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Id3 Controls Cell Death of 2B4+ Virus-Specific CD8+ T Cells in Chronic Viral Infection

Alexandra J. Menner,*† Katharina S. Rauch,*† Peter Aichele,‡ Hanspeter Pircher,‡ Christian Schachtrup,§ and Kristina Schachtrup*†

Sustained Ag persistence in chronic infection results in a deregulated CD8+ T cell response that is characterized by T cell exhaustion and cell death of Ag-specific CD8+ T cells. Yet, the underlying transcriptional mechanisms regulating CD8+ T cell exhaustion and cell death are poorly defined. Using the experimental mouse model of lymphocytic choriomeningitis virus infection, we demonstrate that the transcriptional regulator Id3 controls cell death of virus-specific CD8+ T cells in chronic infection. By comparing acute and chronic infection, we showed that Id3- virus-specific CD8+ T cells were less abundant, whereas the absolute numbers of Id3+ virus-specific CD8+ T cells were equal in chronic and acute infection. Phenotypically, Id3- and Id3+ cells most prominently differed with regard to expression of the surface receptor 2B4; although Id3- cells were 2B4-, almost all Id3+ cells lacked expression of 2B4. Lineage-tracing experiments showed that cells initially expressing Id3 differentiated into Id3- 2B4+ cells; in turn, these cells were terminally differentiated and highly susceptible to cell death under conditions of persisting Ag. Enforced Id3 expression specifically increased the persistence of 2B4+ virus-specific CD8+ T cells by decreasing susceptibility to Fas/Fas ligand–mediated cell death. Thus, our findings reveal that the transcriptional regulator Id3 promotes the survival of virus-specific CD8+ T cells in chronic infection and suggest that targeting Id3 might be beneficial for preventing cell death of CD8+ T cells in chronic infection or for promoting cell death of uncontrolled, hyperactive CD8+ T cells to prevent immunopathology. *The Journal of Immunology, 2015, 195: 000–000.

Cytotoxic CD8+ T cells are crucial for an effective immune response against viral infections (1, 2). Naive CD8+ T cells recognize viral Ag rapidly expand and differentiate into potent effector cells producing cytokines and effector molecules, which enable the killing of infected cells (1). Upon Ag clearance, immune homeostasis is re-established by T cell contraction, and the majority of virus-specific CD8+ effector T cells undergo apoptosis (3). However, some cells remain as long-lived memory cells that are able to respond rapidly to secondary infection. In contrast, in the presence of high Ag load, virus-specific CD8+ T cells can become dysfunctional or can be deleted, resulting in restricted expansion of virus-specific CD8+ T cells and subsequent chronic infection (2, 4). The transcriptional control of this aberrant differentiation process, called “CD8+ T cell exhaustion,” and the mechanisms leading to deletion of virus-specific CD8+ T cells are incompletely understood (2).

Exhaustion and deletion of virus-specific CD8+ T cells can be observed in humans with chronic infections, such as HIV, hepatitis B virus, or hepatitis C virus, or in cancer (2, 4). Many features of the deregulated antiviral response in patients can be recapitulated in the experimental model of lymphocytic choriomeningitis virus (LCMV) infection of mice by using a high dose of the fast-replicating strain LCMV Docile, which results in virus persistence (5). CD8+ T cell exhaustion is a stepwise process, in which virus-specific T cells gradually lose the ability to produce IL-2, TNF-α, and IFN-γ. CD8+ T cell exhaustion is accompanied by the sustained expression of a variety of surface receptors, such as PD-1 and 2B4, which contribute to the dysfunction of these cells (2, 6). Most importantly, exposure to high Ag loads leads to cell death of virus-specific CD8+ T cells contributing to virus persistence (7, 8). Apoptosis of CD8+ T cells is controlled by the intrinsic (or mitochondrial) pathway, which is mediated by activation of the proapoptotic BH3-only proteins (e.g., Bcl-2–interacting mediator of death [Bim]), and the extrinsic pathway, which is mediated by ligation of death receptors (e.g., Fas or TNFR) (3, 9). The Bim-mediated intrinsic pathway controls both T cell contraction in acute infection and the persistence of Ag-specific CD8+ T cells in chronic infection (3, 10–15). In acute infections, Fas/Fas ligand (FasL)-triggered cell death is dispensable for CD8+ T cell contraction (13, 16); however, it could compensate for the loss of Bim in another infection model (14), and both Bim and Fas were shown to cooperate in controlling the cell death of Ag-stimulated CD8+ T cells in chronic infection (12, 17–19).

CD8+ T cell-differentiation pathways upon acute and chronic infections are controlled, in part, by distinct sets of transcription factors (2). In addition, there are context-dependent roles for key transcription factors, such as T-bet, Blimp-1, or Foxo3, resulting

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Abbreviations used in this article: Bim, Bcl-2–interacting mediator of death; FasL, Fas ligand; HLH, hemophagocytic lymphohistiocytosis; Id, inhibitor of DNA binding; Id3 RV, Id3-expressing RV; LCMV, lymphocytic choriomeningitis virus; MFI, mean fluorescence intensity; mAb, monoclonal antibody; p.I, postinfection; P14, P14 TCR transgenic; RV, retrovirus; wt, wild-type.

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in different functions in acute versus chronic infections (20–22). The inhibitor of DNA binding (Id) proteins are important transcriptional regulators of CD8^+ T cell differentiation upon acute infection (23), yet the role of these proteins in chronic infection has not been studied. Among the four mammalian Id proteins (Id1–4), Id2 and Id3 were described to regulate CD8^+ T cell function. Upon bacterial or viral infections, Id2 promotes the differentiation into short-lived KLRG-1^+ effector CD8^+ T cells, whereas it restricts the differentiation of cells with long-term memory potential (24). In addition, upon Listeria infection, but not in influenza infection, Id2 promotes the survival of CD8^+ T cells by restricting Bim expression (24–26). In contrast, very early postinfection (p.i.), high Id3 expression marks cells with a higher potential to become long-lived memory cells, whereas Id3 expression is absent in short-lived effector cells (27). In accordance, Id3-deficient CD8^+ T cells do not properly differentiate into long-lived memory cells upon acute infection, and CD8^+ T cells with an enforced Id3 expression show increased persistence due to increased expression of genes that regulate DNA replication and repair (27, 28). Id proteins function as negative regulators of the basic helix-loop-helix E protein transcription factors E2A (with its splice variants E12 and E47), HEB, and E2-2 (23). Although E proteins are able to regulate target gene expression through DNA binding as homo- or heterodimers with other basic helix-loop-helix transcription factors, Id proteins form heterodimers with any of the E proteins and, due to the lack of the DNA-binding region, prevent target gene regulation. CD8^+ T cells lacking both E2A and HEB differentiate preferentially into terminally differentiated KLRG-1^+ effector CD8^+ T cells, whereas the differentiation into memory precursor cells is delayed (29). Thus, the balance of Id and E proteins is an important regulator of memory precursor formation in acute infection.

In this study, we show that, in chronic infection, the transcriptional regulator Id3 controls cell death of virus-specific CD8^+ T cells by reducing surface expression of Fas. We find that, in chronic LCMV infection, Id3^− virus-specific CD8^+ T cells were eliminated early in the response, contributing to the deregulated expansion of virus-specific CD8^+ T cells. We also show that Id3^− virus-specific CD8^+ T cells predominantly expressed the receptor 2B4 and that Id3 overexpression selectively prevented deletion of these 2B4-expressing virus-specific CD8^+ T cells. Thus, we propose that 2B4^+ cells are more susceptible to Fas/FasL-mediated cell death compared with 2B4^− virus-specific CD8^+ T cells and that Id3 controls the Fas/FasL-mediated cell death of virus-specific CD8^+ T cells during sustained Ag presence.

Materials and Methods

Mice and viral infections

LD3GF+/− (B6.Id3tm2.Lcmv) (30) and Thy1.1 P14 TCR-transgenic (P14) mice (B6.D2-Tg [Tcr-LCMV] 318SlD2/JDv3) specific for the gp33 epitope of the LCMV glycoprotein (31) were housed and bred in specific pathogen–free conditions. Experiments were performed on 8–19-wk-old mice. For LCMV experiments, mice were infected i.v. with 200 or 10^6 PFU LCMV Docile, as indicated. LCMV Docile was grown in MDCK cells and titered on MC57G fibrosarcoma cells by a standard focus-forming assay, as described (32). All animal experiments were approved and performed in accordance with the guidelines of the local animal care and use committees and the Regierungsräumisdu Freiburg.

Flow cytometry and cell sorting

Cells were isolated from the isolated organs, and single-cell suspensions were prepared. Splenic erythrocytes were eliminated using RBC lysis buffer. Cells were stained at 4°C for 15 min in PBS supplemented with 1% FCS, 2 μM EDTA, and various fluorochrome-conjugated Abs. Propidium iodide (PI; 1 μg/ml; Sigma) was used to exclude dead cells. Prior to cell surface staining, GP33 Dextramer (Immudex) staining was performed for 10 min at room temperature and 20 min at 4°C. For intracellular staining, 10^6 splenocytes were restimulated with gp33 peptide for 4 h, with GolgiPlug added after 1 h. The cells were stained with the Cytofix/Cytoperm Plus solution kit (BD Biosciences). Data were collected using an LSR Fortessa (BD Biosciences) or Gallios (Beckman Coulter) flow cytometer and analyzed with FlowJo software (TreeStar).

Abs with the following specificities were used: CD8a (53-6.7, CD4 (RM4-5), CD3e (145-2C11), Thy1.1 (HS51), Thy1.2 (53-2.1), Vε (B20.1), 2B4 (eBio244F4), PDI (RMP30-1), CD20L (MEL-14), CD44 (IM7), IFN-γ (XMG1.2), TNF-α (MP6-XT22), KLRG1 (2F1), B220 (RA3-6B2), MHC class II (M5/114.15.2), CD11b (M1/70), Ter119 (TER-119), and CD49b (DX5) (all from eBioscience). Fas (Jo2) was purchased from BD Biosciences. TNFR1 (55R-286), TNFR2 (TR75-89), and 2B4 (m2B4 (B6.4584.1) were purchased from BioLegend.

Specific cell populations were sorted on a BD FACS Aria III (BD Biosciences) or a FACSAria III (BD Biosciences) or were isolated by MACS (Miltenyi Biotec) with anti-biotin or anti-CD8a MicroBeads, according to the manufacturer’s protocol.

Retrovirus production and CD8^+ T cell transduction

Retrovirus (RV) was produced by transfection of HEK-293T cells with 15 μg the indicated pMIG-derived plasmids and 5 μg pCL-Eco using Promofectin (PromoKine). After 48 h, the virus-containing supernatant was harvested and used immediately for transduction. Retroviral particles were collected from transduced CD8^+ T cells, which had been activated overnight on anti-CD3–coated (2C11; 2 μg/ml) and anti-CD28–coated (37.51; 1 μg/ml) plates, were spin-infected twice by resuspending them in the retroviral supernatant and centrifuging at 2500 rpm at 30°C for 60 min in the presence of Polybrene (4 μg/ml; Sigma) and IL-2 (100 U/ml; PeproTech).

Adoptive cell transfer and biotin injection

Retrovirally transduced P14 CD8^+ T cells were transferred immediately after the retroviral transduction by i.v. injection into recipient mice that were infected with LCMV 24 h prior. A total of 5 × 10^7 P14 cells, as determined by FACS analysis for expression of the transgenic TCR (CD8^+ Vα2^+), was transferred. For biotin labeling, N-hydroxysulfo succinimidie biotin (EZ-Link Sulfo-NHS-LC-LC-biotin; Pierce) was injected i.v. at 20 mg/ml in PBS (0.2 ml/mouse), as described (33). For adoptive transfer of sorted cells in the lineage-tracing analysis, 4–5 × 10^6 cells were FACS sorted from spleens of mice 8 d.p.i. with 10^6 PFU LCMV Docile and injected i.v. into infection-matched recipient mice. In all adoptive-transfer experiments, donor and host T cells were distinguished by differential expression of the surface markers Thy1.1 and Thy1.2, respectively.

Western blotting

P14 CD8^+ T cells transduced with an empty RV or Id3 RV were cultured for 6 d in the presence of IL-2 (100 U/ml; PeproTech). Protein extracts were separated by 15% SDS-PAGE, followed by Western blotting using anti-Id3 (clone 6-1; CalBioreagents), anti-β2-microglobulin (clone AC-15; Sigma), HRP-conjugated goat anti-rabbit IgG (sc-2004), and HRP-conjugated goat anti-mouse IgG (sc-2005; both from Santa Cruz Biotechnology).

FasL apoptosis assay

Whole CD8^+ T cells from mice that had received adoptive transfer of empty RV– or Id3 RV–transduced P14 CD8^+ T cells were isolated from spleens using anti-CD8a MicroBeads at day 10 p.i. with 10^6 PFU LCMV Docile. A total of 10^6 cells was cultured at 37°C in 96-well plates in the presence of IL-2 (500 U/ml; PeproTech) and stimulated for 24 h using increasing concentrations of soluble FasL (ALX-522-001) and a cross-linking enhancer (ALX-804-034-C050; both from Enzo Life Sciences). Analysis was performed by FACS using PI staining to identify viable cells. Differences in cell survival between empty RV– or Id3 RV–transduced cells were calculated by determining the frequency of GFP^+ cells among viable (FT^−) transduced P14 cells.

Quantitative RT-PCR

Total RNA was isolated using the RNaseasy Mini Kit (Qiagen), including DNase I treatment. cDNA was prepared with oligo(dT)18 primers using the First-Strand cDNA Synthesis Kit (Thermo Scientific). Quantitative PCR was performed with the Fast Start Universal SYBR Green Master Kit (Roche) on the Mastercycler ep Gradient S (Eppendorf) with gene-specific primers. Hprt1 transcript levels were used for normalization. The following primers were used: Binz1 forward, 5′-GCCCTGCGCCCTTTTG-3′ and Binz1 reverse, 5′-CCCGAGCAGAGAGAGAGGATG-3′; Bcl2-1 forward, 5′-GAGAGCGCTCAACAGGGAGATG-3′ and Bcl2-1 reverse, 5′-CCACGGCT-CCTAGTACCTGGA-3′; Bcl2 forward, 5′-CTCCGAAGATGATGCGAGATG-3′ and Bcl2 reverse, 5′-TGGTCGACATCTGTGGTGCAGT-3′; Bax forward,
5'-TGAAGACAGGGCCCTTTTTG-3' and Bsa reverse, 5'-AATTCGCCGGAGACACTCG-3'; and Hprt forward, 5'-GTTAAGCAGTACAGCCCCAAA-3' and Hprt reverse, 5'-AGGGCATATCCAACAACAAACTT-3'.

**Statistics**

Statistical analyses were performed with the two-tailed Student *t* test or one-way ANOVA using Prism 6 software (GraphPad Software). All *p* values < 0.05 were considered significant.

**Results**

**Differential abundance of Id3^−3^-virus-specific CD8^+^ T cells in acute and chronic infection**

Expression of the transcriptional regulator Id3 is downregulated in CD8^+^ T effector cells upon acute infection (27). To examine the role of Id3 during chronic viral infection, we compared its expression in virus-specific CD8^+^ T cells from mice infected with either 200 or 10^6^ PFU of the Docile strain of LCMV. Infection with 200 PFU LCMV Docile evokes a potent CD8^+^ T cell response, leading to virus clearance between days 8 and 14 p.i. and, thus, served as a model for acute infection. In contrast, infection with 10^6^ PFU LCMV Docile leads to reduced numbers of virus-specific CD8^+^ T cells at day 8 p.i., functional exhaustion of virus-specific CD8^+^ T cells, and virus persistence and, thus, was used as a model for chronic infection (Supplemental Fig. 1A, 1B, data not shown) (5). For in vivo monitoring of Id3 expression we used Id3^GFP/+^ reporter mice expressing GFP under the control of the endogenous Id3 locus (30). Virus-specific CD8^+^ T cell response and viral titers did not differ in Id3^GFP/+^ mice compared with wild-type (wt) control mice (Supplemental Fig. 1C, 1D). Although naive CD8^+^CD62L^hi^CD44^lo^ T cells express intermediate levels of Id3, polyclonal CD8^+^CD44^hi^ T cells in uninfected mice can be separated into Id3^−^ and Id3^+^ expressing (Id3^+^) cell populations (Fig. 1A) (30). To study Id3 expression in virus-specific CD8^+^ T cells, we analyzed Id3 expression in splenic CD8^+^ T cells recognizing either LCMV gp33 or the nucleoprotein epitope of aa 396–404 (np396). We confirmed that after acute viral infection using 200 PFU LCMV Docile, Id3 expression was downregulated during chronic infection compared with acute LCMV infection. (A) Id3 expression of splenic naive CD8^+^CD62L^hi^CD44^lo^ (shaded graph) and CD8^+^CD44^hi^ (solid line) T cells of uninfected mice using Id3-GFP reporter (Id3^GFP/+^) mice. GFP fluorescence of naive CD8^+^CD62L^hi^CD44^lo^ T cells of uninfected wt mice is shown as control (dashed line). (B) Id3 expression, using flow cytometry, of splenic CD8^+^H-2Db^gp33^+ T cells at the indicated time points p.i. of Id3^GFP/+^ and wt mice with 200 PFU (upper panels) or 10^6^ PFU (lower panels) LCMV Docile. Total cell numbers of Id3^−^ (left panels) or Id3^+^ (right panels) CD8^+^H-2Db^gp33^+ (C) and CD8^+^H-2Db^np396^+ (D) T cells in the spleen at days 8, 14, and 35 p.i. with 200 or 10^6^ PFU LCMV Docile. Each symbol represents an individual mouse, and horizontal lines are the means. **p < 0.01, ***p < 0.001, two-tailed unpaired Student *t* test. ns, not significant.
in the majority of gp33- and np396-specific CD8+ T cells, and only a small gp33- and np396-specific CD8+ T cell population expressed high levels of \( \text{Id}3 \) (Fig. 1B, data not shown) (27). In contrast, at day 8 p.i. with 10^6 PFU LCMV Docile, we observed as many as 50% \( \text{Id}3 \) cells among gp33-specific CD8+ T cells and \( \sim25\% \) \( \text{Id}3 \) cells among np396-specific CD8+ T cells (Fig. 1B, data not shown). This higher percentage of \( \text{Id}3 \) cells among virus-specific CD8+ T cells after 10^6 PFU LCMV Docile infection could represent a greater abundance of \( \text{Id}3 \) cells or the specific depletion of \( \text{Id}3 \) cells. To distinguish between these two possibilities, we calculated absolute cell numbers in the spleens of mice at days 8, 14, and 21 p.i. with 200 or 10^6 PFU LCMV Docile. At day 8 p.i., the number of \( \text{Id}3 \) gp33-specific CD8+ T cells was comparable in mice infected with 200 or 10^6 PFU LCMV Docile. In contrast, the absolute numbers of \( \text{Id}3 \) gp33-specific CD8+ T cells were dramatically decreased in mice infected with 10^6 PFU LCMV Docile versus 200 PFU LCMV Docile (Fig. 1C). The absolute numbers of \( \text{Id}3 \) or \( \text{Id}3 \) gp33-specific CD8+ T cells were similar at all other time points analyzed (Fig. 1C). These observations were comparable for np396-specific CD8+ T cells, because there was a dramatic decrease in \( \text{Id}3 \) np396-specific CD8+ T cells at day 8 p.i. in mice infected with 10^6 PFU LCMV Docile versus 200 PFU LCMV Docile, which was still evident at day 14 p.i., and there was only a slight decrease in \( \text{Id}3 \) np396-specific CD8+ T cells at

**FIGURE 2.** \( \text{Id}3 \)-2B4+ virus-specific CD8+ T cells are terminally differentiated and deleted during chronic infection. (A) Analysis, using flow cytometry, of \( \text{Id}3 \) (GFP) and 2B4 expression on splenic CD8+H-2Dbgp33+ T cells at day 8 (upper panels) and day 35 (lower panels) p.i. of \( \text{Id}3 \)GFP/+ mice with 10^6 PFU LCMV Docile. Frequency of 2B4+ cells among \( \text{Id}3 \) (\( \text{Id}3 \)GFP/+ upper panels) or \( \text{Id}3 \)GFP/- (upper panels) splenic CD8+H-2Dbgp33+ and CD8+H-2D2np396+ T cells at day 8 and day 35 p.i. of \( \text{Id}3 \)GFP/+ mice with 10^6 PFU LCMV Docile. Each symbol represents an individual mouse, and horizontal lines represent the means. ***, p < 0.001, ****p < 0.0001, two-tailed paired Student \( t \) test. (B) CD8+CD44hi T cells were sorted using FACS based on their expression of 2B4 and \( \text{Id}3 \), at day 8 p.i. with 10^6 PFU LCMV Docile, from the spleen and lymph nodes of \( \text{Id}3 \)GFP/+ mice (upper panels). A total of 4–5 \( \times \) 10^5 cells of the indicated phenotype was adoptively transferred into infection-matched hosts. Flow cytometric analysis of 2B4 and \( \text{Id}3 \) (GFP) expression on donor-derived gp33-specific CD8+ T cells isolated from the spleen at day 7 posttransfer (lower panels). (C) Amount of recovered transferred CD8+ T cells (Thy1.2+) in the spleens of host mice (Thy1.1) that received adoptive transfer of \( \text{Id}3 \)-2B4+, \( \text{Id}3 \)-2B4-, or \( \text{Id}3 \)-2B4- CD8+CD44hi T cells at day 7 posttransfer. Each symbol represents the mean value of one to three mice analyzed in one experiment; bars are the mean ± SEM of two to four independent experiments. ***, p < 0.001, ****p < 0.0001, two-tailed unpaired Student \( t \) test.
FIGURE 3. Id3 overexpression increases the persistence of Ag-specific CD8+ T cells during chronic infection. (A) Schematic overview of the experimental set-up. P14 CD8+ T cells were isolated from spleens and lymph nodes using MACS purification, stimulated overnight using anti-CD3- and anti-CD28-coated plates, and retroviral transduced on day 1. Immediately after transduction, 5 x 10^5 P14 CD8+ T cells were adoptively transferred into wt hosts that were infected with 10^6 PFU LCMV Docile on day 0. Donor P14 CD8+ T cells were distinguished from host CD8+ T cells by differential expression of the surface markers Thy1.1 and Thy1.2, respectively, and were analyzed on days 8, 10, 14, and 21 p.i. (B) Immunoblot analysis of P14 CD8+ T cells, not transduced (-) or transduced with empty RV or Id3 RV 6 d after transduction, using an Id3-specific Ab. β-actin served as loading control. (C) Flow cytometric analysis of the percentage of transduced cells (GFP+) among transferred P14 CD8+ T cells in the spleen at different time points after LCMV infection. (D) Frequency of GFP+ cells among transferred P14 CD8+ T cells in spleen, blood, and lung at the indicated time points after 10^6 PFU LCMV Docile infection. Empty RV–transduced cells; ■, Id3 RV–transduced cells. Data are mean ± SEM of at least four independent experiments, with one to nine mice/group. *p < 0.05, **p < 0.01, ***p < 0.001, two-tailed unpaired Student t test. (E) Total cell numbers of empty RV–transduced (○) or Id3 RV–transduced (■) GFP+ P14 CD8+ T cells in spleen and lung at day 14 after 10^6 PFU LCMV Docile infection. Each symbol represents the mean of one to four recipient mice of an independent experiment, and the lines connect values obtained within one experiment. *p < 0.05, two-tailed paired Student t test. ns, not significant.
day 8 p.i. (Fig. 1D). Taken together, after high-dose infection virus-specific CD8+ T cells lacking expression of Id3 are either selectively depleted or not generated, whereas Id3+ virus-specific CD8+ T cells are generated and maintained.

Id3+ virus-specific CD8+ T cells lack expression of 2B4 and differentiate into Id3−2B4+ CD8+ T cells under conditions of Ag persistence

Next, we were interested in the phenotype and lineage relationship of Id3− and Id3+ virus-specific CD8+ T cells under conditions of Ag persistence. Upon chronic Ag stimulation, CD8+ T cells express multiple distinct surface receptors, such as PD-1, 2B4, Lag3, and CD160 (6), and we confirmed that virus-specific CD8+ T cells have increased expression of these same surface receptors upon infection with 10^6 PFU LCMV Docile compared with 200 PFU (Supplemental Fig. 2A). Next, we investigated PD-1, 2B4, Lag3, and CD160 expression in Id3− and Id3+ virus-specific CD8+ T cells after infection with 10^6 PFU LCMV Docile. Most interestingly, we found an inverse correlation between Id3 and 2B4 expression in splenic gp33- and np396-specific CD8+ T cells (Fig. 2A). In contrast, PD-1, Lag3, and CD160 expression levels did not differ on Id3+ compared with Id3− virus-specific CD8+ T cells at days 8 and 35 p.i. Id3+ gp33-specific CD8+ T cells had slightly lower surface expression of PD-1, but equal levels of Lag3 and CD160, compared with Id3− gp33-specific CD8+ T cells (Supplemental Fig. 2B). Because the severity of functional exhaustion was correlated with the concurrent expression of multiple of these surface receptors (6), we analyzed the abundance of PD-1 and 2B4 double-positive, single-positive, or double-negative cells among Id3− and Id3+ gp33-specific CD8+ T cells (Supplemental Fig. 2C). This analysis revealed that Id3− cells were primarily double positive and contained very few double-negative cells, whereas Id3+ cells were predominantly PD-1−2B4+ or double negative at day 8 p.i. (Supplemental Fig. 2C). We also showed that, at day 8 p.i. with 10^6 PFU LCMV Docile, virtually all virus-specific CD8+ T cells already were functionally exhausted, as determined by their lack of IFN-γ and TNF-α expression upon restimulation (Supplemental Fig. 1B). Thus, Id3 expression did not distinguish functionally exhausted from nonexhausted cells; however, Id3+ and Id3− virus-specific CD8+ T cells have a distinct cell surface phenotype. Although Id3-expressing virus-specific CD8+ T cells lack surface expression of 2B4, the majority of Id3− virus-specific CD8+ T cells are 2B4+.

To determine the lineage relationship between Id3+ and Id3− cells, we sorted Id3−2B4−, Id3−2B4+, and Id3−2B4+CD8+ CD44hi T cells by FACS at day 8 p.i. with 10^6 PFU LCMV Docile (Fig. 2B, Supplemental Fig. 3A) and transferred the same numbers of sorted cells into mice that had been infected with 10^6 PFU LCMV Docile on the same day as the donor mice. Recovery and phenotype of these cells were analyzed 7 d posttransfer using differential expression of Thy1.1 and Thy1.2. To investigate the phenotype of virus-specific CD8+ T cells, we analyzed gp33-specific CD8+ T cells among the transferred cells. Interestingly, transferred gp33-specific Id3−2B4− CD8+ T cells remained Id3−2B4−, whereas gp33-specific Id3−2B4+ CD8+ T cells had differentiated into Id3−2B4+CD8+ T cells and gp33-specific Id3−2B4+CD8+ T cells differentiated into Id3−2B4− and Id3−2B4+CD8+ T cells at day 7 posttransfer (Fig. 2B). This indicates that Id3−2B4− CD8+ T cells differentiate into 2B4+CD8+ T cells by first downregulating Id3 and then upregulating 2B4. Furthermore, although we were able to recover a reasonable amount of transferred cells upon transfer of Id3+2B4− CD8+ T cells, the recovery of Id3−2B4− and Id3+2B4−CD8+ T cells was greatly reduced at day 7 posttransfer (Fig. 2C). This difference was already apparent, although not significant, in the blood 2 d after transfer, indicating that cellular engraftment was similar and that the Id3−2B4− cells died rapidly (Supplemental Fig. 3B). These data indicate that, under conditions of persistent Ag, virus-specific Id3+2B4−CD8+ T cells downregulate Id3 and differentiate into short-lived Id3−2B4+ cells.

Ectopic expression of Id3 enhances the persistence of virus-specific CD8+ T cells

So far, we showed that Id3− virus-specific CD8+ T cells were less abundant in chronic infection compared with acute infection and that Id3+ virus-specific CD8+ T cells were less abundant compared with Id3+ virus-specific CD8+ T cells upon adoptive transfer into chronically infected hosts. Thus, we wondered whether, in chronic infection, high Id3 expression is instructive for an increased abundance of virus-specific CD8+ T cells. Therefore, we overexpressed Id3 by retroviral transduction in P14 CD8+ T cells that specifically recognize the LCMV gp33–41 epitope and de-

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**FIGURE 4.** Id3 overexpression does not influence proliferation of virus-specific CD8+ T cells during chronic infection. (A) Flow cytometric analysis of biotin labeling of transduced (GFP+) or nontransduced (GFP−) donor P14 CD8+ T cells after adoptive transfer and 10^6 PFU LCMV Docile infection. Lymphocytes were labeled with biotin by i.v. injection at day 9 p.i. and were analyzed 15 min later in the peripheral blood by streptavidin staining and flow cytometry (upper panels). Control mice (No Biotin) did not receive biotin or donor P14 CD8+ T cells. Analysis was performed on total host CD8+ T cells. Flow cytometric analysis of biotin labeling of empty RV−or Id3 RV−transduced cells by gating on nontransduced (GFP−) and transduced (GFP+) donor P14 CD8+ T cells of the spleen 24 h after biotin labeling at day 10 p.i. (lower panels). (B) MFIs of biotin in nontransduced (GFP−) or transduced (GFP+) P14 CD8+ T cells of the spleen and blood, as analyzed in (A), at 24 h after biotin labeling. Data are the mean ±SEM of two independent experiments, with three or four mice/group.
Id3 overexpression does not influence cell proliferation in vivo

The increased abundance of Id3-overexpressing P14 T cells between days 8 and 14 p.i. with 10^6 PFU LCMV Docile led us to investigate whether Id3 promotes the proliferation of CD8^+ T cells. To study in vivo whether Id3 overexpression leads to enhanced proliferation of virus-specific CD8^+ T cells upon 10^6 PFU LCMV Docile infection, we retrovirally transduced P14 CD8^+ T cells with empty RV or Id3 RV and transferred these cells into mice that had been infected with 10^6 PFU LCMV Docile 24 h before, as described above (Fig. 3A). At day 9 p.i. mice received one i.v. injection of 4 mg of N-hydroxysulfosuccinimide biotin, which efficiently biotinylates the membrane proteins of all hematopoietic cells in peripheral blood, spleen, thymus, and bone marrow (33). In contrast to BrdU labeling, this method does not require cell permeabilization for intranuclear staining during analysis, which we found to significantly interfere with the GFP fluorescence used to track retrovirally transduced cells. To track differences in cell proliferation in Id3 RV–transduced P14 T cells compared with control P14 T cells, we determined surface biotin abundance 24 h after biotin labeling using streptavidin staining on transduced (GFP^+) or nontransduced (GFP^-) transduced P14 CD8^+ T cells by flow cytometry. We confirmed that 15 min after biotin injection, all P14 T cells were uniformly labeled, as determined by FACS analysis of peripheral blood (Fig. 4A). Cell proliferation was determined by the decrease in biotin abundance on the cell surface, because this is distributed to the daughter cells upon division and, thus, decreases with each division (Fig. 4A) (33). We could not detect significant differences in the mean fluorescence intensity (MFI) of biotin on transferred P14 T cells, isolated from spleen or blood, which were transduced with either empty RV or Id3 RV compared with nontransduced cells in the same animal (Fig. 4). Thus, Id3 overexpression does not increase proliferation of CD8^+ T cells.

**Ectopic expression of Id3 selectively enhances the persistence of 2B4^+ virus-specific CD8^+ T cells**

Because Id3 overexpression did not increase cell proliferation, Id3 either prevents cell death of virus-specific CD8^+ T cells or promotes the differentiation into a cell subset that is less prone to cell death. Specifically, we hypothesized that Id3 overexpression either prevents cell death of short-lived virus-specific 2B4^+CD8^+ T cells or drives the differentiation of 2B4^-CD8^+ T cells into 2B4^-CD8^+ T cells, which we showed to have a longer lifespan, despite persistent Ag stimulation (Fig. 2C). To
isolated by FACS at days 8 and 14 p.i. with 10^6 PFU LCMV Docile. Data are the mean ± SEM of at least three independent experiments. (A) Analysis of *Bcl-2*, *Bim*, *Bad*, and *Bax* mRNA expression levels on splenic transferred P14 CD8+ T cells that were transduced (GFP+) with an empty RV or Id3 RV or not transduced (GFP-) and isolated by FACS at days 8 and 14 p.i. with 10^6 PFU LCMV Docile. Data are the mean ± SEM of at least three independent experiments. (B) Analysis of *Bim* mRNA expression levels on 2B4+ and 2B4+ splenic transferred P14 CD8+ T cells, sorted using FACS, at day 14 p.i. with 10^6 PFU LCMV Docile. Data are the mean ± SEM of six mice from two independent experiments. *p < 0.05; two-tailed paired Student t test. ns, not significant.

**FIGURE 6.** Changes in the intrinsic apoptosis pathway are not responsible for the prosurvival function of Id3. (A) Analysis of *Bcl-2*, *Bim*, *Bad*, and *Bax* mRNA expression levels on 2B4+ virus-specific CD8+ T cells in the cell population that was transduced with the Id3 RV, as shown by the increased percentages of 2B4+ cells among Id3-transduced transferred cells (GFP+; Id3 RV) compared with either control-transduced cells in different hosts (GFP-; empty RV) or nontransduced transferred cells in the same host (GFP-; Id3 RV) (Fig. 5). Thus, Id3 specifically delays cell death of 2B4+ virus-specific CD8+ T cells under conditions of Ag persistence.

The intrinsic apoptosis pathway, including *Bim*, is not involved in the prosurvival effect of Id3 in chronic infection

Because Id3+2B4+ virus-specific CD8+ T cells are highly susceptible to cell death compared with Id3+2B4+ cells under conditions of Ag persistence, we wondered whether Id3 regulates the expression of key components of the intrinsic apoptosis pathway, such as *Bcl-2*, *Bim*, *Bad*, and *Bax*. Using the Id3-overexpression and adoptive-transfer model, we first analyzed mRNA expression of *Bcl-2*, *Bim*, *Bad*, and *Bax* in GFP+ and GFP- P14 cells that had been transduced with empty RV or Id3 RV and sorted by FACS. Interestingly, we found a selective accumulation of 2B4+ P14 T cells in the cell population that was transduced with the Id3 RV, as shown by the increased percentages of 2B4+ cells among Id3-transduced transferred cells (GFP+; Id3 RV) compared with either control-transduced cells in different hosts (GFP-; empty RV) or nontransduced transferred cells in the same host (GFP-; Id3 RV) (Fig. 5). Thus, Id3 specifically delays cell death of 2B4+ virus-specific CD8+ T cells under conditions of Ag persistence.

**Id3 overexpression decreases Fas surface expression on CD8+ T cells and reduces their sensitivity to FasL-mediated cell death**

Next, we analyzed surface expression of the death receptors of the extrinsic apoptosis pathways Fas (*CD95*), TNFR1 (*p55*), and TNFR2 (*p75*) on adoptively transferred P14 cells at day 10 p.i. with 10^6 PFU LCMV Docile. Both Fas and TNFR2 were highly expressed on 2B4+ and 2B4+ P14 CD8+ T cells at day 10 p.i., whereas TNFR1 was only weakly expressed (data not shown). To determine whether overexpression of Id3 alters surface expression of these receptors, we retrovirally transduced P14 CD8+ T cells with empty RV or Id3 RV, adoptively transferred these P14 T cells into hosts that had been infected with 10^6 PFU LCMV Docile, and analyzed Fas, TNFR1, and TNFR2 expression at day 10 p.i. Most interestingly, we found that Id3 overexpression markedly reduced surface expression of Fas on both 2B4+ and 2B4+ P14 CD8+ T cells (Fig. 7A). In contrast, surface expression of TNFR1 and TNFR2 was unaffected by Id3 overexpression (data not shown). Additionally, we found that Id3+2B4+ gp33-specific CD8+ T cells had lower Fas surface expression levels compared with Id3+2B4+ gp33-specific CD8+ T cells at day 5 p.i. with 10^6 PFU LCMV Docile (Fig. 7B), a time point just prior to the observed decrease in Id3+ virus-specific CD8+ T cells.

We speculated that reduced Fas surface expression on Id3-overexpressing cells caused the accumulation of Id3-overexpressing CD8+ T cells due to a reduced sensitivity to Fas/FasL-mediated cell death. To test this, we isolated CD8+ T cells at day 10 p.i. from the spleens of mice that had received P14 CD8+ T cells transduced with empty RV or Id3 RV and performed an in vitro FasL-mediated killing assay. As expected, stimulating cells with increased concentrations of FasL resulted in an overall increase in the numbers of dead CD8+ T cells, as determined by PI staining (data not shown). To determine whether Id3-overexpressing cells have a selective survival advantage compared with control vector-
transduced cells, we determined the frequency of transduced (GFP+) cells among viable (PI−) transferred P14 CD8+ T cells. As expected, the frequency of GFP+ cells remained constant in empty RV or Id3 RV as indicated in the spleen at day 10 p.i. with 10⁶ PFU LCMV Docile. Dotted line depicts the fluorescence-minus-one (FMO) control, lacking the Fas Ab. Each symbol represents the mean of one to four mice from three independent experiments, and the lines connect paired values obtained from one experiment. (B) Flow cytometric analysis and MFI of Fas expression among Id3+2B4+ or Id3−2B4− splenic CD8+H-2Dbgp33T cells at day 5 p.i. of Id3/GFP+ mice with 10⁶ PFU LCMV Docile. Each symbol represents one mouse from two independent experiments, and the lines connect paired values obtained from one mouse. (C) In vitro FasL-mediated apoptosis assay. Total CD8+ T cells isolated from the spleen of mice that received adoptive transfer of empty RV− or Id3 RV−transduced P14 CD8+ T cells were isolated at day 10 p.i. with 10⁶ PFU LCMV Docile and cultured for 24 h with increasing amounts of FasL. The percentages of GFP+ cells among viable (PI−) P14 CD8+ T cells were determined by flow cytometry. Symbols represent the mean ± SEM of three or four independent experiments. *p < 0.05, **p < 0.01, two-tailed paired Student t test. ns, not significant.

FIGURE 7. Id3 decreases Fas surface expression and sensitivity to FasL-mediated cell death. (A) Flow cytometric analysis and MFI of Fas expression among 2B4+ or 2B4− transferred P14 CD8+ T cells that were transduced (GFP+; shaded line graph; •) or non-transduced (GFP−; open line graph; ○) with empty RV or Id3 RV as indicated in the spleen at day 10 p.i. with 10⁶ PFU LCMV Docile. Dotted line depicts the fluorescence-minus-one (FMO) control, lacking the Fas Ab. Each symbol represents the mean of one to four mice from three independent experiments, and the lines connect paired values obtained from one experiment. (B) Flow cytometric analysis and MFI of Fas expression among Id3−2B4+ or Id3+2B4− splenic CD8+H-2Dbgp33+ T cells at day 5 p.i. of Id3/GFP− mice with 10⁶ PFU LCMV Docile. Each symbol represents one mouse from two independent experiments, and the lines connect paired values obtained from one mouse. (C) In vitro FasL-mediated apoptosis assay. Total CD8+ T cells isolated from the spleen of mice that received adoptive transfer of empty RV− or Id3 RV−transduced P14 CD8+ T cells were isolated at day 10 p.i. with 10⁶ PFU LCMV Docile and cultured for 24 h with increasing amounts of FasL. The percentages of GFP+ cells among viable (PI−) P14 CD8+ T cells were determined by flow cytometry. Symbols represent the mean ± SEM of three or four independent experiments. *p < 0.05, **p < 0.01, two-tailed paired Student t test. ns, not significant.

Id3 overexpression does not increase cytokine production of virus-specific CD8+ T cells

Chronic infection results in reduced numbers of virus-specific CD8+ T cells and in functional exhaustion, such as reduced cytokine production, ultimately leading to virus persistence (2). To determine whether Id3 overexpression alters CD8+ T cell function, we compared cytokine expression of empty RV− or Id3 RV−transduced P14 CD8+ T cells isolated at days 8 and 14 posttransfer from mice infected with 10⁶ PFU LCMV Docile and restimulated them with gp33 peptide in vitro. Id3-overexpressing P14 CD8+ T cells did not have increased expression of IFN-γ or TNF-α compared with empty RV-transduced cells (Fig. 8A). Finally, we determined the viral titers in the liver and blood of mice infected with 10⁶ PFU LCMV Docile that received adoptive transfer of either empty RV− or Id3 RV−transduced P14 CD8+ T cells. Again, no changes in viral titers were noted in mice that received Id3 RV−transduced cells compared with empty RV−transduced cells (Fig. 8B). Thus, although Id3 delays cell death of virus-specific CD8+ T cells, it does not affect the functional exhaustion of these cells.
In this study, we showed that the transcriptional regulator Id3 contributes to controlling the deletion of virus-specific CD8+ T cells in chronic infection. Mechanistically, we found that Id3 overexpression reduced Fas surface expression on virus-specific CD8+ T cells and rendered these cells less susceptible to Fas/FasL-mediated death. Most interestingly, we found that Id3 overexpression selectively prevented cell death of virus-specific CD8+ T cells expressing high levels of the inhibitory receptor 2B4. Together with our data showing that 2B4-expressing cells were terminally differentiated and quickly disappeared after transfer into chronically infected mice, we propose that the susceptibility of 2B4+ virus-specific CD8+ T cells to Fas/FasL-mediated cell death under conditions of persistent Ag is regulated by Id3.

In acute infection, Ag recognition leads to vigorous Ag-specific CD8+ T cell expansion and viral clearance. In contrast, under conditions of high viral loads, virus-specific CD8+ T cells may become functionally exhausted and physically deleted, resulting in virus persistence (2, 5). Although therapeutic efforts aimed at reversing CD8+ T cell exhaustion yielded virus clearance (34), in some cases functional exhaustion might be beneficial to the host in that it prevents severe immunopathology. This is evident in immunodeficient patients with defects in components of the cytolytic machinery of T and NK cells, resulting in defective viral clearance and hemophagocytic lymphohistiocytosis (HLH), a life-threatening disorder of severe hyperinflammation (35). Using different mouse models of HLH and blockade of the inhibitory receptors PD-1 and Lag3, it was shown that functional exhaustion of CD8+ T cells contributes to disease severity, whereas reduced functional exhaustion results in fatal HLH (35). In this study, we show that increased Id3 expression rescues exhausted virus-specific CD8+ T cells from Fas/FasL-mediated cell death; however, it does not instruct the dedifferentiation of exhausted CD8+ T cells into functional effector cells, because Id3 overexpression did not result in regained effector functions, as determined by expression of IFN-γ and TNF-α. Consequently, we also did not observe a decrease in viral titers in mice that received transfer of Id3 RV–transduced P14 CD8+ T cells. These findings are in agreement with other recent studies demonstrating that Fas-CD8+ T cells are more resistant to deletion, but Fas deficiency in CD8+ T cells does not prevent functional exhaustion (17). Thus, for therapeutic options in chronic infection, it is necessary to supplement targeting of the Id3–Fas axis with molecules that target T cell exhaustion to increase the abundance of functional T cells. In addition, targeting the Id3–Fas axis might prove to be beneficial for preventing immunopathology in syndromes caused by hyperactivation of CD8+ T cells, such as HLH. In this case, increased Id3 expression might be responsible for an increased persistence of these hyperactivated CD8+ T cells, and reducing Id3 levels might lead to increased Fas/FasL-mediated cell death and reduced immunopathology.

Cell death of virus-specific CD8+ T cells in chronic infection is induced cooperatively by the Bim-mediated intrinsic apoptotic pathway and the Fas/FasL-mediated cell death receptor pathway (12). Importantly, Fas/FasL-mediated death contributes to the elimination of virus-specific CD8+ T cells in chronic LCMV infection in a T cell–intrinsic manner (17), yet the molecular mechanism of Fas/FasL-mediated cell death is complex. Although in some cases the absolute levels of Fas surface expression correlate well with susceptibility to FasL-mediated death (36), Fas membrane localization, efficiency of receptor signaling complex...
assembly and activation, and cross-talk with members of the intrinsic apoptosis pathway also influence susceptibility to FasL-mediated death (37). In this article, we report that overexpression of Id3 reduces Fas surface expression levels in virus-specific CD8+ T cells and renders them less susceptible to Fas/FasL-induced cell death. Thus, we believe that downregulation of Fas surface expression contributes to the increased persistence of Id3-overexpressing 2B4+CD8+ T cells during chronic infection. However, somewhat counterintuitive is our finding that 2B4+ virus-specific CD8+ T cells also have high levels of surface Fas expression but are less susceptible to cell death in vivo compared with their 2B4- counterparts. This indicates that high Fas surface expression levels, per se, do not lead to cell death in chronic infection. Rather, we postulate that other mechanisms contribute to the differential death susceptibility of 2B4- and 2B4+ virus-specific CD8+ T cells. One very intriguing mechanism of provoking susceptibility to Fas/FasL-induced cell death is the TCR-mediated translocation of Fas to lipid rafts (38); thus, we speculate that membrane localization of Fas might differ between 2B4- and 2B4+ cells. Additionally, there might be other unknown intrinsic signaling requirements that change upon differentiation of 2B4- into 2B4+ cells, rendering 2B4+ cells more susceptible to Fas/FasL-mediated death. Finally, cross-talk with members of the intrinsic apoptosis pathway also influences susceptibility to FasL-mediated death (37). In line with the finding that Bim cooperates with Fas in controlling the death of CD8+ T cells in chronic mouse γ-herpes virus infection (12), we observed increased Bim mRNA in 2B4- cells compared with 2B4+ cells. Thus, we suggest that, in chronic LCMV Docile infection, both Fas- and Bim-mediated death also contribute to the deletion of virus-specific CD8+ T cells and that cross-talk between the intrinsic and extrinsic apoptosis pathways may ultimately tip the balance of survival and death.

Several transcription factors, such as T-bet or Blimp-1, regulate the differentiation of CD8+ T cells in chronic infection (2, 20, 39, 40). Additionally, Id2 and Id3 are important transcriptional regulators of CD8+ T cell differentiation and survival in acute infections (23). In this study, we showed that, as in acute infection (27), Ag-specific CD8+ T cells also separate into Id3- and Id3+ subpopulations in chronic infection. In acute infections, Id3+ CD8+ T cells had increased memory potential, and their gene-expression profile correlated substantially with that of long-lived memory cells (27). In accordance, we also found that Id3+ virus-specific CD8+ T cells are maintained longer than Id3- T cells after transfer into chronically infected hosts, indicating that cells with long-term memory-like potential also would be found within the Id3+ subpopulation in chronic infection (41). Thus, as proposed for acute infections, we also provide evidence for a prosurvival role for Id3 in CD8+ T cells. In contrast to acute infection, in which Id3 overexpression rescued cell death of highly proliferative CD8+ T effector cells by inducing higher expression of genes involved in DNA replication and repair (28), we showed in this study that Id3 functions in chronic infection by decreasing the susceptibility of exhausted virus-specific CD8+ T cells to Fas/FasL-mediated killing. Id3 generally functions by interacting with the E protein transcription factors E2A and HEB and preventing their gene-regulatory activity; in acute infection, E2A and HEB regulate CD8+ T cell differentiation and survival (24, 28, 29). In silico analysis of the Fas gene using the rVISTA tool revealed the presence of three conserved E2A binding sites located in the promoter and the first intron of the Fas gene, suggesting that E protein transcription factors might directly activate Fas expression in CD8+ T cells, which would be affected by changes in the balance of E proteins and Id3. Interestingly, the transcriptional repressor Blimp-1 was shown to directly suppress Id3 expression in acute infection (28) and to regulate CD8+ T cell exhaustion in chronic infection (40). Yet, although there is a modest increase in virus-specific CD8+ T cells in Blimp-1-deficient mice, the main phenotype is the lower expression of inhibitory surface receptors on virus-specific CD8+ T cells (40). Additionally, the transcription factor FOXO3 limits CD8+ T cell survival in chronic LCMV infection without affecting CD8+ T cell differentiation (22). Thus, it will be interesting to determine whether Id3 acts in a common pathway with Blimp-1 or FOXO3 in regulating CD8+ T cell survival and differentiation in chronic infection.

In conclusion, the current study identified Id3 as a regulator of virus-specific CD8+ T cell survival in chronic infection. Upon high-dose LCMV infection, virus-specific CD8+ T cells are exhausted and physically deleted, resulting in a severely decreased CD8+ T cell response compared with low-dose infection. We showed that cells expressing Id3 were selectively protected from this deletion, and using Id3 overexpression we found that this delayed death is mediated by a reduced susceptibility to Fas/FasL-mediated death. Most interestingly, we demonstrated that virus-specific Id3+ 2B4+ CD8+ T cells were more susceptible to cell death compared with their Id3-2B4- counterparts and that Id3 overexpression led to a selective accumulation of 2B4+ virus-specific CD8+ T cells. Thus, preventing the downregulation of Id3 in virus-specific CD8+ T cells might rescue the severe deletion of virus-specific CD8+ T cells in chronic infections.

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Disclosures
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