A Major Subset of Mutated CLL Expresses Affinity-selected and Functionally Proficient Rheumatoid Factors

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One-third of chronic lymphocytic leukemias (CLL) can be grouped into stereotyped B-cell receptor (BCR) subsets based on similarity of their expressed variable heavy chain complementarity-determining regions 3 (VH-CDR3).[1] Previously, we and Kostareli et al identified 2 novel stereotyped CLL subsets with somatically mutated immunoglobulin heavy chain variable region (IGHV), each representing 0.2% of CLL, expressing IGHV3-7-encoded and IGHV4-59-encoded rheumatoid factors (RFs).[2,3] Interestingly, stereotypic V4-59-RFs are identical to CLL BCR subset no. 13.[2] Importantly, these stereotypic IgM antibodies display monoreactive RF activity, not to be confused with polyreactive CLL IgMs, which show beside RF activity, low-affinity binding to many other (self) antigens.[4,5]

To extend these findings, we tested a comprehensive panel of CLL IgM for RF activity. Primary CLL cells of 152 cases were induced to plasmacytic differentiation and IgM secretion in vitro by costimulation of 500,000 CLL cells with 50,000 γ-irradiated CD40L-expressing L-cells, the TLR7 ligand R848 (1 μg/mL) and IL-21 (50 ng/mL), as described.[6] Six of the 152 CLL culture supernatants specifically reacted with purified human IgG in ELISA (Figure 1A). The equilibrium dissociation constant (K_D) values of the recombinant IgM for soluble IgG,

determined by surface plasmon resonance as described,[7] ranged between 26 nM and 7 nM (Figure 1B). The 6 supernatants were unreactive with several other antigens, that is, insulin, actin, myosin, vimentin, LPS *E. coli* O55-B5, and pustulan in ELISA (Figure 1A), a microarray of sequence-defined glycans and a microarray representative of human tissues (not shown). Of the remaining 146 CLL IgM supernatants, 50 displayed polyreactivity, whereas 96 did not bind any of the tested antigens (not shown).

Of the 6 RF-expressing CLLs (CLL115, CLL189, CLL226, CLL243, CLL253, CLL282), the IGHV and immunoglobulin kappa variable region (IGKV) genes were amplified and sequenced, unveiling somatic mutations in all 6 cases. Five CLLs expressed an IGHV1-69 rearrangement and CLL282 expressed an IGHV3-30-encoded IGHV (Figure 2A). The VH-CDR3 amino acid (aa) sequences of the RF CLLs were blasted against VH-CDR3 aa sequences present in GenBank (release 228), using protein BLAST (https://blast.ncbi.nlm.nih.gov/). Homology was defined as 60% aa VH-CDR3 sequence identity/similarity, allowing a maximal gap of 3 aa.[9,10] Using these criteria, we unveiled that CLL189 is homologous to stereotypic V1-69-RFs (Supplemental Digital Content, Figure 1A, http://links.lww.com/HS/A143) and that CLL243 and CLL253 are homologous to so-called stereotypic WOL-RFs (Supplemental Digital Content, Figure 1B, http://links.lww.com/HS/A143). As yet, these stereotypic RFs have not been described in CLL. The CLL226 harbored VH-CDR3 homology with both an IGHV1-69-expressing salivary gland mucosa-associated lymphoid tissue (MALT) lymphoma and an IGHV4-4-expressing ocular-adnexal MALT lymphoma (Figure 2C). Intriguingly, all 6 RF CLLs coexpressed a rearranged IGKV3-20, which is also typically found in stereotypic V1-69-RFs and WOL-RFs (Figure 2A). Four of the 5 IGHV1-69-expressing RF CLLs shared a S36R replacement mutation (Figure 2B and Supplemental Digital Content, Figure 2A, http://links.lww.com/HS/A143). CLL243 and CLL253, both homologous to the stereotypic WOL-RF, shared 3 replacement mutations (S31R, S32T, and P41L) (Supplemental Digital Content, Figure 2B, http://links.lww.com/HS/A143).

To address the question of whether precursor clones of the CLL had indeed been selected on the basis of their IgG-binding capacity, we reverted the somatic mutations in the IGHV of CLL115 and CLL189 to the germline configurations (CLL115-VH-GL and CLL189-VH-GL). The reverted RFs and the native, somatically mutated RFs of CLL115 and CLL189 were produced as recombinant IgM, using a

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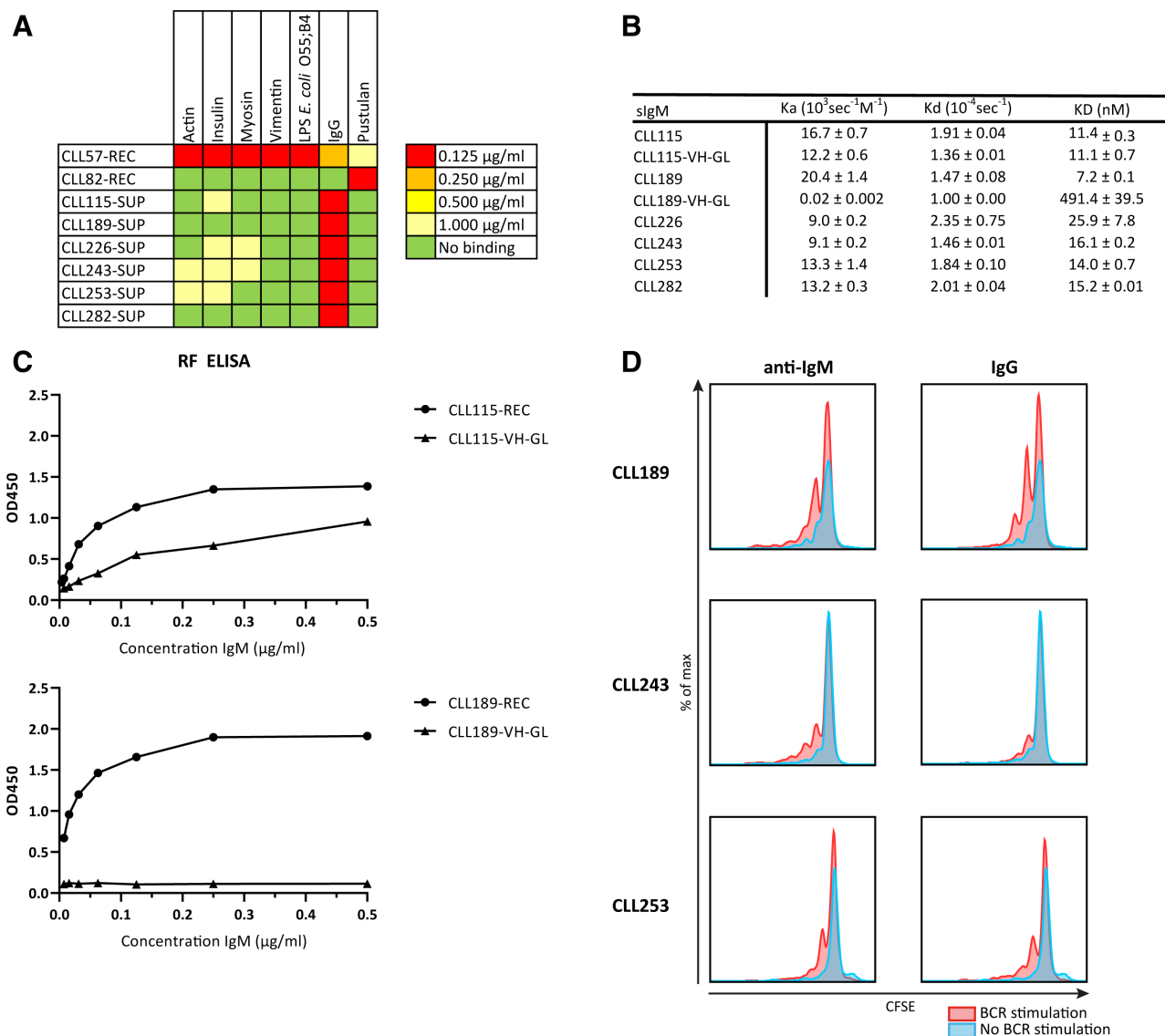Supplemental Digital Content is available for this article.

Figure 1. The BCRs of CLLs with RF reactivity are affinity-selected and mediate tumor cell proliferation in response to ligation by human IgG. (A), Six out of 152 CLL IgM specifically bind IgG in ELISA. Controls are IgM of CLL57, which is polyreactive, and IgM (V3-7Sh-1) of CLL82, which is specific for β-(1,6)-glucan.[8] (B), IgG-binding affinities of IgMs derived from the CLLs, including CLL115 and CLL189, and their IGHV-germline-reverted variants (CLL115-VH-GL, CLL189-VH-GL) as measured by surface plasmon resonance (IBIS MX96, IBIS Technologies, The Netherlands).[7] (C), Binding characteristics of IgMs from CLL115, CLL115-GL, CLL189, and CLL189-GL with coated IgG in ELISA. (D), Primary cells of CLL189, CLL243, and CLL253 proliferate upon interaction with their cognate antigen (IgG) or with anti-IgM monoclonal antibodies. Fresh CLL cells were labelled with CFSE, cultured for 8 days and analyzed by flow cytometry. BCRs = B-cell receptors; CFSE = carboxyfluorescein succinimidyl ester; CLLs = chronic lymphocytic leukemias; IGHV = immunoglobulin heavy chain variable region; RF = rheumatoid factor.

eukaryotic expression system.[11] The modified RF of CLL115-VH-GL had a slightly reduced IgG binding in ELISA, which was explained by the lower association constant (K_a) than the K_a of the native CLL115 RF (Figure 1B,C). The reduced binding was not reflected in the K_D values of the native and GL-reverted CLL115 IgM. The modified RF of CLL189-VH-GL displayed a complete loss of IgG binding in ELISA. Accordingly, the K_D of the native RF and the GL-reverted CLL189 antibodies were 7 nM and 491 nM, respectively (Figure 1B-C). To also address the contribution of the IGKV somatic mutations to the RF reactivity of CLL189, we produced CLL189 IgM either encoded by IGHV mutated/IGKV germline or by IGHV germline/IGKV germline (designated CLL1189-VH/VK-GL and CLL189-VH-GL/VK-GL, respectively). CLL189-VH/VK-GL IgM displayed a similar IgG binding curve as the native CLL189 IgM and, as might be expected, the CLL189-VH-GL/VK-GL IgM showed alike

CLL189-VH-GL IgM, a very low RF reactivity. In addition, we produced CLL115 IgM variants using the reverted VK3-20/JK2 of CLL189, which as compared with the VK-CDR3 (109S and 116Y in CLL189 versus 109T and 116R in CLL115). Intriguingly, CLL115-VH-GL/VK-GL displayed a similar binding curve as compared to the native CLL115 IgM and both CLL115-VH/VK-GL and CLL115-VH-GL variants showed very similar binding curves (Supplemental Digital Content, Figure 3, http://links.lww.com/HS/A143).

Primary carboxyfluorescein succinimidyl ester-labeled tumor cells of CLL189, CLL243, and CLL253 were costimulated with γ-irradiated CD40L-expressing L-cells, R848, CpG-2006, IL-2, IL-4, and IL-21 (Figure 1D). Concomitant BCR ligation with either anti-IgM monoclonal antibodies or coated IgG induced a similar degree of proliferation in all 3 CLLs after 8 days of culturing (Figure 1D). Proliferation
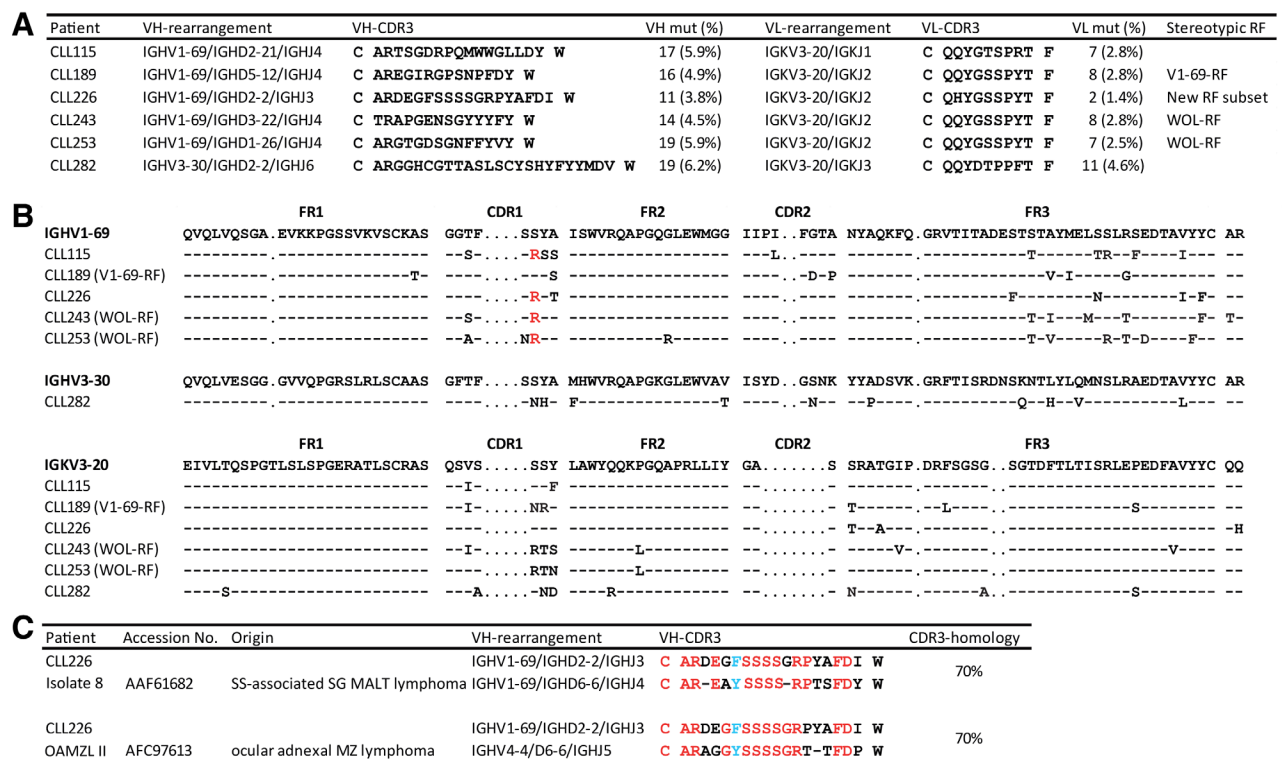
A

Patient	VH-rearrangement	VH-CDR3	VH mut (%)	VL-rearrangement	VL-CDR3	VL mut (%)	Stereotypic RF
CLL115	IGHV1-69/IGHD2-21/IGHJ4	C ARTSGDRPQMWWGLLDY W	17 (5.9%)	IGKV3-20/IGKJ1	C QQYGTSPRT F	7 (2.8%)	
CLL189	IGHV1-69/IGHD5-12/IGHJ4	C AREGIRGPSNPFDY W	16 (4.9%)	IGKV3-20/IGKJ2	C QQYGSSPYT F	8 (2.8%)	V1-69-RF
CLL226	IGHV1-69/IGHD2-2/IGHJ3	C ARDEGFSSSSGRPYAFDI W	11 (3.8%)	IGKV3-20/IGKJ2	C QHYGSSPYT F	2 (1.4%)	New RF subset
CLL243	IGHV1-69/IGHD3-2/IGHJ4	C TRAPGENSGYYYFY W	14 (4.5%)	IGKV3-20/IGKJ2	C QQYGSSPYT F	8 (2.8%)	WOL-RF
CLL253	IGHV1-69/IGHD1-26/IGHJ4	C ARGTGDSGNFFYVY W	19 (5.9%)	IGKV3-20/IGKJ2	C QQYGSSPYT F	7 (2.5%)	WOL-RF
CLL282	IGHV3-30/IGHD2-2/IGHJ6	C ARGGHCGTTASLSCYSHYFYYMDV W	19 (6.2%)	IGKV3-20/IGKJ3	C QQYDTPPFT F	11 (4.6%)	

B

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                    FR1                        CDR1       FR2              CDR2       FR3
IGHV1-69           QVQLVQSGA.EVKKPGSSVKVSCKAS  GGTF....SSYA ISWVRQAPGQGLEWMGG IIPI..FGTA NYAQKFQ.GRVTITADESTSTAYMELSSLRSEDTAVYYC AR
CLL115             ---------.---------------  --S-....-RSS -----------------   ---L..---- -------.-----------T------TR--F----I--- --
CLL189 (V1-69-RF)  ---------.-------------T-  ----....---S -----------------   ----..-D-P -------.-----------V-I-----G--------- --
CLL226             ---------.---------------  ----....-R-T -----------------   ----..---- -------.----------F--------N--------I-F- --
CLL243 (WOL-RF)    ---------.---------------  --S-....-R-- -----------------   ----..---- -------.-----------T-I---M---T-------F- T-
CLL253 (WOL-RF)    ---------.---------------  --A-....NR-- ----------R------   ----..---- -------.-----------T-V-----R-T-D----F-- --

IGHV3-30           QVQLVESGG.GVVQPGRSLRLSCAAS  GFTF....SSYA MHWVRQAPGKGLEWVAV ISYD..GSNK YYADSVK.GRFTISRDNSKNTLYLQMNSLRAEDTAVYYC AR
CLL282             ---------.---------------  ----....-NH- F--------------T   ----..-N-- --P----.----------Q--H--V----------L--- --

                    FR1                       CDR1      FR2              CDR2      FR3
IGKV3-20           EIVLTQSPGTLSLSPGERATLSCRAS QSVS.....SSY LAWYQQKPGQAPRLLIY GA.......S SRATGIP.DRFSGSG..SGTDFTLTISRLEPEDFAVYYC QQ
CLL115             ------------------------- --I-.....--F ----------------- --......- -------.------.-------------------- --
CLL189 (V1-69-RF)  ------------------------- --I-.....NR- ----------------- --......- T------.--L----.---------S-------- --
CLL226             ------------------------- ----.....--- ----------------- --......- T--A---.------.-------------------- -H
CLL243 (WOL-RF)    ------------------------- --I-.....RTS -------L--------- --......- -----V-.------.-------------V---- --
CLL253 (WOL-RF)    ------------------------- ----.....RTN -------L--------- --......- -------.------.-------------------- --
CLL282             ----S-------------------- ---A.....-ND ----R----------- --......- N------.------A.-----------S-------- --
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C

Patient	Accession No.	Origin	VH-rearrangement	VH-CDR3	CDR3-homology
CLL226			IGHV1-69/IGHD2-2/IGHJ3	C ARDEGFSSSSGRPYAFDI W	70%
Isolate 8	AAF61682	SS-associated SG MALT lymphoma	IGHV1-69/IGHD6-6/IGHJ4	C AR-EAYSSSS-RPTSFDY W	
CLL226			IGHV1-69/IGHD2-2/IGHJ3	C ARDEGFSSSSGRPYAFDI W	70%
OAMZL II	AFC97613	ocular adnexal MZ lymphoma	IGHV4-4/D6-6/IGHJ5	C ARAGGYSSSSGRT-TFDP W	

Figure 2. The configuration of IGHV and IGKV of RF CLL. (A), IGHV and IGKV rearrangements and VH-CDR3 of 6 RF CLLs. (B), Alignment of amino acid sequences of the IGHV1-69-, IGHV3-30-, and IGKV3-20-expressing CLLs. Indicated in red a S36R shared between 4 CLL. (C), Comparison of the VH-CDR3 amino acid sequences expressed by CLL226, a salivary gland MALT lymphoma, and an ocular adnexal MALT lymphoma, respectively. VH-CDR3 homology is calculated as the fraction of identical/similar amino acids. Identical amino acids are depicted in red and similar amino acids are depicted in blue. CLLs = chronic lymphocytic leukemias; IGHV = immunoglobulin heavy chain variable region; IGKV = immunoglobulin kappa chain variable region; RF = rheumatoid factor.

on coated IgG was not observed in the non-RF-expressing CLL159 (Supplemental Digital Content, Figure 4, http://links.lww.com/HS/A143). It is noted that, under the culture conditions chosen, 11 of 17 CLLs proliferated upon anti-IgM stimulation (not shown).

In man, a total of 5 stereotypic RF groups have been identified, designated V1-69-RF, WOL-RF (both known as RFs of the Wa idiotype), V3-7-RF, V4-59-RF, and V4-59/JH5-RF.[3,10–13] Stereotypic RF BCRs are frequently expressed by gastric- and Sjögren's syndrome-associated salivary gland MALT lymphoma, hepatitis C virus (HCV)–related B-cell lymphoma and more rarely by ocular adnexal MALT lymphoma, splenic marginal zone B-cell lymphoma, and diffuse large B-cell lymphoma.[10,11,13–15] The fact that a substantial fraction of CLL cases (6/152) express BCRs specific for IgG is surprising. In comparison, the frequency of the RF CLL subgroup within the current cohort (4%), defined not only on basis of their structural similarities but foremost on basis of their antigenic specificity, is higher than the largest stereotyped VH-CDR3 homology CLL subset no. 2 (expressing IGHV3-21), which accounts for about 3% of CLLs.[1] Finally, since CLL226 shares VH-CDR3 homology with both a salivary gland- and an ocular adnexal-MALT lymphoma, this BCR can be considered as a newly identified stereotypic RF.

The binding affinities of the recombinant RF CLL BCRs for IgG are relatively high and comparable with RF of MALT lymphomas and HCV-related lymphomas (K_D ranging between 91 nM and 4 nM).[9,10] The complete loss of IgG-binding of the CLL189 IgM after reversion of the IGHV mutations strongly supports the notion that this CLL originates from a B cell that had been selected for its capacity to bind IgG. The fact that reversion of the IGKV mutations did not result in loss or diminished RF activity demonstrates that for CLL189 IgM, the mutated IGHV is pivotal for its capacity to bind IgG and that the mutations in the IGKV gene, however did not augment, but also not lower the affinity for IgG and thus still may be the result of a stringent selection process. In contrast, the IgG-binding capacity of CLL115 was only slightly affected by reversion of the somatic IGHV and IGKV mutations. Apparently, the somatic IGHV/IGKV mutations of CLL115 contribute only marginally to the RF affinity. Again however, it still may indicate that the somatic mutations in this CLL had been selected not to impede the binding of the originally selected naïve BCR. Recently, based on our findings in similar IGHV/IGKV mutation reversion experiments in 4 stereotypic RFs of salivary gland MALT lymphomas, we also assessed the highly variable contribution of somatic mutations in IGHV and IGKV in the affinity for IgG.[9]

Recently, a model has been proposed of ligand-independent autonomous CLL signaling through homotypic binding of a BCR-intrinsic VH-FR2 VRQ motif.[16] Notably, in spite of the fact that CLL189, CLL243, CLL253 and the previously reported V3-7-RF CLL,[3] all harbor the VH-FR2 VRQ motif, they did not proliferate in response to secondary signals alone, that is, in the absence of extrinsic BCR ligation. Our observation that the proliferative responses induced in the newly defined subset of RF-monospecific CLLs critically depend on the combination of extrinsic BCR engagement and secondary signals corroborates that of others[17] and is in apparent contrast to the concept of BCR-intrinsic signaling of VH-FR2-VRQ-harboring CLL. Notwithstanding, it is possible that the large group of CLLs that express polyreactive BCRs might well proliferate without extrinsic BCR ligation. Our study demonstrates that the BCRs of the RF CLLs are functionally proficient and potentially able to mediate tumor cell growth in vivo driven by IgG, most likely in antigen-IgG immune complexes.

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Disclosures

The authors have no conflicts of interest to disclose.

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