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## Immature CD4<sup>+</sup>CD8<sup>+</sup> Thymocytes and Mature T Cells Regulate Nur77 Distinctly in Response to TCR Stimulation

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# Immature CD4<sup>+</sup>CD8<sup>+</sup> Thymocytes and Mature T Cells Regulate Nur77 Distinctly in Response to TCR Stimulation<sup>1</sup>

Nicole R. Cunningham,<sup>2</sup> Stephen C. Artim,<sup>2</sup> Christen M. Fornadel,<sup>2</sup> MacLean C. Sellars,<sup>2</sup> Samuel G. Edmonson, Grant Scott, Frank Albino, Akriti Mathur, and Jennifer A. Punt<sup>3</sup>

The orphan steroid receptor, Nur77, is thought to be a central participant in events leading to TCR-mediated clonal deletion of immature thymocytes. Interestingly, although both immature and mature murine T cell populations rapidly up-regulate Nur77 after TCR stimulation, immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes respond by undergoing apoptosis, whereas their mature descendants respond by dividing. To understand these developmental differences in susceptibility to the proapoptotic potential of Nur77, we compared its regulation and compartmentalization and show that mature, but not immature, T cells hyperphosphorylate Nur77 in response to TCR signals. Nur77 resides in the nucleus of immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes throughout the course of its expression and is not found in either the organellar or cytoplasmic fractions. However, hyperphosphorylation of Nur77 in mature T cells, which is mediated by both the MAPK and PI3K/Akt pathways, shifts its localization from the nucleus to the cytoplasm. The failure of immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes to hyperphosphorylate Nur77 in response to TCR stimulation may be due in part to decreased Akt activity at this developmental stage. *The Journal of Immunology*, 2006, 177: 6660–6666.

Immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes are screened against autoreactivity during their development in the thymus via a variety of mechanisms. Clonal deletion is perhaps the best appreciated of these processes and results in apoptosis of cells expressing T cell receptors that bind thymic self-Ags with high affinity. Thymic deletion can be mimicked *in vitro* by exposing autoreactive T cells to APCs expressing cognate Ag (1) or by engaging both the TCR and CD28 molecules with immobilized Abs (2–4). Notably, the same high-affinity TCR/CD28 costimulatory interaction that kills CD4<sup>+</sup>CD8<sup>+</sup> (double-positive, DP)<sup>4</sup> thymocytes leads to proliferation and differentiation of mature T cells (5). The molecular basis for the markedly different outcomes of TCR/CD28 signaling cascades in immature and mature T cells is unknown; however, investigators have identified multiple molecules that are important players in the proapoptotic signaling pathway.

The transcription factor, Nur77, appears to play a key role in TCR-mediated death of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. Thymocytes constitutively expressing Nur77 undergo apoptosis (6) and dominant negative Nur77 constructs can inhibit TCR-mediated apoptosis in T cell hybridomas (7, 8) and Ag-induced apoptosis in thymocytes (6, 9). Consistent with the need for costimulatory signals to induce clonal deletion, up-regulation of Nur77 *in vitro* requires TCR and CD28 coengagement (10, 11).

The activity of Nur77 is regulated transcriptionally and by post-translational modification, specifically phosphorylation (12–17). Both the MAPK and PI3K/Akt signaling circuits have been shown to induce Nur77 phosphorylation in a variety of tissues, influencing both its subcellular location and function (15, 18, 19). MAPK phosphorylation of the N-terminal region may regulate the nuclear export of Nur77 (16, 20). ERK2 can directly phosphorylate Nur77 at Thr142 in the N-terminal region, although the functional significance of this event is unclear (21). Akt phosphorylation of the A-box serine residue in the DNA binding domain is thought to antagonize the DNA binding ability of Nur77 and may promote its association with cytoplasmic 14-3-3 in T cell lines (17, 18). Other kinases, including pp90<sup>RSK</sup>, also have the capacity to phosphorylate Nur77 at the same A-box residue (15, 19).

How Nur77 regulates apoptosis remains controversial. Some studies suggest that its transcriptional activity is critical, while others suggest that its interactions with mitochondrial proteins are responsible for its prodeath activity. Specifically, investigators have shown that TCR-mediated T cell apoptosis is abrogated when the ability of Nur77 to bind DNA is impaired (22). Studies showing that interactions with the retinoic acid receptors, RAR $\alpha$  and RXR $\alpha$ , inhibit both the trans-activation activity of Nur77 and its ability to induce death are consistent with the implication that Nur77 regulates apoptosis via its ability to regulate gene transcription (23, 24). Finally, Nur77 overexpression has also been associated with the transcription of proapoptotic genes in thymocytes (23).

However, Nur77 may also promote apoptosis by translocating to the mitochondria, interacting with Bcl-2 and provoking cytochrome *c* release (25–30). This phenomenon was first described among cancer cell lines, but it may also apply to some lymphocytes (31).

To improve our understanding of the role Nur77 plays in thymic development and to provide insights into the divergent signaling pathways that control cell fate, we compared Nur77 regulation in immature and mature T cells. Our results reveal that, while both immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes and mature T cells up-regulate Nur77 in response to TCR/CD28 costimulation, they regulate its

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<sup>4</sup> Abbreviations used in this paper: DP, double positive; SP, single positive; RT, room temperature; GSK-3, glycogen synthase kinase-3.

phosphorylation and compartmentalization distinctly. Specifically, we show that Nur77 is hyperphosphorylated by PI3K- and MAPK-mediated signals in mature, but not immature, T cells, and that this phosphorylation induces a shift in localization of Nur77 from the nucleus to the cytoplasm. Nur77 remains underphosphorylated in immature thymocytes and resides in their nuclei, rather than their mitochondria. We also find that developmental differences in TCR-mediated phosphorylation of Nur77 reflect developmental differences in upstream signaling pathways, specifically those related to Akt.

## Materials and Methods

### Antibodies

mAbs anti-CD2 (RM2-5), anti-CD28 (37.51), anti-TCR  $\beta$ -chain (H57-597), FITC anti-IgG1 (A85-1), anti-Nur77 (12.14), anti-human CD9 (M-L13), anti-E2F1, anti-actin, and anti-Bim were purchased from BD Biosciences. Anti-14-3-3 was purchased from Abcam. Anti-HSP-60 (N-20) was obtained from Santa Cruz Biotechnology. Anti-Akt, P-Akt, P-glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), and phospho-Akt substrate Abs were purchased from Cell Signaling Technology.

### Mice

Female C57BL/6 mice were purchased from Taconic Farms, and tissue was harvested from mice <6 mo of age. Our studies have been reviewed and approved by Haverford College's Institutional Animal Care and Use Committee.

### Isolation of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes and T cells

CD4<sup>+</sup>CD8<sup>+</sup> thymocytes and T cells were purified by magnetic cell sorting (Miltenyi Biotec). Harvested thymocytes were labeled with anti-mouse CD8 $\alpha$  (Ly-2) MicroBeads and positively selected via a MidiMACS separator. Harvested lymphocytes and splenocytes were labeled with mouse anti-CD90 (Thy1.2) MicroBeads and positively selected. Recovered cells were >90% CD4<sup>+</sup>CD8<sup>+</sup> and >95% T cells, respectively.

### Cell stimulation

Twenty-four- or 6-well plates were coated with anti-TCR (10  $\mu$ g/ml), anti-CD2 (10  $\mu$ g/ml), and anti-CD28 (50  $\mu$ g/ml), as indicated. Note that we include anti-CD2 or anti-CD4 in our stimulation mixture of immobilized Abs so that Ick, which is sequestered away from the TCR-signaling complex in freshly isolated thymocytes, can be recruited to provide optimal stimulation of DP thymocytes (32, 33). For stimulation lasting 1 h or longer, cells were suspended at a concentration of  $10 \times 10^6$ /ml in culture medium (RPMI 1640, 10% FCS, 2 mM L-glutamine, 1 mM penicillin/streptomycin, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and  $5 \times 10^{-5}$  M 2-ME). A total of  $1 \times 10^6$  cells and  $10 \times 10^6$  was distributed per well in 24- and 6-well plates, respectively, and was incubated for indicated times (37°C, 5% CO<sub>2</sub>) in the presence or absence of inhibitors: 5  $\mu$ M dequelin (Sigma-Aldrich), 50  $\mu$ M LY294002 (Calbiochem), 50  $\mu$ M PD98059 (Calbiochem), and 10  $\mu$ M U0126 (Promega). For acute stimulation, purified cells were rested in culture medium for 1–2 h at 37°C. Cells were then washed in PBS/1% BSA, resuspended at a concentration of  $10 \times 10^6$  cells per ml, and incubated at 4°C for 20 min with a mixture of biotinylated mAbs (anti-TCR $\beta$ , anti-CD2, and anti-CD28; 5  $\mu$ g/ml each). Streptavidin (20  $\mu$ g/ml) was added to cross-link the Abs, and cell suspensions were mixed and incubated at 37°C for the times indicated.

### Nur77 Staining for flow cytometry

Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) while agitating (30 min, room temperature (RT)). Fixed cells were washed twice with staining medium, permeabilized with 0.1% Triton X-100 in PBS (15 min, RT), washed three times with internal staining medium (10% FBS, 0.05% NaN<sub>3</sub> in PBS), and incubated overnight at 4°C with anti-Nur77. Cells were incubated with FITC mAb anti-mouse IgG1 for 30 min at RT, washed, then resuspended in staining medium. Cells were analyzed on a FACSCalibur with CellQuest software (BD Biosciences).

### Cell fractionation

Fractionation experiments were performed using the ProteoExtract subcellular proteome extraction kit (Calbiochem) according to the manufacturer's instructions, using  $5 \times 10^6$  cells per sample.

### Western blot analysis

Cells were lysed at  $2 \times 10^8$  cells per ml with SDS lysis buffer (0.5% w/v SDS, 0.05 M Tris-Cl (pH 8.0), 100 mM DTT, 100 mM Na<sub>3</sub>VO<sub>4</sub>, protease inhibitor mixture, 10  $\mu$ M calyculin A). After 10-min incubation on ice, each lysate was sonicated and centrifuged (14,000  $\times$  g at 4°C). Lysates were separated by SDS-PAGE and transferred to Immobilon-Blot polyvinylidene difluoride membrane (Bio-Rad). Blots were blocked with Blotto (5% nonfat dry milk, 0.001% Tween 20 in PBS) for 1 h at RT, then washed with PBS-Tween (0.001% Tween 20 in PBS) and incubated with primary Abs in PBS-Tween with 1% BSA, rocking overnight at 4°C. Blots were washed with PBS-Tween and incubated with HRP-conjugated secondary Abs for 30 min at RT. Blots were visualized by ECL (Amersham Biosciences) and exposed on Hyperfilm (Amersham Biosciences) or using Supersignal West Femo substrate (Pierce), and the FluorChem SP (Alpha Innotech).

### Phosphatase assay

Cells were lysed with SDS lysis buffer, sonicated, and clarified before being diluted in phosphatase buffer (50 mM Tris-HCl, 100 mM NaCl, 2 mM DTT, 0.1 mM EDTA, and 0.01% Brij 35), and concentrated using Vivaspin concentrators (Vivascience). Lysates were incubated in the presence or absence of 400 U  $\lambda$ -phosphatase (New England Biolabs) for 15 min at 30°C.

### Akt kinase assay

Akt activities were analyzed using the Akt kinase assay kit (Cell Signaling Technology) according to the manufacturer's instructions. Briefly, after stimulation, cells were incubated in ice-cold cell lysis buffer plus protease inhibitors on ice for 10 min. Lysates were centrifuged (14,000  $\times$  g at 4°C), and supernatants were incubated with immobilized Akt Ab slurry (2.5 h at 4°C with gentle rocking). Suspensions were centrifuged and pellets washed with lysis buffer. Pellets were resuspended in kinase buffer with 200  $\mu$ M ATP and 1  $\mu$ g of GSK-3 fusion protein and incubated (30 min at 30°C) for the kinase reaction. Reactions were terminated with the addition of SDS sample buffer, centrifuged (14,000  $\times$  g at 4°C), and supernatants were harvested for Western blot analysis.

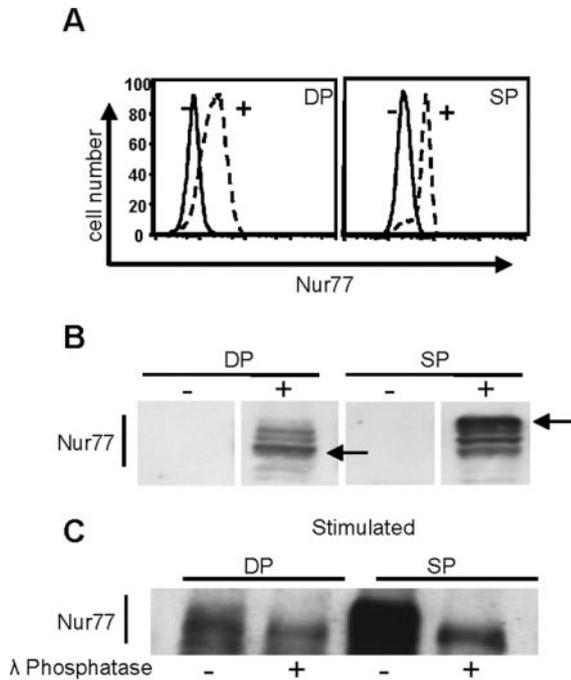
## Results

### Both immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes and mature T lymphocytes up-regulate Nur77 upon TCR and CD28 costimulation

Given that Nur77 has been shown to be a critical mediator of apoptosis and negative selection, we anticipated that its regulation would differ between immature thymocytes, which die upon TCR/CD28 costimulation, and mature T cells, which proliferate and differentiate in response to the same signals. We first compared the ability of immature and mature T cells to up-regulate Nur77. Both cell types express little if any Nur77 expression before stimulation and both rapidly up-regulate Nur77 after TCR/CD28 costimulation (Fig. 1A). We have shown previously that Nur77 expression peaks 2 h after TCR/CD28 stimulation of immature CD4<sup>+</sup>CD8<sup>+</sup> (DP) thymocytes and disappears by 5 h (10). Nur77 expression also peaks and declines with similar kinetics in stimulated mature single-positive (SP) T cells (data not shown). We therefore focused on this relatively early period after receptor stimulation in our comparative analysis of Nur77 regulation.

### Nur77 is differentially modified in mature SP T cells vs immature DP thymocytes

To explore the possibility that immature and mature T cells regulate Nur77 differently at the posttranslational level, we compared Nur77 expression by Western blot analysis (Fig. 1B). Our results show a distinct difference in the mobility of Nur77 in each subset of cells. Specifically, the dominant form of Nur77 in stimulated mature SP T cells migrates more slowly than the dominant form in stimulated immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes (Fig. 1B). Given that Nur77 is known to be the target of multiple kinases, these developmental differences in mobility suggested differences in



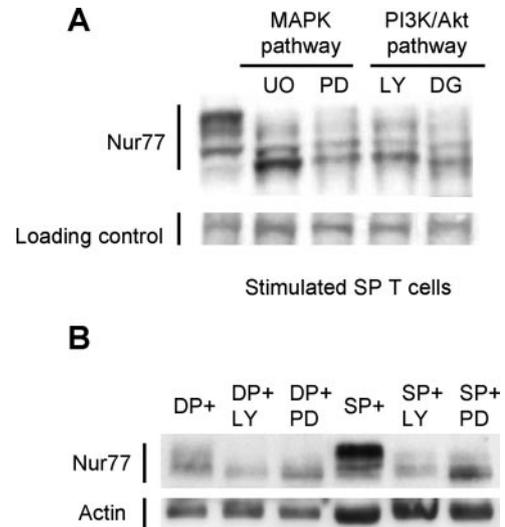
**FIGURE 1.** TCR/CD28 costimulation up-regulates Nur77 expression in both immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes and mature T cells, but results in different patterns of phosphorylation. *A*, Purified immature DP and mature SP T cells cultured for 2 h in the presence or absence of plate-bound anti-TCR/CD28 Abs were fixed, permeabilized, and stained with mAb specific for Nur77, followed by FITC-conjugated anti-mouse Abs, then analyzed by flow cytometry. Histogram plots of stimulated (+) and unstimulated (-) immature CD4<sup>+</sup>CD8<sup>+</sup> (DP) thymocytes and mature (SP) T cells are shown. *B*, Lysates from stimulated and unstimulated DP and SP cells were separated by SDS-PAGE (7%), and Western blots were probed with Abs to Nur77. Arrows indicate an upper band that dominates in SP T cells and a lower band that dominates in DP thymocytes. *C*, Purified immature DP thymocytes and mature SP T cells were stimulated with plate-bound TCR/CD28 for 2 h. Cell lysates were incubated in the presence (+) or absence (-) of  $\lambda$ -phosphatase. Western blots were performed and probed with anti-Nur77 Abs.

phosphorylation. Indeed, all bands compress to the fastest migrating form after lysates from stimulated T cell subsets were treated with  $\lambda$ -phosphatase (Fig. 1C).

#### *Akt and MAPK signaling pathways both contribute to Nur77 phosphorylation in mature T cells*

A number of kinases associated with the PI3K/AKT and MAPK signaling pathways are capable of phosphorylating Nur77. To determine whether either of these signaling cascades played a role in the differential phosphorylation of Nur77 in primary immature thymocytes and mature T cells, we evaluated Nur77 mobility after stimulating cells in the presence of multiple kinase inhibitors. Treatment of stimulated mature T cells with LY294002, a specific inhibitor of PI3 kinase, or deguelin, a relatively new PI3K/Akt inhibitor (34), inhibits the phosphorylation of Nur77. Inhibitors of MEK, PD98059 (35) and UO126 (36), also abrogate Nur77 phosphorylation (Fig. 2A). These data indicate that both Akt and MAPK pathways cooperate to phosphorylate Nur77 in mature T cells.

MAPK and PI3K signaling cascades may also regulate Nur77 in CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. When gel resolution is optimal, we observe a small increase in Nur77 mobility in CD4<sup>+</sup>CD8<sup>+</sup> thymocytes stimulated in the presence of either the PI3K inhibitor (LY) or the MEK inhibitor (PD) (Fig. 2B and data not shown). These



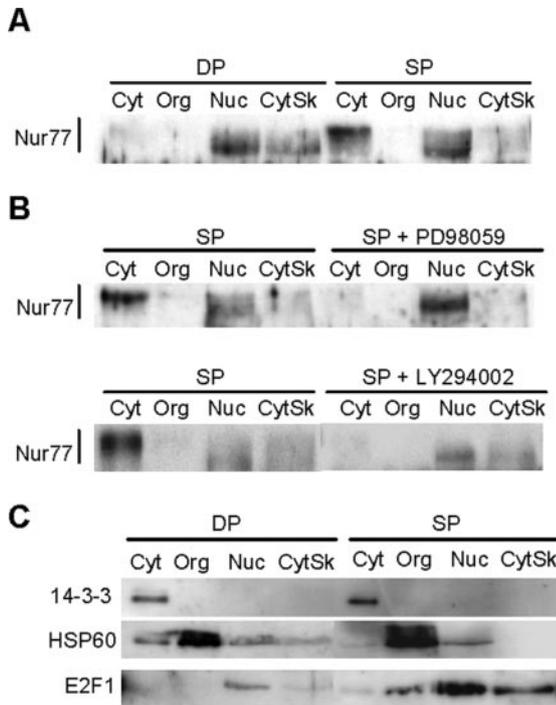
**FIGURE 2.** Phosphorylation of Nur77 in mature T cells is dependent on both MAPK and PI3K/Akt signaling pathways. *A*, Purified SP T cells were stimulated for 2 h with plate-bound anti-TCR/CD28 Abs in the presence or absence of inhibitors of the MAPK pathway (UO0126 (UO) and PD98059 (PD)) or the PI3K/Akt pathway (LY290042 (LY) and deguelin (DG)). Lysates were analyzed by Western blot and probed with anti-Nur77 Abs. A nonspecific band (at ~125 kD) serves as a loading control. *B*, Lysates from DP and SP T cells stimulated in the presence of LY290042 (LY) and PD98059 (PD) were run on the same SDS-PAGE gel and analyzed by Western blotting probed with Abs to Nur77. Blots were also probed with anti-actin Abs, which indicate that, although more protein was loaded in the SP vs DP samples, protein loading within each subgroup was similar. Results show that PI3K and MAPK inhibitors also influence Nur77 mobility DP thymocytes, albeit less dramatically.

observations suggest that Nur77 is also phosphorylated in immature thymocytes, albeit less extensively. We therefore refer to the dominant form of Nur77 in mature T cells as hyperphosphorylated and the dominant form of Nur77 in immature T cells as hypophosphorylated or underphosphorylated.

#### *The phosphorylation status of Nur77 influences its intracellular localization*

To explore the functional consequences of phosphorylation of Nur77, we compared the subcellular localization of Nur77 in immature and mature T cells (Fig. 3A). Underphosphorylated forms of Nur77 are present in the nuclear fractions of stimulated CD4<sup>+</sup>CD8<sup>+</sup> thymocytes and mature T cells. In contrast, hyperphosphorylated Nur77 is found only in the cytoplasmic fraction of mature T cells. Its localization in the cytoplasm is dependent on phosphorylation: when mature T cells are stimulated in the presence of either a PI3K or MAPK inhibitor, Nur77 remains nuclear (Fig. 3B). The validity of the fractionation was verified by blotting fractions from immature and mature T cells for proteins known to be in the indicated subcellular compartments (Fig. 3C).

The absence of Nur77 from the organellar fraction of stimulated immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes was notable, given speculation that it exerts its proapoptotic activity by interacting with members of the bcl-2 family members at the outer mitochondrial membrane. To examine the possibility that its subcellular localization shifts over time, we performed a time-course experiment, examining cell fractions for Nur77 over the full 4-h period during which Nur77 is present after stimulation (Fig. 4). Our data confirm that Nur77 expression peaks at ~2 h after costimulation, declining thereafter. Most importantly, they indicate that the compartmentalization of

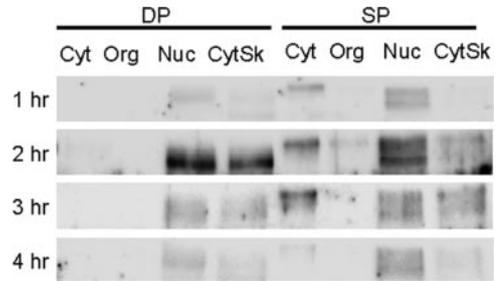


**FIGURE 3.** Subcellular localization of Nur77 differs between immature and mature T cells and is regulated by phosphorylation. *A*, T cells were stimulated with plate-bound anti TCR/CD28 for 2 h, and lysates were separated into cytoplasmic (Cyt), membrane/organelle (Org), nuclear (Nuc), and cytoskeletal (CytSk) fractions. Samples were analyzed by Western blotting and probed with Abs against Nur77. Results indicate that the hyperphosphorylated version of Nur77 is found in the cytoplasm of mature T cells, and that the underphosphorylated version is found in the nuclear (and cytoskeletal) fractions of both mature and immature T cells. *B*, Mature T cells were stimulated for 2 h in the presence or absence of MAPK inhibitor PD98059 or the PI3K inhibitor LY290042. Cell fractions were analyzed by Western blotting and probed with Abs to Nur77. Results indicate that hyperphosphorylation of Nur77 via PI3K and MAPK pathways is required for its translocation to the cytoplasm in mature T cells. *C*, Subcellular fractions from mature SP and immature DP cells were blotted with Abs against 14-3-3, HSP60, and E2F1, controls for cytoplasmic, mitochondrial, and nuclear fractionation, respectively.

Nur77 does not change in immature (or mature) T cells over time; specifically, Nur77 does not appear in the organellar fraction that includes the mitochondria.

It is important to note that we variably observe hypophosphorylated Nur77 in the cytoskeletal fractions of both the immature and mature T cells. Although this may suggest that Nur77 can associate with cytoskeletal elements, the significance of this finding is questionable. This fraction is the last to be isolated in the protocol and may be the least pure of the group. Indeed, as data in Fig. 3C show, proteins known to be confined to the nucleus (e.g., E2F1) can also be detected in this cytoskeletal fraction in both immature and mature T cells.

These results showing phosphorylation-dependent compartmentalization are consistent with previous observations that Akt and MAPK signaling encourages translocation of Nur77 from the nucleus to the cytoplasm. They also suggest that Nur77's proapoptotic effect in immature thymocytes is due to its activity in the nucleus, an observation consistent with experiments showing that Nur77's DNA binding capacity is critical to its proapoptotic function among thymocytes.



**FIGURE 4.** Subcellular compartmentalization of Nur77 does not change over time after TCR costimulation of immature and mature T cells. Purified DP and SP T cells were stimulated with plate-bound anti-TCR/CD28 for the times indicated and lysates were separated into cytoplasmic (Cyt), membrane/organelle (Org), nuclear (Nuc), and cytoskeletal (CytSk) fractions. Samples were analyzed by Western blotting and probed with Abs against Nur77. Results indicate that Nur77's subcellular distribution does not change over time.

#### *Akt is more active in mature T cells than in immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes*

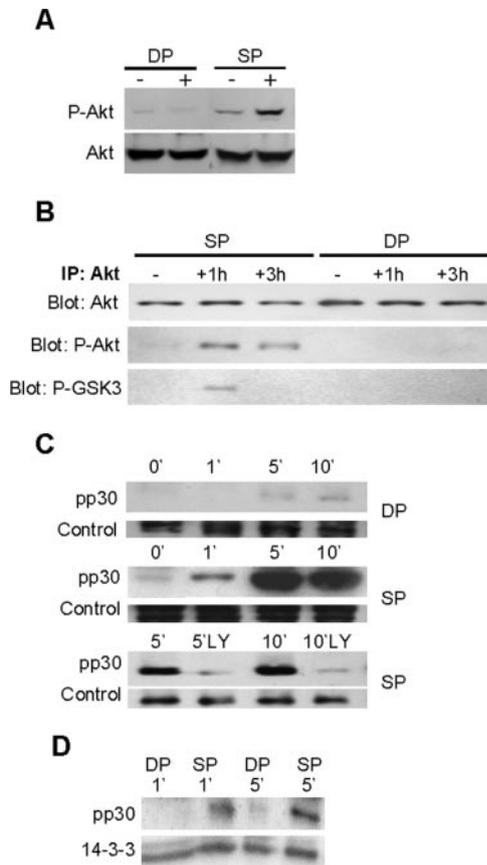
Not only can differences in Nur77 phosphorylation reveal clues about its function in developing T cells, but they may also point to key differences in upstream signaling pathways that lead to the very distinct fates of immature and mature T cells. We focused our attention on the activity of the Akt pathway in immature and mature T cells. In addition to its role in phosphorylating Nur77 in mature T cells, Akt plays a well-characterized global role in antagonizing apoptosis and enhancing metabolic processes that encourage cell survival.

Akt activation is dependent on phosphorylation, first at Ser473, then at Thr308. To assess Akt activity, we first compared Ser473 phosphorylation in TCR/CD28 stimulated immature and mature T cells. Our results show that, although Akt is present at comparable levels in immature and mature T cells, mature T cells phosphorylate it more efficiently after stimulation (Fig. 5A). To determine whether differences in phosphorylation truly reflected differences in activity, we performed an *in vitro* kinase assay by incubating an Akt substrate, recombinant GSK-3 $\beta$ , with Akt immunoprecipitated from stimulated mature T cell and immature CD4<sup>+</sup>CD8<sup>+</sup> thymocyte lysates. Phosphorylated GSK-3 is observed only in Akt samples from stimulated mature T cells (Fig. 5B). As expected, immunoprecipitated Akt is also more efficiently phosphorylated at Ser473 in mature T cells than in immature thymocytes.

A general decrease in Akt activity would presumably lead to a decrease in phosphorylation of targets other than Nur77. Consistent with this prediction, Western blots with Abs specific for phosphorylated Akt substrates reveal that a 30-kDa substrate, identified by others as S6 ribosomal protein (37), is phosphorylated in a PI3K dependent manner in stimulated mature T cells, but not immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes (Fig. 5, C and D). Preliminary data also indicate that Bim, a proapoptotic BH3 family member known to play a key role in thymocyte clonal deletion and shown to be phosphorylated at Ser<sup>87</sup> by Akt (38), is more efficiently phosphorylated in mature vs immature thymocytes (data not shown).

## Discussion

Our results show that Nur77 is up-regulated by TCR/CD28 costimulation in both mature T cells and immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, but is hyperphosphorylated only in mature T cells. Phosphorylation of Nur77 depends on signaling through both the PI3K/Akt and MAPK pathways and results in a change in subcellular compartmentalization: whereas the hypophosphorylated form of



**FIGURE 5.** Akt is differentially activated in mature T cells vs immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. *A*, Western blots with Abs specific for phosphorylated Akt (Ser473) (*upper panel*) or Akt (*lower panel*) were performed on lysates from purified immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes (DP) and mature T cells (SP) cultured for 1 h in the presence (+) or absence (-) of anti-TCR/CD28 plate-bound Abs. Mature SP T cells phosphorylate Akt more effectively than immature DP thymocytes. *B*, Akt was immunoprecipitated from lysates of DP and SP cells cultured in the presence (+) or absence (-) of plate-bound anti-TCR/CD28 Abs for the times indicated. A direct Akt kinase assay was performed with immunoprecipitates using recombinant GSK-3 $\beta$  fusion protein as substrate. Lysates were separated by 10% SDS-PAGE, and Western blot analyses were performed on the reaction mixture with Abs specific for Akt (*top panel*), phosphorylated Akt (*second panel*), and phosphorylated GSK-3 $\beta$  (*third panel*). *C*, Lysates from purified DP and SP cells stimulated with plate-bound anti-TCR/CD28 Abs for the times indicated were analyzed by Western blotting and probed with anti-Akt substrate Ab. The 30-kDa substrate, identified as S6 ribosomal protein, was more efficiently phosphorylated in SP (*middle panel*) vs DP (*upper panel*) cells. The dependency of this event on the Akt pathway was verified by performing Western blotting on lysates from SP T cells stimulated in the presence or absence of the PI3K inhibitor LY240092 (LY). Nonspecific bands in the 70- to 90-kDa range serve as loading controls. *D*, Lysates were prepared as described in *C* and blotted with anti-Akt substrate Ab and anti-14-3-3 as a more rigorous loading control.

Nur77 is found in the nucleus of both mature and immature T cells, hyperphosphorylation of Nur77 in mature T cells induces its translocation to the cytoplasm.

These data are consistent with elegant experiments performed with T cell hybridomas showing that Akt-mediated phosphorylation of Nur77 resulted in its sequestration to the cytosol (17). Our data extend these findings by showing that primary T cells also phosphorylate Nur77 via Akt- and MAPK-associated pathways; furthermore, they reveal that phosphorylation and compartmentalization of Nur77 are actually developmentally regulated in primary immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes and mature T cells.

Which kinase or kinases are specifically responsible for phosphorylating Nur77 in primary T cells? Akt and ERK2 are attractive candidates: both are known to target Nur77 in various cell types and our findings that PI3K and MAPK inhibitors abrogate Nur77 phosphorylation are consistent with their potential involvement. Given that both sets of inhibitors influence Nur77 identically, increasing its mobility so that it runs at the level of what we presume is an unphosphorylated form, our results raise the possibility that Akt and MAPK pathways converge on a downstream kinase that phosphorylates Nur77 directly. pp90<sup>rsk</sup> is a particularly appealing contender for this role: it is capable of phosphorylating Nur77 and may require both MAPK and PI3K/Akt signaling to be fully activated (15). Data indicating that PI3K pathways can regulate ERK activity in thymocytes (39) also raises the possibility that ERK, alone, is responsible for the posttranslational modifications seen in Nur77. This pathway could conceivably be responsible for the hint of PI3K- and MAPK-dependent Nur77 phosphorylation that we see in immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes.

Interestingly, thymocytes expressing overactive forms of Akt (i.e., from a gag-Akt transgenic (40) and the floxed PTEN mutant crossed to CD4-Cre (41)) did not hyperphosphorylate Nur77 when stimulated; instead, they exhibited the same phosphorylation pattern as wild-type thymocytes (data not shown). These data are consistent with our indications that the Akt and ERK pathways cooperate to phosphorylate Nur77.

Although other studies have indicated that Nur77 can mediate apoptosis by translocating to the mitochondria, our data definitively show that Nur77 remains in the nucleus of TCR/CD28 stimulated CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. Our own immunofluorescence studies are consistent with this conclusion; we see no evidence for the colocalization of Nur77 with the mitochondria (data not shown).

Together, our data suggest a model in which Nur77 induces and/or enhances TCR-mediated cell death in immature thymocytes by regulating a proapoptotic transcriptional program. Our results also suggest that in mature T cells, multiple phosphorylation events inhibit DNA binding by inducing Nur77 translocation to the cytosol. We propose that phosphorylation at the A-box serine (Ser<sup>350</sup>) is of particular importance in mature T cells. Matsuyama et al. (17) have, in fact, shown that phosphorylation at Ser<sup>350</sup> is responsible for sequestering Nur77 in the cytosol by facilitating its interaction with cytosolic 14-3-3 in T cell hybridomas (an association that we have not yet been able to detect in primary T cells). They have also shown that Akt could no longer rescue rat fibroblast lines from Nur77-induced cell death when Ser<sup>350</sup> was mutated, confirming the relationship between Nur77 phosphorylation and apoptosis (17). We have made an effort to determine whether the hyperphosphorylated version of Nur77 is regulated at this key site using Abs described as specific for phospho-Nur77 (Ser<sup>350</sup>); however, we have not found these Abs to be reliable. Definitive determination of the importance of Ser<sup>350</sup> in mediating TCR-induced apoptosis will clearly require biochemical analysis of Nur77 isolated from primary T cells and the development of genetic mouse models.

Given Nur77's proapoptotic influence, why might it be expressed in mature T cells at all? It is important to note that Nur77 does not invariably act as a promoter of apoptosis. In some cellular contexts, Nur77 overexpression positively regulates cell proliferation (26, 27, 42), suggesting that Nur77 may not be a committed executioner, but instead may act more variably on the orders of other signaling molecules to enforce cell fate. The Nur77 that remains in the nucleus of mature SP T cells could complex with distinct proteins and bind to different promoter elements. On the other hand, it is also possible that, by keeping Nur77 available yet

inactive, mature T cells provide themselves with a quick way to abort a proliferative program.

Developmental differences in Nur77 phosphorylation may not only improve our understanding of the downstream events that specifically cause TCR-mediated apoptosis and negative selection. Our data also indicate that they can provide us with clues about the developmentally regulated differences in upstream signaling cascades and may help reveal the molecular basis for the dramatic difference in immature and mature T cell fate following TCR stimulation.

ERK activity has been studied extensively in CD4<sup>+</sup>CD8<sup>+</sup> thymocyte populations, and a consensus is emerging that negative selection signals result in a strong but short burst in ERK activity, whereas positive selection signals result in weaker but more sustained ERK activity (5). Our preliminary data (not shown) also suggest that TCR/CD28 stimulation inspires distinct patterns of ERK activity in immature CD4<sup>+</sup>CD8<sup>+</sup> vs mature SP T cells.

We focused our experimental attention on another principal regulator of Nur77 phosphorylation, Akt, which has been less thoroughly studied in developing T cells. We provide direct evidence that Akt activity differs between TCR-stimulated immature and mature T cells: CD4<sup>+</sup>CD8<sup>+</sup> thymocytes do not phosphorylate or activate Akt as efficiently as mature T cells in response to TCR/CD28 stimulation. These observations have broad implications because of Akt's central role in cell growth, metabolism, and survival.

Developmental differences in Akt activity could be due to differences in the activity of upstream signaling cascades (e.g., PI3K, PDK1, PDK2) and/or differences in activity of key negative regulators (e.g., phosphatases). Previous data from our lab showing that immature thymocytes do not efficiently polarize lipid microdomains, which are enriched for the lipid substrates of PI3K that regulate localization of Akt and PDK2, are consistent with the former possibility (43). However, the role of phosphatases may also be critical, and we are currently investigating their influence on Akt activation in developing T cells. Given that differences in ERK activity may also underlie developmental distinctions in responsiveness to T cell signaling, it may be particularly important to focus on regulators that have a more general effect on signaling networks.

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## Disclosures

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