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Original article

Effective T-cell immune responses in the absence of the serine/threonine kinase RIP2

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Abstract

The serine/threonine kinase RIP2 has been reported to be essential for Nod1 and Nod2 mediated cell activation, and has been suggested to play a role in the signaling cascade downstream of the T-cell receptor. We sought to ascertain the exact role of RIP2 in T-helper cell differentiation and $CD8^+$ T-cell effector function *in vivo* and *in vitro*. In contrast to previous reports, we found that RIP2-deficient T cells did not exhibit impaired proliferation upon TCR engagement *in vitro*, and differentiation to cytokine producing Th1 or Th2 cells was normal in the absence of RIP2. These results were confirmed *in vivo*, as wild-type and RIP2-deficient virus-specific $CD8^+$ T cells expanded comparably in mice after LCMV infection. Wild-type and RIP2-deficient $CD4^+$ and $CD8^+$ T cells from infected mice also showed similar proliferation and cytokine production when restimulated with full or partial agonist peptides *ex vivo*. Furthermore, no significant difference in adaptive T-cell responses could be observed between wild-type and RIP2-deficient mice after *Listeria monocytogenes* infection. Thus contrary to early reports, our data show that RIP2 is not an essential component of the TCR signaling machinery.

Keywords: Receptor-interacting protein 2; T cell

1. Introduction

Protective immune responses against invading pathogens are typically driven by an early innate immune response followed by a more specific T- and B-cell mediated adaptive immune response. Naïve T-cell activation is initiated upon recognition of antigen in the context of MHC by the T-cell receptor (TCR). Once the TCR is triggered, signaling machinery is assembled that leads to the activation of NF-κB; a transcription factor controlling the expression of many genes required for the effector function, proliferation and survival of the T cell. Specifically, TCR engagement leads to Protein Kinase C-θ activation [1], followed by the sequential assembly of a complex formed by the molecules CARMA1, Bcl10 and MALT1 [2]. This complex is involved in the activation of the TRAF molecules TRAF2 and TRAF6, leading to the recruitment of the IKK complex, which regulates NF-κB nuclear translocation by phosphorylating and activating the NF-κB inhibitor, IκB [3]. It has previously been described that TRAF6 directly ubiquitylates the IKKγ subunit [4], whereas the kinase TAK1 has been shown to phosphorylate IKKβ [5], thereby leading to activation of the IKK complex and ultimately NF-κB.

Receptor-interacting protein 2 (RIP2/RICK) is a widely expressed serine/threonine kinase which has recently been shown to be a key effector molecule mediating signaling downstream of the Nod-like receptors, Nod1 and Nod2 [6]. Although RIP2 has previously been implicated in inducing inflammation after Toll-like receptor stimulation, recent data indicate that it is not required for this process [6–9]. RIP2 has also been reported to play a central role in TCR signaling; specifically, RIP2-deficient T cells were shown to have impaired proliferative capacity when stimulated with anti-CD3 antibodies *in vitro* [7,8,10]. An association between RIP2 and several molecules involved in TCR signaling has also

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been suggested in some reports. In particular, RIP2 has been shown to co-precipitate with Bcl10 following TCR stimulation and to mediate its phosphorylation [10]. In addition, several *in vitro* studies suggest that RIP2 can associate with TRAF6 [11,12], IKKγ/NEMO [13,14], as well as TAK1 [15,16]. RIP2 appears therefore to be an important component of the signaling machinery assembled after TCR engagement and its absence has profound implications for T-cell function.

Many studies have produced conflicting results when comparing *in vitro* and *in vivo* findings. In the absence of the serine/threonine kinase PKC-θ for example, T cells exhibit an anergic phenotype *in vitro*, and *in vivo* Th2 immune responses are impaired. In comparison, upon activation in an *in vivo* Th1/CTL inflammatory setting these cells exhibit normal effector function and survival [17–20]. Thus, it is important to rigorously study T-cell responses *in vitro* and *in vivo* before drawing firm conclusions. Our current study aimed to clarify the extent to which RIP2 played a role in inducing T-cell effector function *in vitro* and *in vivo*. In contrast to early reports, our results show that *in vitro* T-cell proliferation as well as T-helper cell differentiation to Th1 or Th2 cell subsets was unaffected by the absence of RIP2. Moreover, *in vivo* effector functions of RIP2-deficient $CD4^+$ and $CD8^+$ T cells upon specific peptide challenge, infection with *Listeria monocytogenes* or lymphocytic choriomeningitis virus was comparable to control wild-type T cells in all conditions tested. Taken together, RIP2 signaling appears to play a redundant role in the development of *in vitro* and *in vivo* T-cell immune responses.

2. Materials and methods

2.1. Mice

C57BL/6 and BALB/c wild-type mice were obtained from Charles River Breeding Laboratories. RIP2-deficient mice were kindly provided by Professor R. Flavell (Yale University, New Haven, CT, USA). SMARTA-2, lymphocytic choriomeningitis virus (LCMV) GP13 TCR transgenic mice (GLNGPD IYKGVYQFKSVEFD) have been described [18]. Both mouse strains were backcrossed to C57BL/6 background for >8 generations. Mice were maintained specific pathogen-free at BioSupport, Zürich in isolated ventilated cages. Animal used in experiments were between 8 and 10 weeks of age. All experiments were performed with permission from the Zürich Animal Ethics Committee.

2.2. Pathogens and reagents

LCMV isolate WE was originally obtained from Professor R.M. Zinkernagel (University Hospital, Zurich) and propagated on L929 cells. *Listeria monocytogenes* was kindly provided by Dr. Maries van den Broek (University Hospital, Zurich). Anti-CD3 and anti-CD28 antibody were purchased from eBioscience. LCMV glycoprotein peptides GP13, GP33, and GP33-V4Y [21,22] were purchased from NeoMPS. Phosphorothioate-modified CpG containing oligonucleotide was synthesized by Microsynth. The following oligonucleotide sequence was used 1668pt (5-TCC ATG ACG TTC CTGAAT AAT-3).

2.3. Proliferation assay

$CD4^+$ T cells were purified from splenic single-cell suspensions by magnetic separation (MACS, Milteny Biotech) and incubated with anti-CD3 antibody or dendritic cells and the specific antigen at different concentrations. Cells were cultured for 72 h at 37 °C with [^{3}H]thymidine added (1 μCi/well) for the last 12 h. Total [^{3}H]thymidine incorporation was measured as an indicator of cell proliferation. Alternatively, purified T cells were incubated with a final concentration of 2.5 μM CFSE (Molecular Probes) for 7 min, followed by extensive washing in media before culture and subsequent analysis of proliferation by CFSE dilution with flow cytometry.

2.4. T cell–DC coculture

$CD4^+$ and $CD8^+$ T cells were isolated from the spleens of transgenic SMARTA2 mice or LCMV infected mice by MACS bead separation. Splenic $CD11c^+$ dendritic cells from naïve mice were also isolated by magnetic separation. Isolated T cells ($6.5–10 \times 10^4$ cells/well) and DCs ($1.4–2 \times 10^4$ cells/well) were cultured in 96-well plates in the presence of GP13, GP33 or GP33-V4Y peptide at the indicated concentrations. Proliferation was assessed on day 3 as described in the previous section. Additionally, cells were activated on day 3 in the presence of PMA and ionomycin for 4 h (with the addition of Brefeldin A for the last 2 h). Interferon (IFN)-γ and interleukin (IL)-4 production was determined by intracellular cytokine staining and flow cytometry.

2.5. Staining of LCMV-GP33 specific $CD8^+$ T cells

Blood was collected in heparin containing tubes. After red blood cell lysis, cells were incubated with 10 μg/ml $GP33^-$ PE-conjugated MHC class I tetramers at 4 °C for 45 min. APC-labeled anti-CD8 monoclonal antibody (mAb) (eBioscience) was subsequently added for 20 min at 4 °C. Cells were washed and analyzed by flow cytometry.

2.6. Intracellular cytokine staining and FACS analysis

After stimulation with PMA/ionomycin, cells were washed with PBS/0.1% BSA and surface stained with FITC- or PercP-labeled anti-CD4 or PE-labeled anti-CD8 mAb (eBioscience). Next, cells were washed with PBS and fixed with 2% paraformaldehyde for 20 min at room temperature. Fixed cells were incubated in permeabilization buffer (0.5% saponin/PBS/0.5% BSA) containing PE-labeled anti-IL-4, APC-labeled anti-IFN-γ or FITC-labeled anti-tumor necrosis factor (TNF)-α mAb (eBioscience) for 30 min at room temperature. After washing in permeabilization buffer, cells were resuspended in PBS/0.1% BSA and analyzed by flow cytometry