + CD4 + or Thy1.2 + CD8 + splenocytes or those stimulated with anti-CD3 antibodies for the times indicated.

defects as well as mutations in genes that might be involved in chromatin condensation. Remarkably, mice harbouring a point mutation of *kleisin*- β , a subunit of the condensin II complex, resulted in such a phenotype (Gosling *et al*, 2007,

2008). Mice homozygous for the *nessy* allele of *kleisin*- β have relatively normal numbers of DN thymocytes; however, beginning at the DP stage they have drastically reduced cell numbers, presumably due to increased apoptosis during

Figure 6 Detection of histones in peripheral T lymphocytes by western blot. **(A)** Purified naive T cells were left untreated or stimulated with anti-CD3 antibodies for 1, 3, 6, 12 or 24 h. Half of each sample was either acid extracted overnight or lysed using NP40 buffer (see Materials and methods). Modified histone H3 (mono-, di- or tri-methylated H3K4, tri-methylated H3K9/27, acetylated H3) or unmodified histone H3 or the H3.3 variant were detected by western blot. **(B)** Purified naive T cells were left untreated or stimulated for 24 h with anti-CD3 antibodies. The cells were lysed with NP40 buffer (lysate) and the remaining material (pellet) was acid extracted overnight. Both were resolved by SDS-PAGE and detection of H3K4me2, total histone H3, Stat5 and Actin was assayed by western blot. **(C)** Cells were treated as in **(A)** and antibodies recognizing histones H1, H2A, H2B and H4 were used to assess the status of the remainder of the nucleosome.

negative selection (Gosling *et al*, 2007). We analysed chromatin condensation during T-cell differentiation in these mice and found that they were defective in their ability to condense chromatin in the DN/DP transition (Figure 8A and C), suggesting that failure to properly condense chromatin could contribute to the increased apoptosis and resulting dramatic reduction in cell number seen at the DP stage in these animals. Consistent with these findings, the CD4⁺ and CD8⁺ SP mature thymocytes also had a more open chromatin configuration (Figure 8A and C) and drastically reduced cell number compared with their wild-type littermates (Gosling *et al*, 2007). Developing thymocytes must also undergo β -selection, which occurs at the DN2–DN3 transition. As with negative selection, the outcome of this process is dependent on proper response to TCR engagement. While wild-type cells undergo a dramatic condensation event as the cells transition from DN2 to DN3, *nessy* cells appear to lag behind (Figure 8C). Taken together, these data suggest that chromatin condensation is a critical step that is required for proper TCR-mediated selection events in the thymus.

We next examined the effects of the *nessy* mutation on peripheral T cells, specifically chromatin accessibility and the ability to express Stat5-target genes. Consistent with our findings in the thymus, peripheral Thy1.2⁺ CD4⁺ and CD8⁺ cells from mutant animals had a more open chromatin configuration than wild-type littermates (Figure 8B and C). Interestingly, there was no difference in Thy1.2⁺ CD4⁺ CD8⁺ cells, suggesting that $\alpha\beta$ T cells, and not other T cells, are sensitive to perturbation of kleisin- β function. Transmission electron microscopic analysis of CD25⁺ peripheral T cells from *nessy* mice revealed that there were cells with varying degrees of chromatin condensation. A minority of cells

appeared to have condensed chromatin, similar to naive wild-type cells (Figure 8D, left panel). The majority of cells, however, displayed an open chromatin configuration with a range of phenotypes (Figure 8D, centre and right panels). To test whether decondensation of chromatin was sufficient for Stat5-target gene expression, we assessed *Cis* expression levels in CD25⁺ peripheral T cells from wild-type and mutant animals. As illustrated in Figure 8E, the cells from mutant animals already expressed *Cis* at high levels that are comparable to activated, proliferating wild-type T cells, without the need for IL-2 stimulation. Consistent with this finding, *nessy* peripheral T cells have abnormal expression of the activation markers CD44 and CD25 that is different than what is seen in the thymus (Gosling *et al*, 2007; Supplementary Figures S5 and S6). Taken together, these data support the hypothesis that chromatin condensation status is important for both T-cell differentiation in the thymus and appropriate signal transduction in the periphery and that proper chromatin condensation depends on proper function of the condensin II complex.

Discussion

These studies were directed at determining the mechanism for the simple observation that IL-2 is unable to induce Stat5-target genes in naive T cells. As illustrated, this was not a result of the inability of IL-2 to induce tyrosine phosphorylation of Stat5, the ability of Stat5 to acquire DNA-binding activity (Gatzka *et al*, 2006) or to translocate to the nucleus. Rather, Stat5 was unable to access the promoter of the target gene, *Cis* and, although not examined, regulatory sequences of other known and unknown genes critical for

Figure 7 Chromatin condenses rapidly during thymocyte development. **(A)** Thymocytes were isolated and stained with DAPI and with antibodies to mono-, di- and tri-methylated histone H3K4, tri-methylated histone H3K9/27 and acetylated histone H3 to detect modification of histone H3. Antibodies to N-terminal and C-terminal epitopes of unmodified histone H3 are also shown. **(B)** Flow cytometric analysis of chromatin condensation during thymocyte development using PE- conjugated H3K4me1 antibodies as a marker for chromatin accessibility. Subpopulations of Thy1.2+ thymocytes were delineated based on surface expression of CD4 and CD8.

T-cell proliferation. The ability to access the promoters required TCR signalling and appeared rapidly following engagement of the TCR. As the proliferation of peripheral T cells is absolutely dependent upon Stat5 function (Moriggi *et al*, 1999a, b), the requirement for TCR signalling to allow Stat5 to access its target genes provides an essential control to insure that only antigen-specific T cells will be amplified.

A recent study suggests that formaldehyde crosslinking may be insufficient to capture weak or transient transcription factor DNA binding (Schmiedeberg *et al*, 2009). Thus, it is possible that TCR signalling would induce a modification that increases affinity permitting detection via formaldehyde

crosslinking methods. However, as several approaches demonstrate that the chromatin of naive T cells is highly condensed and that the ability of Stat5 to be detected on the promoter correlates with decondensation, our interpretation is the most likely.

Importantly, naive peripheral T cells are not totally refractory to IL-2-induced gene expression since genes such as *Fos* are rapidly induced by IL-2. It is of note, however, that the genes that can be induced in naive T cells by IL-2 are generally not induced by IL-2 in activated T cells. It will be important to determine whether the accessibility of the relevant transcription factors to those promoters changes in

Figure 8 The condensin II complex is required for proper condensation of chromatin and the silencing of IL-2-target genes. (A) Thymocytes from 6-week-old *nessy* and wild-type littermates were analysed for surface expression of Thy1.2, CD4 and CD8 in combination with intracellular H3K4me1 staining. (B) Splenocytes from 6-week-old *nessy* and wild-type littermates were analysed for surface expression of Thy1.2, CD4 and CD8 in combination with intracellular H3K4me1 staining. (C) Mean fluorescence intensity of H3K4me1 staining on thymocytes and splenocytes from mice as in (A, B). Subpopulations of thymocytes were determined by surface staining for Thy1.2, CD4, CD8, CD25 and CD44. Subpopulations of splenocytes were determined by surface staining for Thy1.2, CD4 and CD8. NS, not significant difference between WT and *nessy*. (D) Representative images from TEM analysis of purified CD25⁺ T cells from *nessy* mice. (E) Quantitative RT-PCR analysis of *Cis* expression from purified CD25⁺ T cells from 6-week-old *nessy* and wild-type littermate controls. Expression is normalized to HPRT.

a reciprocal manner relative to the access of Stat5 to its promoters. One intriguing possibility is that the relevant transcription factors are at the promoters and signalling pathways activate them *in situ*. Another possibility is that a small fraction of the chromatin remains accessible and permissive to induction of transcription (Spector, 2003; Fraser and Bickmore, 2007). Another possibility is a change in nucleosome occupancy at Stat5-binding sites since changes in global nucleosome positioning in naive versus activated T cells has been reported (Schones *et al*, 2008). We therefore interrogated their database to examine the nucleosome positions of a number of known Stat5-target genes and compared with non-activated with activated CD4⁺ T cells. For none of the genes were there differences in the locations of nucleosomes around the Stat5-binding sites in the promoters and/or 1500 base pairs around the transcriptional start site.

The basis for the inability of Stat5 to access its target genes could be envisioned to result from a number of properties of the relevant areas of chromatin including DNA methylation or histone modification, both of which have been implicated in regulation of gene expression in lymphocytes.

As demonstrated, the absence of any changes in DNA methylation at the *Cis* locus would rule out this mode of regulation. A variety of studies have recently focused on the role of histone modifications in the regulation of gene expression in lymphocytes (Chang and Aune, 2007; Araki *et al*, 2009; Karlic *et al*, 2010). Therefore, we sought to determine whether there were changes in histone modifications at the *Cis* locus. However, it became obvious that there were a number of technical issues, unique to naive T cells, which had not been previously considered. Specifically, we noted that the extent of sonication required to fragment the DNA of naive T cells was dramatically greater than any other cells we have examined. In addition, chromatin from naive T cells was considerably more resistant to micrococcal nuclease digestion than that from activated T cells (data not shown). As a consequence, we examined a number of parameters that evaluated, globally, the accessibility of chromatin. Specifically, electron microscopy illustrated dramatic changes in DNA condensation rapidly following TCR engagement with kinetics comparable to the ability of Stat5 to access its target genes. More strikingly, the simple ability of antibodies against a variety of

histone modifications to access their epitopes in fixed cells by immunofluorescence again dramatically changed following TCR engagement with comparable kinetics. Together, the results strongly support the hypothesis that TCR engagement is essential for the decondensation of chromatin thereby allowing access of Stat5 to genes that are essential for T-cell proliferation. While our studies have focused on Stat5, the global nature of the condensation/decondensation raises the intriguing possibility that other transcription factors activated by signalling pathways are comparably affected. For example, it has been demonstrated that TCR signalling is required for TGF- β -induced expression of *Foxp3* (Selvaraj and Geiger, 2007).

Our studies should be considered in the context of a number of the published studies detailing changes in histone modifications during lymphocyte development. Namely, when using naive T cells, it is essential to consider the accessibility of the chromatin to the antibodies being used to determine the extent of modifications present. For example, most studies have noted a dramatic increase in histone methylation marks during activation, both globally and at specific loci (Barski *et al*, 2007; Chang and Aune, 2007; Wang *et al*, 2008; Araki *et al*, 2009; Wei *et al*, 2009). However, our data suggest the possibility that the lack of detection of any histone modifications in naive cells is not due to the absence of the modifications, but rather the inability to detect the modifications.

As the highly condensed chromatin of naive T cells can be hypothesized to be an important regulation of cytokine responsiveness, one could speculate that the acquisition of this condensed chromatin is a key step in T-cell differentiation and may have a role in selection. As illustrated, the chromatin condensation occurs specifically at the DN/DP transition. This transition is characterized, in part, by the termination of cellular proliferation and the beginning of positive and negative selection. Since cell fate is determined by signals originating from the TCR, promoting either survival or apoptosis, it can be envisioned that inappropriate cytokine signalling could distort the outcome. By globally restricting cytokine signalling, appropriate selection that is solely regulated by the TCR, can occur. In this regard, it should also be noted that previous studies have shown SOCS1, a potent inhibitor of cytokine signalling, is specifically upregulated and highly expressed at the double-positive stage (Chong *et al*, 2003). Based on this and the consequences of its deletion, it has also been hypothesized that SOCS1 contributes to blocking cytokine rescue during selection (Marine *et al*, 1999).

The above would suggest that an inability to properly condense chromatin during differentiation would have the consequence of allowing cells to retain the ability to respond to cytokines. This, in turn, could affect selection by allowing cells to survive despite a lack of TCR signalling by providing survival signals or to spare cells receiving strong TCR signalling by protecting against apoptosis. While the deletion or mutation of a variety of genes have the consequence of affecting selection and altering peripheral T-cell function, it was particularly striking that a mutation in the *kleisin- β* gene of the condensin II complex had this phenotype. As illustrated here, this mutation affects chromatin condensation during T-cell development and, most importantly, peripheral T cells show an activated phenotype and constitutively express the Stat5-target gene *Cis*.

A number of studies have documented the role of cytokines, particularly IL-7, in the survival of peripheral T cells (reviewed in Surh and Sprent, 2008). In particular, IL-7 is required for the survival of naive T cells and the question arises of the effects of nuclear condensation on this function. Our previous studies have shown that while Stat5 function is absolutely essential for the proliferation of naive T cells following TCR stimulation and cytokine addition, maintenance of peripheral T cells is not affected by deletion of Stat5a and Stat5b (Moriggl *et al*, 1999b). Thus, other receptor-activated pathways contribute to survival and, indeed, our studies demonstrate that there are additional IL-2-activated pathways, requiring Jak3 activation, that induce the expression of genes such as *Fos*. IL-7 is also required for the proliferation of lymphoid progenitors; however, this proliferation does not require Stat5a or Stat5b, suggesting that early in development proliferation is driven by mechanisms different from those that drive the proliferation of naive T cells. Alternatively, early progenitors have mechanisms driving proliferation that are redundant to the ability of Stat5 proteins to drive proliferation.

More recently, studies (Park *et al*, 2010) have demonstrated a unique role for IL-7 signalling in the thymus for the differentiation of CD8⁺, cytotoxic lineage cells and provided evidence that cytokine-induced *Runx3* expression is critical for this differentiation. Importantly, the ability to generate CD8⁺ T cells was only reduced by 50% by the deletion of *Stat5a* and *Stat5b* but was more significantly reduced when both *Stat5* genes as well as the *Stat6* gene were deleted. As our studies have demonstrated chromatin condensation occurs during thymocyte differentiation and consequently it will be of interest to determine more precisely when this occurs during the differentiation of CD8⁺ T cells. The requirement for Stat transcription factor induced gene expression would suggest that it occurs late in thymic CD8⁺ T-cell differentiation.

Condensin and cohesin are related protein complexes that contain a heterodimer of the structural maintenance of chromosomes (SMC) proteins and associated proteins including members of the kleisin family (Haering and Nasmyth, 2003; Hagstrom and Meyer, 2003; Hirano, 2006). Cohesin is considered the glue that tethers sister chromatids during mitosis while condensin is involved in the chromatin compaction. Compaction is thought to involve an ATP-dependent opening and closing of the SMC 'V' structure to allow locking on DNA strands and compacting the captured strands.

The concept that chromatin condensation has a critical role in suppressing gene expression is established in several cellular contexts. For example, during erythrocyte differentiation, nuclear chromatin becomes increasingly condensed and global transcription is repressed. In chicken erythrocytes, which retain the nucleus in the fully differentiated state, a unique non-histone protein (mature erythrocyte nuclear termination stage protein) promotes chromatin condensation (Grigoryev *et al*, 1992). In mammalian cells, more than blood, a condensin II subunit, has been implicated in condensation and represses E-box transcription (Xu *et al*, 2006). In *Caenorhabditis elegans*, a condensin-like complex mediates both chromosome-wide gene repression as well as gene-specific repression (Hagstrom and Meyer, 2003).

Our studies demonstrate that the ability of Stat5 to access its target genes appears rapidly following TCR engagement

and has kinetics comparable to decondensation of chromatin as assessed in several approaches. However, the TCR-mediated signals that lead to rapid chromatin decondensation are unknown. It has been demonstrated that the BAF complex proteins (Brahma-related gene associated factor or Brahma gene associated factors) becomes stably associated with chromatin within 10 min of TCR engagement and have been hypothesized to mediate chromatin remodelling by a PIP2-dependent change in actin organization (Rando *et al*, 2002). It will important to explore the possible changes in condensin complexes during lymphocyte activation and the possible role of remodelling complexes, such as the BAF complex, in these changes.

Materials and methods

T-cell isolation and culture

Spleens from 6- to 8-week-old C57B/6, *Stat5*^{-/-} or *nessy* mice were processed into a single-cell suspension. Total T cells were isolated by negative selection via AutoMACS using the mouse Pan T kit (Miltenyi Biotech) following the manufacturer's protocol. Subpopulations of peripheral T cells were isolated by supplementing the Pan T kit with biotin-labelled antibodies to CD25, CD4 or CD8 (BD Biosciences). Purities always exceeded 95% on the live cell gate. Purified T cells were cultured in T-cell media consisting of RPMI 1640 media supplemented with 10% FBS, 10 mM Hepes (pH 7.0), 2 mM L-glutamine, 1 mM sodium pyruvate, 1 × non-essential amino acids, penicillin, streptomycin (all from Life Technologies) and 50 μM β-mercaptoethanol (Sigma). In all experiments, cells were seeded at 2 × 10⁶/ml in T-cell media. In all IL-2 response assays, naive T cells were stimulated with 1000 U/ml recombinant human IL-2 (Chiron) to bypass the requirement of the IL-2R α chain (Willerford *et al*, 1995). In all assays, anti-CD3 antibodies (2C11; BD Biosciences) were used at 1 μg/ml. To generate activated T cells, splenocytes were stimulated with anti-CD3 antibodies and 200 U/ml of rhIL-2 for 24 h. The cells were then washed twice in PBS containing 2% FBS and grown in T-cell media supplemented with 200 U/ml rhIL-2 for 5 to 7 days. Cells were growth arrested by overnight IL-2 withdrawal. Cycloheximide studies were performed by pretreating cells with 10 μg/ml Cycloheximide for 1 h.

Western blot and immunofluorescence

Whole-cell extracts were prepared by incubating cells in NP40 buffer (1% NP40, 50 mM Tris, pH 7.5, 100 mM NaCl, 50 mM NaF, 40 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM EDTA) supplemented with Complete Mini protease inhibitors (Roche) for 30 min at 4°C. Cell pellets were removed by centrifugation and equal volume of Laemmli sample buffer was added to the supernatants. In some experiments, pellets were reserved for subsequent acid extraction.

Chromatin-bound histones were isolated by acid extraction. Briefly, cells were lysed in Triton extraction buffer (TEB; PBS containing 0.5% Triton X-100, 2 mM PMSF, 0.02% Na₃N) for 10 min at 4°C. Pellets were washed in TEB and histones were extracted overnight in 0.2 N HCl at 4°C. pH of the supernatants was neutralized with NaOH and supplemented with Laemmli sample buffer. Either 40 μg (whole-cell extracts) or 5 μg (acid extracts) of protein were resolved via SDS-PAGE. Proteins were transferred to nitrocellulose membranes and blocked in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.1% Tween 20) containing 5% non-fat dry milk. Blots were incubated with primary antibodies overnight at 4°C followed by 1 h at room temperature, washed in TBST and then incubated with anti-rabbit IgG HRP-linked antibody (Cell Signaling Technologies) for 1 h. Proteins were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

For immunofluorescence, cells were allowed to adhere to microscope slides then fixed with 4% paraformaldehyde for 5 min. Cells were permeabilized with PBS containing 0.1% Triton X-100 and 2% FBS for 45 min at room temperature. Slides were stained with primary antibodies and then counterstained with AlexaFluor 488 donkey anti-rabbit or AlexaFluor 594 goat anti-mouse antibodies (Invitrogen). Slides were mounted with Vecta-shield mounting medium containing DAPI (Vector Labs).

Intracellular staining of H3K4me1 was performed in conjunction with surface staining for T-cell markers (CD4, Thy1.2 and CD25 all from BD Biosciences and TCRβ, CD44 and CD8 from eBioscience). Briefly, following surface staining, cells were washed 2 × with PBS + 2% FBS and fixed in 4% paraformaldehyde. After washing 2 × with PBS + 2% FBS cells were permeabilized/blocked in PBS + 2% FBS, + 0.02% Triton, + 0.2 μl/ml normal rabbit serum (Sigma). Cells were incubated with PE-coupled H3K4me1 antibodies. Following two washes with PBS + 2% FBS, cells were analysed on a FACSCalibur or LSRII (BD Biosciences).

The following additional antibodies were used in these studies: CD3ε (553057) from BD Biosciences; Thy 1.2 (ab49991), H3K4me1 (ab8895), H3K4me2 (ab7766), H3K4me3 (ab8580), H3K9/27me3 (ab8898), histone H3 (ab1791), histone H3 [Y173] (ab32151) and histone 3.3 (ab62642) all from Abcam; Acetyl H3 (06-599) from Upstate Biotechnologies; Pan-Actin (4968) and phospho-Stat5 (9531) from Cell Signaling Technologies. The H3K4me1 antibody was coupled to PE using the Lynx rapid RPE antibody conjugation kit following the manufacturer's instructions (AbD serotec). The Stat5a antiserum was previously described (Quelle *et al*, 1996). Antiserum recognizing Stat5b was prepared by immunizing rabbits with a synthetic peptide corresponding to amino acids 773–786 of Stat5b. Antisera for Stat5a and Stat5b were affinity purified using columns containing these peptides coupled to sepharose. The purified Stat5a and Stat5b antisera were used as a cocktail to recognize total Stat5.

All RNA were isolated using the RNeasy mini kit with Qiashredder and on-column genomic DNA digest (Qiagen) following the manufacturer's protocol. Quantitative RT-PCR was done using TaqMan EZ RT-PCR reagents (Applied Biosystems) on either an ABI Prism 7700 sequence detector (Applied Biosystems) or an iCycler (Bio-Rad). Taqman Gene Expression Assays (Applied Biosciences) used in these studies are listed in Supplementary Figure S7. All amplifications were performed in triplicate with 25 ng total RNA. A standard curve consisting of five-fold serial dilutions (ranging from 250 to 0.4 ng) of total RNA from growing cultures of T cells was included in each assay.

Chromatin immunoprecipitation

Cells (20 × 10⁶) were crosslinked by addition of 1% formaldehyde and washed 2 × with PBS containing 2% FBS and complete proteinase inhibitors (Roche) and resuspended in 400 μl of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) supplemented with proteinase inhibitors. Lysates were sonicated (15 μm amplitude) on an MSE Soniprep 150 to generate fragments between 200 and 700 bp, verified by agarose gel electrophoresis (for naive cells this required 11 × 15 s pulses; for activated cells, this required 5 × 10 s pulses). Supernatants were diluted 1:10 in dilution buffer (0.01% SDS 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl) supplemented with proteinase inhibitors and divided into triplicate IPs, 10 μl was reserved as input. Each IP was precleared with 40 μl of Salmon Sperm DNA/Protein A 50% agarose bead slurry (Upstate) and normal rabbit serum (Sigma). The supernatants were incubated with antibodies overnight at 4°C and then incubated for 1 h with 60 μl of agarose bead slurry at 4°C. The beads were then washed and immune complexes were eluted according to the Chip Assay Kit protocol (Upstate). Crosslinks were reversed by incubating with 4 μl of 5 M NaCl for 4 h at 65°C. In all, 2 μl of 0.5 M EDTA, 4 μl of 1 M Tris-HCl, pH 6.5 and 2 μl of 20 mg/ml proteinase K (Fisher BioReagents) was added to each eluate and incubated at 45°C for 1 h. DNA was purified from the eluates using the PCR-Clean up kit (Qiagen). Inputs and immunoprecipitated DNA samples were quantified in triplicate by real-time PCR using SYBR Green chemistry (Qiagen) compared with a standard curve generated using 10-fold serial deletions of sonicated genomic DNA. PCR conditions were 15 min at 95°C followed by 40 cycles of 95°C (15 s), 60°C (30 s) and 72°C (30 s). Primers used in these studies are listed in Supplementary Figure S7. Data are represented as the ratio of immunoprecipitated DNA to that of input DNA. Binding specificity to the *Cis* locus was verified via amplification of a non-specific site downstream of the *Cis* promoter.

Sonication assay

Cells (20 × 10⁶) were treated as in ChIP, except all samples were sonicated with 5 × 10 s pulses. Following sonication, crosslinks were reversed by addition of 8 μl 5 M NaCl and incubated 4 h at 65°C. Samples were then extracted with PCI (Fisher Scientific) and

DNA was precipitated in EtOH and resuspended in 30 µl of TE. In all, 3 µl of 20 ng/ml RNaseA was added for 30 min at room temperature prior to resolving on a 2% agarose gel. DNA fragments were visualized by ethidium bromide staining.

Transmission electron microscopy

In all, 1×10^7 cells were fixed in sodium cacodylate buffer containing 2.5% glutaraldehyde (Tousimis Research Corporation) for 2 h at room temperature. Cells were then washed with 0.2 M cacodylate buffer in 5% sucrose, postfixed in 1% OsO₄, embedded, sectioned and viewed by TEM.

Bisulfite sequencing

Genomic DNA was isolated and bisulfite modified using the Epitect Bisulfite Kit (Qiagen) following the manufacturer's instructions. The *Cis* proximal promoter was then amplified via nested PCR. Primers used in these studies are listed in Supplementary Figure S7. Cycling conditions were 10 min 95°C followed by 40 cycles of 95°C (30 s), 48°C (30 s) and 72°C (1 min) followed by 10 min at 72°C. One-tenth of the reaction was used in a second round of PCR amplification. The PCR conditions were the same as the first round, except there were 35 cycles and the annealing temperature was 46°C. PCR products were purified, TOPO cloned (Invitrogen) and individual clones were sequenced.

Statistical methods

Error bars in gene expression studies indicate standard deviation. Error bars in ChIP and flow cytometry experiments indicate

standard error of the means. All *P*-values were calculated using paired, two-tailed Student's *t* test.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: JSR designed experiments, performed experiments, analysed the data and co-wrote the paper; MG provided the micro-array data; PGT designed experiments, performed experiments and analysed the data; JN1 designed experiments, analysed the data and co-wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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