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Dysfunctional memory CD8+ T cells after priming in the absence of the cell cycle regulator E2F4

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ABSTRACT

The transcriptional repressor E2F4 is important for cell cycle exit and terminal differentiation in epithelial cells, neuronal cells and adipocytes but its role in T lymphocytes proliferation and memory formation is not known. Herein, we investigated the function of E2F4 protein for the formation of functional murine memory T cells. Murine transgenic CD8+ T cells were infected *in vitro* with lentivirus vector expressing a shRNA targeted against E2F4 followed by *in vitro* stimulation with SIINFEKL antigenic peptide. For *in vivo* assays, transduced cells were injected into congenic mice which were then infected with HSV-OVA. The primary response, memory formation and secondary stimulation were determined for CD8+ lentivirus transduced cells. In the absence of E2F4 cell cycle repressor, activated CD8+ T cells underwent intensive proliferation *in vitro* and *in vivo*. These cells had the ability to differentiate into memory cells *in vivo*, but they were defective in recall proliferation. We show that transient suppression of E2F4 during CD8+ T cell priming enhances primary proliferation and has a negative effect on secondary stimulation. These findings demonstrate that the cell cycle repressor E2F4 is essential for the formation of functional memory T cells. A decrease in CD8+ T-lymphocyte compartment would diminish our capacity to control viral infections.

1. Introduction

Activated CD8+ T cells normally undergo many cycles of proliferation before differentiation into cytotoxic T lymphocytes (CTL). CTL deliver host protection through both cytokine synthesis and cytotoxic killing of antigen-expressing targets. At the end of the primary response, the majority of these CTL undergo apoptosis but a subset persists as memory cells. Memory cells appear to be derived mostly from cycling blast cells, which has given rise to the so-called "linear model" of CTL differentiation [1]. It follows from this model that more primary proliferation is always good for memory CD8+ T cell formation.

The link between antigen-driven proliferation and maturation to a memory phenotype has recently been questioned. First, long-lived CD8+ T cells that have undergone spontaneous proliferation in antigen-free hosts acquire some of the markers and properties of memory cells [2]. Second, during the immune response to the bacterium *Listeria monocytogenes*, H3M2- (non-classical MHC class I)-restricted CTL can form memory T cells without proliferation [3]. Memory-like activity may also be induced by the brief engagement of the T cell receptor, insufficient to cause clonal proliferation [4]. This argues that proliferation may not always be strictly necessary for the development of memory T cells.

The progression of cells through the G1 phase and into the S phase of the cycle operates through the phosphorylation of the pocket proteins (p130, p107 and pRb). In the resting state, two inhibitory transcription factors, E2F4 and E2F5, are complexed with a DNA-binding protein (DP) and a hypo-phosphorylated pocket protein, either p130 or p107. The activity of cyclin-dependent kinases (cdk) phosphorylates the pocket proteins, releasing the E2F4 or E2F5 and the DP. These molecules harbor nuclear export sequences, and are thus expelled from the nucleus, to be replaced by the activating factors E2F1, E2F2 and E2F3 [5]. The decision of an individual cell to enter S phase emerges from a titration of E2F4/5-containing complexes against E2F1/2/3 containing complexes. Quiescent cells exhibit increased levels of E2F4 [6] while actively cycling cells show low levels of E2F4 protein [7]. Overexpression of E2F4 induces growth arrest and apoptosis in mammalian cells [8].

Besides cell cycle, E2F4 is also involved in cell differentiation. We have recently reported that E2F4 is required for early lymphoid lineage cell differentiation [9]. However, the role of E2F4 in naïve

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and mature T cell proliferation and function is unknown. Herein, we have investigated the effect of E2F4 absence on naïve CD8+ T cells division, memory formation and secondary proliferation.

Our findings reveal that E2F4 protein is essential for cell cycle exit of primary CD8+ T cell blasts and for the formation of normal, functional memory CD8+ T cells. Thus, in T cells E2F4 has a dual role, acting both in the regulation of proliferation and in differentiation.

2. Materials and methods

2.1. Mice and cell culture

Mice of C57BL/6J strain (CD45.2) and the congenic B6.SJL-Ptprca Pep3b/BoyJ (termed B6.CD45.1) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). OT1 mice express a transgenic TCR that recognizes the 8-mer SIINFEKL peptide derived from residues 257–264 of OVA. A colony of OT1 mice was maintained on the B6.CD45.1-expressing background and was bred in house. Mice were maintained in the specific pathogen-free environment in compliance with institutional guidelines for animal care.

OT1 T cells were purified using a CD8+ T cell isolation kit (Miltenyi Biotec, Auburn, CA), from spleens and lymph nodes of 6–8 week old mice, after the depletion of red blood cells using Lympholyte® -M (Cedarlane Laboratories, Ontario, Canada) centrifugation. The purity of OT1 cells was >95% (as determined by CD8 and CD45.1 surface staining). For *in vitro* experiments, OT1 cells were cultured in RPMI1640 cell growth medium (Invitrogen, Carlsbad, CA) containing 10% FBS (Invitrogen), 2 mM L-Glutamine (Invitrogen), 50 μM β-Mercaptoethanol (Sigma–Aldrich, St. Louis, MO), 2 μg/ml penicillin, 2 μg/ml streptomycin. To transduce naïve T cell by Lentiviral vectors, cells were cultured in the above medium with 15 ng/ml murine recombinant IL-7 (Endogen).

2.2. Construction of lentivirus vectors for expression of RNA short hairpin

Block-it™ U6 RNAi entry vector kit was purchased from Invitrogen. Single stranded oligonucleotides against E2F4 or Lac-Z (LAZ) mRNA were designed using Block-it™ RNAi software and synthesized by Invitrogen. Briefly, the single-stranded oligos were annealed to create double strand oligos *in vitro* in annealing buffer according to the manufacturer's protocol. The double strand oligos were ligated into pENTR™/U6 vector to generate the short hairpin-expressing entry vectors, which were used to screen for effective short hairpin RNA by transient transfection of NIH-3T3 cells. To build a cellular marker for tracking of T cells, DsRed cDNA were amplified from pCMV-DsRed-Express vector (BD Biosciences) using PCR. The primer sequences were:

Forward primer: GTCCCCGCGGGACCGTATTACCGCCATGCATTAG.

Reverse primer: TGGGGTACCCCGCTACAGGAACAGGTGGTGG.

A 952 bp PCR product was digested by *SacII* and *KpnI* and ligated into pLenti6/BLOCK-iT™—DEST vector, which was digested using the same two enzyme to generate the destination Lentivirus vector for SH-RNA targeting either E2F4 or LAZ. Finally, the tested U6 entry vectors were recombined with the destination vector to generate the Lentivirus vector expressing short hairpin RNA, by LR clonase™ reaction performed according to the manufacturer's direction (Invitrogen). The sequences of single-stranded oligonucleotides for E2F4 were:

Top strand: 5′-CACCGCCAGAAACGGCGGATCTACGTTCAAGAG ACGTAGATCCGCCGTTTCTGGC.

Bottom strand: 5′-AAAAGCCAGAAA CGGCGGATCTACGTCTCTT GAACGTAGATCCGCCGTTTCTGGC.

Oligos for LAZ

Top strand: 5′-CACCGCTACACAAATCAGCGATTTCGAAAAATCG CTGATTTGTGTAG.

Bottom strand: 5′-AAAACTACACAAATCAGCGATTTTTCGAAATC GCTGATTTGTGTAGC.

2.3. Generation of Lentivirus

293FT cells (Invitrogen) were transfected with DNA mixture containing E2F4-SH or LAZ-SH-expressing Lentivirus vector and the virus packaging vectors (Invitrogen) using lipofectamine 2000 (Invitrogen). After 48 h, the virus supernatant was harvested and filtered through a 0.48 μM filter. Virus was concentrated by ultracentrifugation at 22,000 rpm for 2 h using Sorvall SW28 rotor, and re-suspended in 200 μl culture medium. Virus stock was frozen at −80 °C. Virus titer was determined by transduction of NIH-3T3 cells.

2.4. Lentivirus transduction of T cells

OT1 T cells were cultured in RPM1640 medium with 15 ng/ml IL-7 for 4 days, washed once and 2×10^6 cells were re-plated into a 12-well plate in 1.8 ml culture medium containing 10 mg/ml polybrene (Sigma). 200 μl Lentivirus (MOI 20) was added and the plate was centrifuged at 1400 rpm for 1 h. Cells were incubated at 37 °C with 5% CO_2 for 24–48 h.

2.5. Generation of and use of HSV-1 amplicon expressing OVA

The HSV-OVA amplicon vector [10] and methods to package helper virus-free HSV amplicon stocks were previously described [11]. Viral pellets were re-suspended in PBS and stored at −80 °C until use. Vectors were titered as described previously [12]. OT1 (CD45.1) cells were injected into congenic C57BL/6 (CD45.2) mice by iv. Twenty-four hours later, animals were inoculated with 1×10^6 IFU HSV amplicon expressing OVA protein by intra-muscular (im) injection into the quadriceps femoris muscle.

2.6. RT-PCR for E2F4 mRNA

T cells were maintained in culture medium for 4 days and infected overnight using Lentivirus expressing SH-RNA targeting E2F4 or LAZ at MOI 20. Cells were washed, then 2×10^6 cells were re-suspended in fresh media in a 12-well plate containing splenocytes pulsed with 1 μM of SIINFEKL peptide. After 48 h, both DsRed positive and negative populations were sorted using a FACS Aria (Becton–Dickinson). RNA from sorted cells was extracted using Trizol reagent (Invitrogen) and quantified. Reverse transcription was performed using SuperScript™ First-Strand Synthesis System (Invitrogen) for RT-PCR.

PCR amplification was carried out using the following conditions: 94 °C denaturation for 3 min for 1 cycle, 94 °C 1 min, 58 °C 1 min, 72 °C 1 min, for 35 cycles. PCR products were confirmed on 1.5% agarose gel and detected with ethidium bromide staining. Primers for E2F4 cDNA: Forward: 5′-ACTCAAGTCTCAGAGGTGGC Reverse: 5′-GCAGCTCGGAGCTCATGCA. Primers for β-actin PCR amplification were obtained from Promega.

2.7. Western blotting

Lentivirus vector-transduced NIH-3T3 cells were lysed in buffer containing 0.5% NP40, 100 mM NaCl, 50 mM Tris–HCl (pH 7.4) and protease inhibitor cocktail (Calbiochem Inc, San Diego, CA) by 3 cycles of free-thaw. Cell lysates were resolved on a 10% SDS–PAGE gel, transferred to Hybond™ ECL™ (Amersham Pharmacia Biotech, Piscataway, NJ) nitrocellulose membranes and blocked with TBS