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CUTTING EDGE

Cutting Edge: Selective Requirement for the Wiskott-Aldrich Syndrome Protein in Cytokine, but Not Chemokine, Secretion by CD4$^+$ T Cells[1]

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***The mechanism of cytokine secretion is not well understood, but cytokines appear to be synthesized and released in a polarized fashion toward an Ag-specific target cell. In this study, we demonstrate that the Wiskott-Aldrich syndrome protein (WASp) is an essential component of the cytokine secretory pathway in CD4$^+$ T cells. Murine WASp-deficient CD4$^+$ T cells fail to polarize cytokines toward a target and show an unexpected and striking block in cytokine secretion. In contrast, chemokine secretion and trafficking of plasma membrane proteins, transported via the constitutive secretory pathway, are unaffected by the lack of WASp. These results suggest that CD4$^+$ T cell cytokines require a specialized, WASp-dependent pathway for cellular traffic and/or vesicle release that is distinct from that required for chemokine release. We propose that the use of different secretory pathways for cytokines and chemokines enables CD4$^+$ T cell activity to be further fine-tuned to serve specialized effector functions. The Journal of Immunology,* 2004, 173: 726–730.**

T cell recognition of Ag-bearing cells is associated with cytoskeletal polarization and the redistribution of membrane and cytosolic molecules toward the cell-cell contact zone known as the immunological synapse (1–3). Such ordered structures are observed during the initial activation of CD4$^+$ T cells (activation synapse) (1, 4) and during their effector stage when cytokines are polarized toward the Ag-bearing target (effector synapse) (5, 6). The exact function of the immunological synapse during these two stages of T cell activity is unclear and remains controversial (7–9). Recent studies with cytotoxic CD8 T cells have revealed the effector synapse to be exquisitely organized with lytic granule secretion occurring in a domain within the synapse distinct from that of the signaling molecules (10). In contrast, CD4$^+$ T cell signals for cytokine synthesis and secretion are thought to be sequential and tightly linked to TCR signals for cytokine transcription (11).

The Wiskott-Aldrich syndrome protein (WASp)[3] connects TCR signals to actin cytoskeleton reorganization (12, 13). This link between the TCR and actin, via WASp, appears critical in the formation of the activating synapse between the Th cell and an APC (14, 15). The Wiskott-Aldrich syndrome (WAS) in humans is a rare xid characterized by recurrent pyogenic and opportunistic infections (16–18). Lymphocytes from WAS patients show signaling and cytoskeletal abnormalities (14, 19), but it is unclear how these defects contribute to poor immunity. We used WASp-deficient mice to evaluate the role of WASp in Th differentiation and demonstrate a novel role for WASp in mediating cytokine, but not chemokine, secretion.

Materials and Methods

Cell purification and in vitro T cell priming

Female wild-type (WT) C57BL/6 mice, 6–8 wk old, were purchased from The Jackson Laboratory (Bar Harbor, ME). WAS$^{-/-}$ mice were provided by K. Siminovitch (University of Toronto). CD4$^+$ T cells were enriched from spleen and lymph node as described (20). Naive T cells (CD62L^{high}CD44^{low}CD4^{high}) were further purified by flow cytometry (FACSVantage cell sorter; BD Biosciences, San Jose, CA). T cell priming was as follows: 10^6 CD4$^+$ T cells/well in culture medium (RPMI 1640 with 10% heat-inactivated FCS, 50 μM 2-ME, 2 mM L-glutamine, and 100 U/ml penicillin and streptomycin) in 24-well plates coated with mAbs to anti-TCRβ (H57.597; 0.5–1 μg/ml) and anti-CD28 (37N51.1; 1–2 μg/ml) and with 40 U/ml recombinant human IL-2 (rhIL-2; National Institutes of Health Research and Reference Reagent Program) (neutral priming). Th1 conditions were as follows: 40 U/ml rhIL-2, 5 ng/ml IL-12, and 20 μg/ml anti-IL-4 mAb (11B11). Th2 conditions were as follows: 40 U/ml rhIL-2, 50 ng/ml recombinant murine IL-4, and 50 μg/ml anti-IFN-γ mAb (XMG1.2). After 5–6 days of primary stimulation (37°C), cells were washed, counted, and restimulated with plate-bound anti-TCRβ mAb (0.5–1 μg/ml). For Ab-coated bead stimulation, surfactant-free white sulfate polystyrene latex beads (Interfacial Dynamics, Portland, OR) were coated at 100 μg of anti-CD3 mAb (2C11) per 500 μl of beads. Coated beads were added to effector T cells at a 1:1 ratio and cultured for a further 10 h.

Cytokine measurements

Secreted IFN-γ and IL-4 were measured by ELISA as described (21). IL-2 mAbs for ELISA were from BD Pharmingen (San Diego, CA). Ab pairs for MIP-1α or RANTES were from R&D Systems (Minneapolis, MN). Intracellular cytokine staining was performed using a BD Pharmingen kit. Brefeldin A

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[3] Abbreviations used in this paper: WASp, Wiskott-Aldrich syndrome protein; WAS, Wiskott-Aldrich syndrome; WT, wild type; rhIL-2, recombinant human IL-2.

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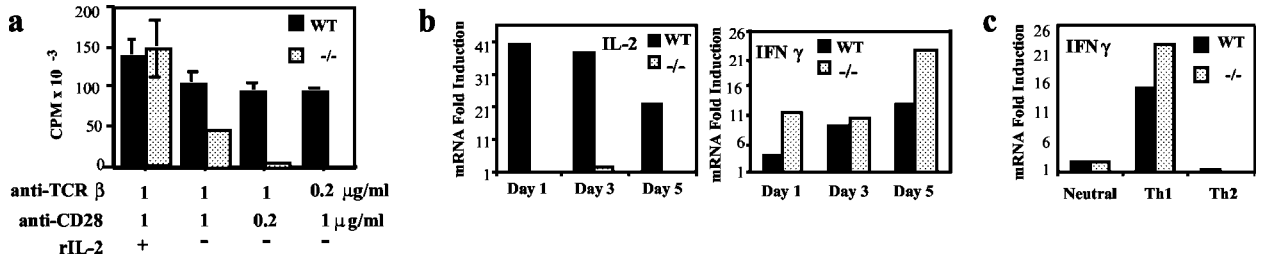

FIGURE 1. Proliferation and cytokine gene expression. *a*, Proliferation at 48 h of naive $CD4^+$ T cells stimulated with plate-bound anti-TCRβ and anti-CD28 mAbs with or without rhIL-2. *b*, $CD4^+$ T cells were stimulated using anti-TCR/CD28 mAbs and rhIL-2 and mRNA purified over the first 5 days of primary stimulation for quantitative RT-PCR. Fold induction of transcripts over unstimulated $CD4^+$ T cells were quantitated by comparison to hypoxanthine phosphoribosyltransferase. *c*, $CD4^+$ T cells were stimulated as in *b* for 5 days, alone (neutral) or under Th1- or Th2-polarizing conditions. Cells were restimulated with anti-TCRβ mAb for 48 h, and mRNA was extracted for quantitation as in *b*. Results represent one of three like experiments.

(Golgi Plug; BD Pharmingen) was added to cultures 4 h before cell harvest. Cells were surface labeled for CD4 (RMA-5) before fixation and permeabilization. SDS-PAGE and Western blots were performed on whole-cell lysates from 48-h-restimulated effector T cells using standard techniques and probed with anti-IFN-γ mAb, XMG1.2. Real-time fluorogenic 5′-nuclease PCR assay was performed as described (22) using the Applied Biosystems (Foster City, CA) Prism 7700 Sequence BioDetector. Each cytokine was analyzed in the same plate using hypoxanthine phosphoribosyltransferase as an endogenous control and mRNA from unstimulated $CD4^+$ T cells as the calibrator.

Immunofluorescence microscopy

Effector $CD4^+$ T cells (48-h restimulated) were isolated by Ficoll gradient centrifugation. Alternatively, primed cells were restimulated with anti-CD3-coated beads as described above. Cells (2×10^5) were placed on poly-L-lysine hydrobromide (Sigma-Aldrich, St. Louis, MO)-coated coverslips and incubated at 37°C for 10 min. Cells were fixed (3% paraformaldehyde), permeabilized (0.3% Triton X-100), and incubated for 1 h at room temperature with anti-IFN-γ mAb (XMG1.2) or anti-IL-4 mAb (11B11) and anti-Giantin (polyclonal rabbit serum, Covance) for *cis*/medial-Golgi detection. Cells were washed and incubated for 1 h at room temperature with respective secondary Abs. Before mounting, cells were incubated for 2 min with 1 nM 4′,6′-diamidino-2-phenylindole (Molecular Probes, Eugene, OR). Coverslips were mounted using Mowial solution with antifade. Cells were imaged using the Axiovert 200M Zeiss (Oberkochen, Germany) microscope and SlideBook software (Intelligent Imaging Innovations, Denver, CO).

Results and Discussion

Previous studies have shown that WASp-deficient T cells have a reduced capacity to proliferate and produce IL-2 (23, 24). The inability of $WAS^{-/-}$ cells to proliferate upon initial stimulation precluded the ability to follow downstream events such as Th differentiation and the production of effector cytokines. We bypassed this initial proliferative block with strong TCR and CD28 stimulatory signals and provision of exogenous IL-2 (Fig. 1*a*). Under these conditions, WASp-deficient $CD4^+$ T cells underwent robust proliferation similar to that seen in WT cells; modulation of any one of these components severely impaired proliferation of $WAS^{-/-}$ cells but not WT cells (Fig. 1*a*). Subsequent experiments used stimulation conditions that supported similar proliferation from $WAS^{-/-}$ and WT cells. We initially focused on induction of cytokine gene expression for IL-2 and the effector cytokine IFN-γ. TCR-mediated signals for *IL-2* gene transcription, and protein secretion, were severely impaired in the absence of WASp (Figs. 1*b* and 2*a*). In contrast, transcriptional activation of the *IFN-*γ gene was intact. Kinetics of gene expression and transcript levels in $WAS^{-/-}$ cells were similar to that seen in WT cells (Fig. 1*b*). In Th differentiation studies, *IFN-*γ gene expression was up-regulated under Th1-skewing conditions and down-regulated under Th2 conditions in both WT and $WAS^{-/-}$ cells (Fig. 1*c*). Thus, at the level of transcription, normal Th1 development occurs in the absence of WASp-mediated signals. Surprisingly, *IL-2* gene activation, but not *IFN-*γ, was sensitive to the absence of WASp. The *IL-2* gene may be particularly reliant on the ability of WASp to enhance NFAT-dependent transcription (25).

Despite normal IFN-γ transcription, we failed to detect IFN-γ in the supernatants of stimulated $WAS^{-/-}$ cells by

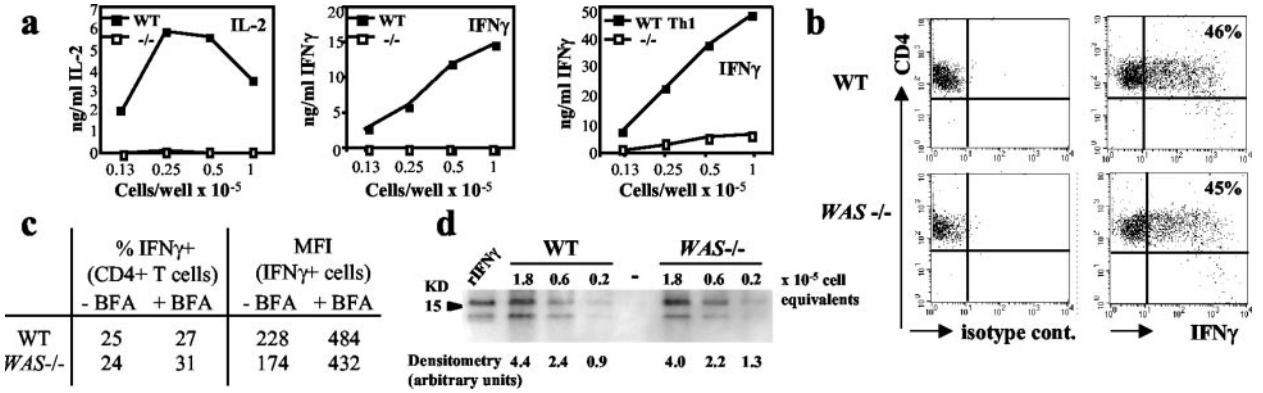

FIGURE 2. Defective IFN-γ secretion in absence of WASp. *a*, $CD4^+$ T cells were stimulated as in Fig. 1*b* for 5 days, or under Th1 conditions (*right panel*), restimulated with anti-TCRβ mAb, and 48-h supernatants were analyzed for IFN-γ by ELISA. For IL-2 detection, supernatants were collected at 48 h of primary stimulation. *b*, $CD4^+$ T cells were primed as in *a* and restimulated for 48 h before staining of intracellular IFN-γ protein and FACS analysis. Percentages refer to the percentage of $CD4^+$ T cells that were IFN-γ positive. Results represent one of five comparable experiments. *c*, Intracellular cytokine staining was performed at 12 h of restimulation as in *b*, with or without the addition of brefeldin A (BFA). *d*, Whole-cell lysates from 48-h-restimulated effector $CD4^+$ T cells were immunoblotted for IFN-γ. *Lane 1* contains rIFN-γ (0.2 μg) as a positive control.

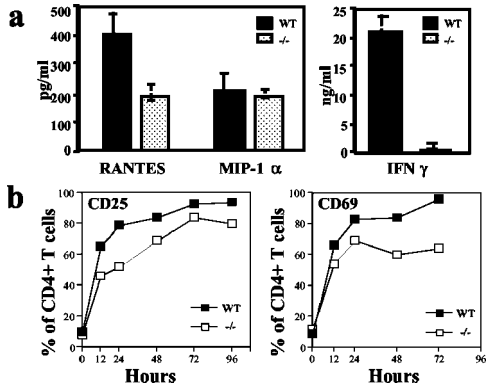

FIGURE 3. Cytokine-specific secretion defect. *a*, $CD4^+$ T cells were stimulated under neutral conditions for 5 days and restimulated for an additional 48 h. Culture supernatants were analyzed for RANTES, MIP-1α, and IFN-γ by ELISA. *b*, $CD4^+$ T cells were stimulated with anti-TCRβ/CD28 mAbs and rhIL-2 for 0–96 h. Cells were collected at various time points for analysis of activation markers. Data show the percentage of $CD4^+$ T cells expressing the given activation marker. Results represent one of three similar experiments.

ELISA (Fig. 2*a*) or ELISPOT (data not shown). This defect in IFN-γ secretion could not be overcome by priming in the presence of strong Th1-promoting conditions (Fig. 2*a*, *right panel*). To determine whether this striking block in cytokine secretion reflected a block in translation of IFN-γ mRNA into protein, we used flow cytometry and Western blot to detect intracellular cytokine in activated Th cells. To our surprise, high levels of intracellular IFN-γ were detected in WASp-deficient $CD4^+$ T cells (Fig. 2, *b* and *d*). The frequency of IFN-γ producers and the amount of IFN-γ per cell were comparable in WT and $WAS^{-/-}$ cells. $WAS^{+/-}$ mice were indistinguishable from WT C56BL/6 mice. Despite the secretion defect, we found no evidence for accumulation/trapping of IFN-γ within the $WAS^{-/-}$ cells when analyzing with and without the protein transport inhibitor brefeldin A (Fig. 2, *c* and *d*), suggesting that cytokines that are not secreted are rapidly degraded. Therefore, the absence of WASp leads to a profound defect in the ability of differentiated Th1 cells to secrete their signature cytokine IFN-γ. These results indicate that Ag-driven signals for cytokine synthesis are qualitatively different from those required for cytokine secretion.

Little is known about the cellular trafficking of cytokine-containing vesicles, but it is thought to occur via the constitutive secretory pathway. Therefore, we asked whether secretion of other proteins via the constitutive secretory pathway was disrupted in the absence of WASp. We examined the secretion of the chemokines RANTES and MIP-1α. Despite disrupted cytokine secretion, RANTES and MIP-1α were readily detected in supernatants from stimulated WT and $WAS^{-/-}$ effector Th cells (Fig. 3*a*). Ag-induced expression of membrane proteins associated with T cell activation, CD69 and the high-affinity IL-2R CD25, was also intact in the absence of WASp (Fig. 3*b*). In addition, previous reports have shown that B cell Ab secretion is unchanged in $WAS^{-/-}$ mice (24). Therefore, WASp deficiency does not disrupt trafficking of a variety of molecules via the constitutive secretory pathway but does lead to a specific block in cytokine secretion. These results suggest that cytokines are sorted into vesicles distinct from those of other secretory proteins, and that their secretion is differentially regulated.

Early studies on CD4 T cells demonstrated that the interaction between T cells and their target cells was accompanied by reorientation of the Golgi apparatus and the microtubule-organizing center toward the point of cell/cell contact (26). The reorganization was a function of Ag dose and led to polarized production of cytokines including IL-2, IL-4, and IFN-γ (5, 27). Polarized cytokine synthesis may impart cytokines with a degree of target specificity, favoring their directed secretion toward the target cell (28). It was proposed that, in the absence of polarization, cytokines would be secreted randomly into the

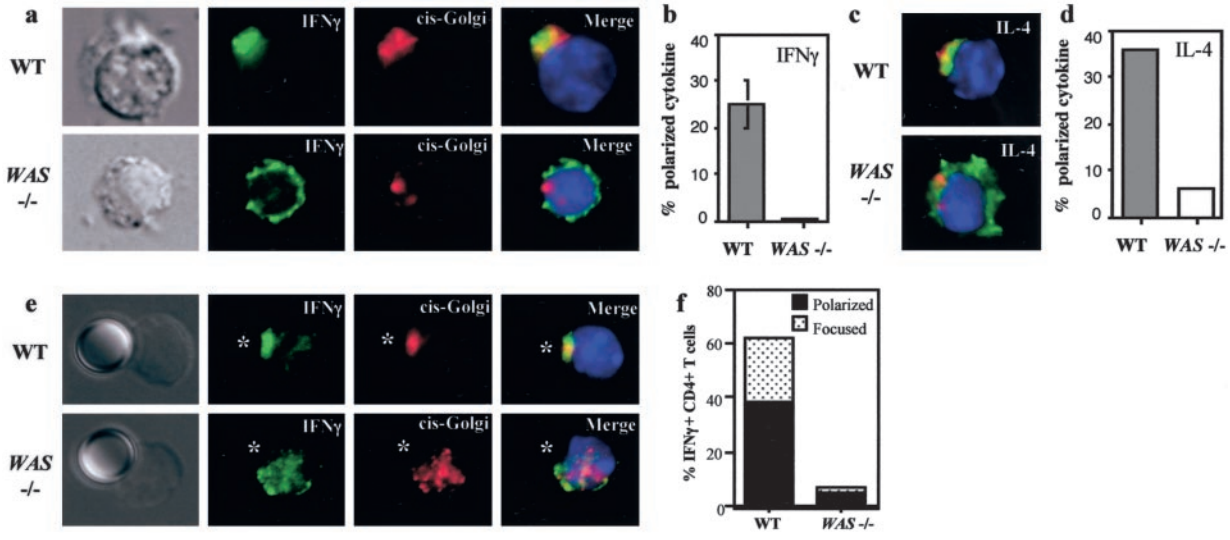

FIGURE 4. Intracellular localization of cytokine. $CD4^+$ T cells were stimulated under neutral conditions for 5 days and restimulated for an additional 48 h as in Fig. 2. The effector cells were collected, placed on poly-L-lysine-coated coverslips, fixed, and permeabilized for staining with mAbs. Before mounting, cells were treated with 4′,6′-diamidino-2-phenylindole to label nuclei (blue). IFN-γ (green) (*a*) and IL-4 (green) (*c*) and *cis*-medial Golgi (Giantin, red) (*a* and *c*). *b* and *d*, Quantitation of percentage of cytokine-positive cells with polarized cytokine, IFN-γ and IL-4, respectively. *e* and *f*, $CD4^+$ effector cells were generated as in *a* and restimulated with anti-CD3-coated beads for 12 h. *f*, Criteria for quantitation: focused, T cells with tight focusing of cytokine to the T/bead synapse (as shown in *e* for WT cells); polarized, T cells where cytoplasm and cytokine had been reoriented toward the bead but not focused to the synapse. Results represent one of three comparable experiments.

surrounding environment. We used immunofluorescence microscopy to look at intracellular cytokine localization in the presence and absence of WASp. Effector Th cells stimulated on Ab-coated plates were fixed and transferred to coverslips for immunohistochemical staining. WT Th cells retained inherent polarity with IFN-γ and the *cis*-medial Golgi apparatus (defined by Giantin expression) localized to one pole of the cell (Fig. 4, *a* and *b*). However, in the absence of WASp, we noticed a prominent change in subcellular localization of cytokine. IFN-γ was dispersed randomly throughout the cell and not localized to areas of the *cis*-medial Golgi (Fig. 4, *a* and *b*). $WAS^{-/-}$ T cells positive for IL-4 had similar changes in subcellular localization of IL-4 (Fig. 4, *c* and *d*) and defective secretion; supernatants from Th2-primed cultures contained 50 ng/ml IL-4 from WT cells and 0.02 ng/ml from $WAS^{-/-}$ cells (although unlike IFN-γ, the frequency of IL-4 producers was reduced in the absence of WASp). The intracellular pool of cytokine (IFN-γ or IL-4) in WASp-deficient cells did not colocalize with the endoplasmic reticulum (binding protein, Bip). In addition, the cytokine appears to exit the *trans*-Golgi network normally in the absence of WASp as treatment with brefeldin A led to relocalization of IFN-γ to the Golgi apparatus in both WT and $WAS^{-/-}$ cells (data not shown). Similarly, the amount of cytokine per cell, as measured by mean fluorescent intensity, increased with brefeldin A treatment in both WT and WASp deficient cells (Fig. 2*c*). Vesicles containing molecules to be released upon cell stimulation (the regulated secretory pathway) can be induced to release their cargo following elevation of intracellular free calcium (29). Release of IFN-γ did not occur following ionomycin treatment from either WT or mutant effector T cell populations, confirming that IFN-γ was not localized to a classical regulated secretory vesicle. To address redistribution of IFN-γ toward a directed target, we used anti-CD3-coated beads as a surrogate for an Ag-bearing target cell. WT Th cells displayed the reported tight focusing of IFN-γ and Golgi apparatus to the cell-bead interface (Fig. 4, *e* and *f*) (5). However, polarization of cytokine and the Golgi apparatus toward the bead and tight focusing of the cytokine to the T cell/bead contact zone was markedly absent in WASp-deficient Th cells (Fig. 4, *e* and *f*). Thus, WASp is required for the polarization of cytokine toward an Ag-bearing target. Unexpectedly, the absence of cytokine polarization is associated with an ablation of cytokine secretion. These observations suggest that polarization of cytokine to the cell/target contact zone may be a prerequisite for cytokine secretion. Mechanistically, this linkage may reflect a requirement for WASp in the redistribution of essential soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor machinery (SNARE) (30) to the effector synapse for cytokine-containing vesicle release. Indeed, loss of cellular polarity in epithelial cells leads to redistribution of target SNAREs to intracellular locations (31).

Our results suggest that cytokine secretion is a regulated process reliant on TCR-dependent WASp activity. Notably, WASp deficiency selectively blocks cytokine secretion but not chemokine secretion. The data imply that, in $CD4^+$ T cells, effector molecules may be differentially sorted into distinct secretory vesicles and secreted via independently regulated pathways. Although WASp plays a role in TCR-mediated signaling for transcription of some cytokines, as demonstrated for IL-2, it is not essential for the activation of all cytokine genes. Importantly, it is not necessary for IFN-γ transcription. Rather, WASp plays an additional role later in the developing effector Th cell response by targeting of effector cytokines to the Th cell-target cell synapse. Surprisingly, without such cellular targeting, cytokines fail to be secreted at all. It is tempting to speculate that the organization of the synapse in Th cells may serve an additional role in mediating cytokine release. An interesting possibility is that the reorganization of molecules to the effector synapse, which aids cell adhesion and local accumulation of signaling molecules, may also serve to focus specific molecules required for cytokine vesicle release. Finally, our work provides a possible mechanistic explanation for the immunodeficiency seen in WAS patients. The pattern of susceptibility to pyogenic, viral, and opportunistic infections in WAS patients is consistent with an inability to liberate Th-derived effector molecules, such as IFN-γ, essential for the clearance of these types of infections.

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