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Healthy Donors Exhibit a CD4 T Cell Repertoire Specific to the Immunogenic Human Hormone H2-Relaxin before Injection

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H2-relaxin (RLN2) is a two-chain peptide hormone structurally related to insulin with a therapeutic potential in multiple indications. However, multiple injections of human RLN2 induced anti-RLN2 Abs in patients, hampering its clinical development. As T cell activation is required to produce Abs, we wondered whether T cells specific for RLN2 might be already present in the human blood before any injection. We therefore quantified the RLN2-specific T cell repertoire using PBMCs collected from healthy donors. CD4 T cells were stimulated in multiple replicates by weekly rounds of stimulation by dendritic cells loaded with RLN2, and their specificity was assessed by IFN-γ ELISPOT. The number of specific T cell lines was used to estimate the frequency of circulating T cells. In vitro T cell response was demonstrated in 18 of the 23 healthy donors, leading to the generation of 70 independent RLN2-specific T cell lines. The mean frequency of RLN2-specific CD4 T cells was similar to that of T cells specific for known immunogenic therapeutic proteins. Using overlapping peptides, we identified multiple T cell epitopes hosted in the N-terminal parts of the α- and β-chains and common to multiple donors, in agreement with their capacity to bind to multiple HLA-DR molecules. Our results provide important clues to the immunogenicity of RLN2 and highlight the weak central immune tolerance induced against this self-hormone. ***The Journal of Immunology*****, 2019, 202: 3507–3513.**

H2-relaxin (RLN2), a two-chain peptide hormone structurally related to insulin, is secreted by the corpus luteum and placenta during pregnancy (1). RLN2 promotes growth of epithelial and stromal cells in the cervix and softens the pelvic ligaments in preparation for parturition. RLN2 also contributes to the cardiovascular changes that occur during pregnancy by increasing cardiac output. In addition, RLN2 has anti-inflammatory, angiogenic, and antifibrotic properties (1). Owing to its broad biological functions, recombinant RLN2 has been tested in multiple acute clinical applications including cervical ripening (2) and heart failure (3) and in chronic conditions in systemic sclerosis (4). Although a single dose or a reduced number of injections are well tolerated, multiple injections of RLN2, as applied in systemic sclerosis, led to a high immunization rate (4, 5). After chronic s.c. administration, anti-RLN2 Abs were detected at 2 weeks in 43% of scleroderma subjects (4). These Abs were nonneutralizing but were associated with increased serum concentrations of recombinant RLN2, which might hamper drug efficacy, owing to the bell-shaped curve effect observed in different preclinical and clinical settings (4, 5).

Immunogenicity issues are frequently observed with replacement enzymes and therapeutic Abs (6) as a result of differences in peptide sequence with endogenously produced counterparts. They have also been encountered for some but not all human recombinant hormones and cytokines. Most diabetic patients develop anti–human insulin Abs that are generally not neutralizing (7) but might prolong insulin lifetime (8). Altered batches of recombinant erythropoietin (EPO) were found to induce autoimmune symptoms (9), whereas IFN-β, GM-CSF, and IL-2 were immunogenic in many patients (6). Thus, multiple lines of evidence indicate that recombinant proteins with a complete human sequence could be immunogenic.

CD4 T cells contribute to humoral responses, including anti-drug Ab responses, by providing cognate signals and cytokines for the B lymphocytes to differentiate into Ab-secreting plasma cells (10, 11). CD4 T cells are selected by self-peptides presented by HLA class II molecules in the thymus. A large number of the autoreactive CD4 T cells are deleted by this process, thus ensuring immune tolerance to many self-proteins. Multiple studies indicate that the amplitude of the CD4 T cell response to foreign Ags (12–15) and therapeutic proteins (16, 17) relies on the size of the naive T cell repertoire resulting from thymic selection. A large naive T cell repertoire is indeed found for immunogenic peptides (12, 14, 15) and proteins (13, 16, 17), whereas in the absence of pre-existing Ag-specific CD4 T cells, Ag injection did not lead to any immune responses, even in the presence of adjuvant (18).

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Abbreviations used in this article: CEA, Commissariat à l'Energie Atomique et aux Energies Alternatives; DC, dendritic cell; EPO, erythropoietin; KLH, keyhole limpet hemocyanin; rh, recombinant human; RLN2, H2-relaxin.

To shed light on the immunogenicity of RLN2, we quantified the RLN2-specific CD4 T cell repertoire in healthy donors and identified the CD4 T cell epitopes that contribute to its immunogenicity. A large T cell repertoire was found to be supported by T cell epitopes hosted by both chains.

Materials and Methods

Proteins and peptides

Keyhole limpet hemocyanin (KLH) was purchased from Thermo Fisher Scientific (Brebières, France). RLN2 (UniProtKB, ID: P04090) was obtained from American Peptide Company (Sunnyvale, CA), and overlapping peptides (Table I) were synthesized by peptides&elephants (Hennigsdorf, Germany).

Generation of RLN2-specific T cell lines

PBMCs were purified from the blood of anonymous healthy donors who gave informed consent (Etablissement Français du Sang, Rungis, France). Monocyte-derived dendritic cells (DCs) were generated from plastic-adherent cells of PBMCs after 5 d of culture in AIM-V medium (Invitrogen, Villebon-sur-Yvette, France) supplemented with 1000 U/ml recombinant human (rh) IL-4 and rhGM-CSF (both from R&D Systems, Lille, France). CD4 T cells were isolated from autologous nonadherent PBMCs by positive selection using magnetic labeling with anti-CD4 mAbs conjugated to magnetic microbeads followed by magnetic cell sorting, as recommended by the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany) (16, 17). DCs were loaded overnight at 37˚C with either RLN2 (2 μM) or KLH (0.25 μM) and matured with LPS (1 μg/ml). A total of 1–3 × 10^4 protein-loaded DCs were added to 1–3 × 10^5 autologous CD4 T cells seeded in multiple round-bottom wells of culture plates in 200 μl of IMDM (Invitrogen) supplemented with 10% human AB serum (Lonza, Levallois-Perret, France), 0.24 mM glutamine, 0.55 mM asparagine, 1.5 mM arginine (all amino acids from Sigma-Aldrich, Saint-Quentin Fallavier, France), 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen) (complete IMDM), and containing 1000 U/ml rh-IL-6 and 10 ng/ml rh-IL-12 (both R&D Systems). CD4 T lymphocytes were restimulated at days 7 and 14 with 1–3 × 10^4 autologous DCs freshly loaded with a protein or peptides, depending on the donor, and were grown in complete IMDM medium supplemented with 10 U/ml rh-IL-2 (R&D Systems) and 5 ng/ml rh-IL-7 (R&D Systems). Each T cell line (expanded T cells contained in one well) was collected at day 21, and its specificity was assessed by IFN-γ ELISPOT.

IFN-γ ELISPOT

MultiScreen hemagglutinin 96-well plates (Merck Millipore, Fontenay sous Bois, France) were coated overnight at 4˚C with 2.5 μg/ml anti-human IFN-γ mAb (1-D1K; Mabtech, Nacka Strand, Sweden) in PBS (Invitrogen). Wells were saturated for 2 h at 37˚C with complete IMDM and washed with PBS. RLN2 (3 μM) or KLH (1 μM) was loaded onto immature DCs in AIM-V for 4 h at 37˚C, whereas peptides (10 μg/ml) were directly added to MultiScreen plates. PBMCs (5 × 10^4 per well) or immature DCs (5 × 10^3 per well) were used as APCs and cocultured in the plates with ~10–25 × 10^3 CD4 T cells in AIM-V supplemented with 0.5 ng/ml rh-IL-7. After overnight incubation at 37˚C and washing, plates were subsequently treated with 0.25 μg/ml biotinylated anti-human IFN-γ mAb (7-B6-1; Mabtech) in PBS/BSA 1%, extravidin-phosphatase (dilution 1:3000 in PBS/Tween 20 0.05%/BSA 1%; Sigma-Aldrich) and NBT/BCIP (Sigma-Aldrich). Spot number was determined by the ELISPOT Reader System (Autoimmun Diagnostika, Ebinger, Germany). CD4 T cell lines were considered specific when a spot count was 2-fold higher in the presence of the protein or peptide than in its absence, with a minimum difference of 25 spots. CD4 T cell precursor frequencies were estimated using the Poisson distribution according to the following formula: Frequency = −ln [(Number of nonspecific CD4 T cell lines/Total number of CD4 T cell lines seeded)]/(Number of CD4 T cells per well).

HLA-DR and -DP specific binding assays

The binding properties of the overlapping peptides to common HLA class II molecules were determined by competitive ELISA, as previously described (15). Each allele is present in a frequency of at least 5% in the European population. Briefly, HLA-DR and -DP molecules were immunopurified by affinity chromatography using monomorphic Abs L243 and B7/21, respectively. Binding assays were performed by diluting HLA class II molecules with an appropriate biotinylated peptide and serial dilutions of competitor peptides. After 24–72 h of incubation and pH neutralization, samples were applied to 96-well MaxiSorp ELISA plates (Invitrogen) previously coated with 10 μg/ml L243 or B7/21. Bound biotinylated peptide was detected by addition of streptavidin–alkaline phosphatase conjugate (GE Healthcare, Buc, France) and 4-methylumbelliferyl phosphate substrate (Sigma-Aldrich). Emitted fluorescence was measured at 450 nm upon excitation at 365 nm. Sequences of the biotinylated reporter peptides and IC_{50} values of their unlabeled forms (reference peptides) were the following: HA306–318 (PKYVKQNTLKLAT) for DRB1*0101 (3 nM), DRB1*0401 (40 nM), DRB1*1101 (20 nM), DRB1*0701 (30 nM), and DRB5*0101 (15 nM); A3 152–166 (EAEQLRAYLDGTGVE) for DRB1*1501 (2 nM); MT 2–16 (AKTIAYDEEARRGLE) for DRB1*0301 (16 nM);

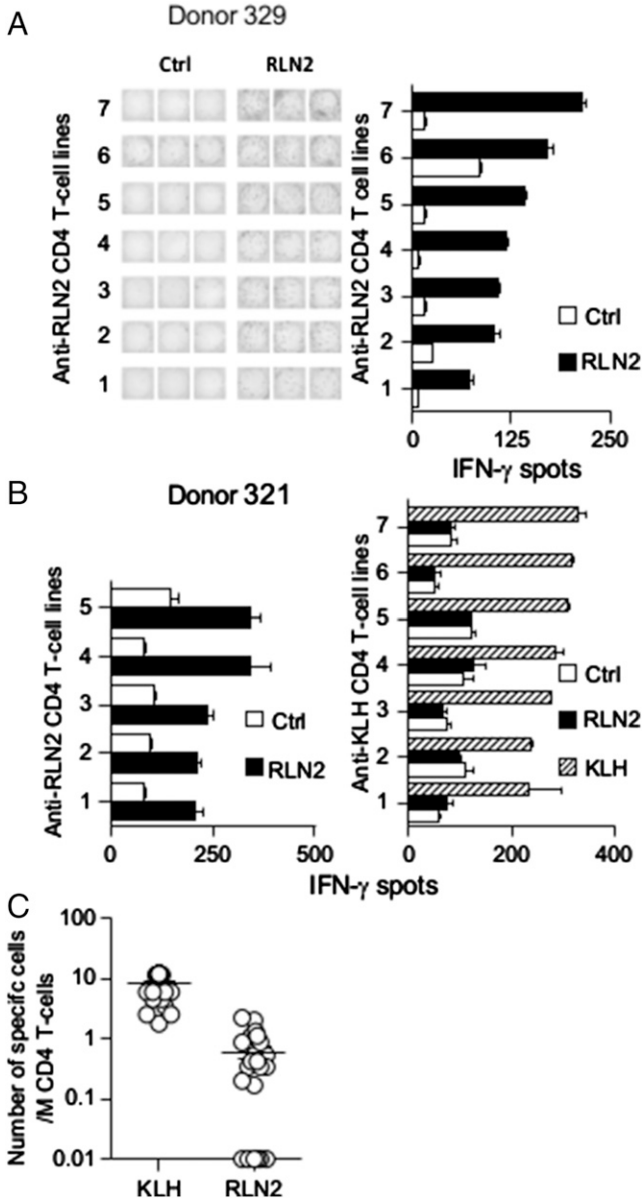

FIGURE 1. Generation of RLN2-specific CD4 T cells from healthy individuals. T cell lines (T cells contained in a single well) were generated by three weekly rounds of stimulation with RLN2 or KLH, and their specificity was tested by IFN-γ ELISPOT with autologous DCs alone (Ctrl) or with DCs previously loaded with RLN2 or KLH. (**A**) RLN2-specific T cell lines generated from donor 329. (**B**) Cross-reactivity analysis between KLH- and RLN2-specific T cell lines. (**C**) Frequencies of CD4 T cells specific for RLN2 and KLH estimated for 23 healthy blood donors. The frequency of CD4 T cell precursors was calculated using the Poisson distribution as described in the *Materials and Methods* section.

Table I. Sequence of RLN2 and overlapping synthetic peptides

Peptides	RLN2 Sequence																										
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
α-Chain	Z	L	Y	S	A	L	A	N	K	C	C	H	V	G	C	T	K	R	S	L	A	R	F	C					
A1–15	Z	L	Y	S	A	L	A	N	K	C	C	H	V	G	C														
A4–18				S	A	L	A	N	K	C	C	H	V	G	C	T	K	R											
A7–21							A	N	K	C	C	H	V	G	C	T	K	R	S	L	A								
A10–24										C	C	H	V	G	C	T	K	R	S	L	A	R	F	C					
β-Chain	D	S	W	M	E	E	V	I	K	L	C	G	R	E	L	V	R	A	Q	I	A	I	C	G	M	S	T	W	S
B1–15	D	S	W	M	E	E	V	I	K	L	C	G	R	E	L														
B4–18				M	E	E	V	I	K	L	C	G	R	E	L	V	R	A											
B7–21							V	I	K	L	C	G	R	E	L	V	R	A	Q	I	A								
B10–24										L	C	G	R	E	L	V	R	A	Q	I	A	I	C	G					
B13–27													R	E	L	V	R	A	Q	I	A	I	C	G	M	S	T		
B16–29																V	R	A	Q	I	A	I	C	G	M	S	T	W	S

Z, pyroglutamic acid.

OXY 271–287 (EKKYFAATQFEPLAARL) for DPB1*0401 (5 nM) and DPB1*0402 (10 nM); and E2/E168 (AGDLLAIETDKATI) for DRB4*0101 (90 nM). Peptide concentrations that prevented binding of 50% of the labeled peptide (IC_{50}) were calculated. Data are expressed as relative activity (ratio of the IC_{50} of the peptide to the IC_{50} of the reference peptide, which is a high binder to the HLA class II molecule).

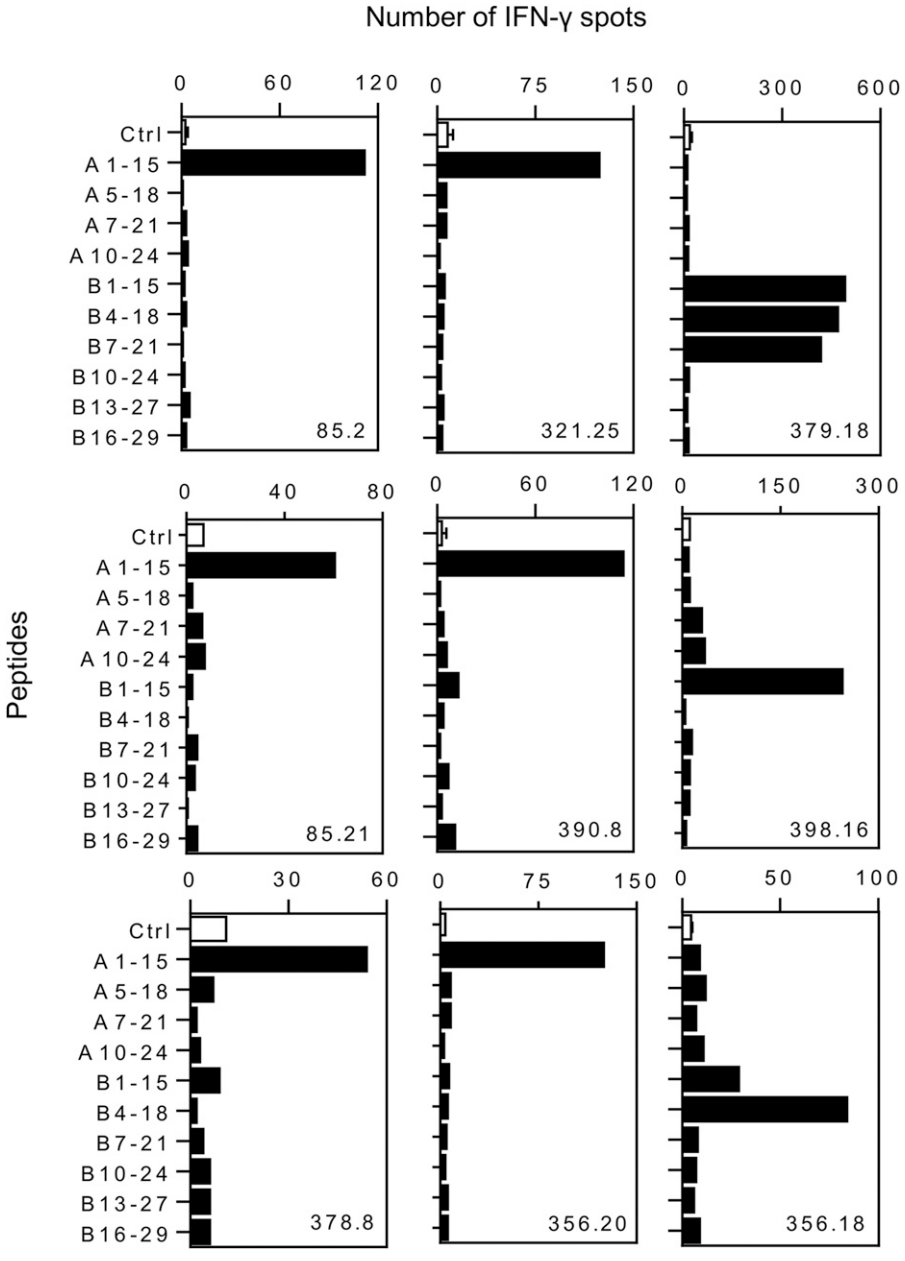

FIGURE 2. Peptide specificity of T cell lines raised against RLN2. RLN2-specific CD4 T cell lines from 14 donors, generated as described in Fig. 1, were tested for recognition of the 10 overlapping RLN2 peptides by IFN-γ ELISPOT. CD4 T cell lines were considered specific in two independent experiments when a spot count was 2-fold higher in the presence of the protein or peptide than in its absence, with a minimum difference of 25 spots. Examples of peptide-specific T cell lines from donors 85, 321, 356, 378, 379, 390, and 398.

Results

A majority of healthy donors exhibited a RLN2-specific T cell repertoire

Because of the low frequency of Ag-specific T cells in healthy donors, we attempted to detect RLN2-specific T cells by generating T cell lines. CD4 T cells collected from healthy donors were stimulated in multiple culture wells by three rounds of stimulation with DCs loaded with RLN2 or KLH, KLH being introduced as a positive control to exclude immunosuppressed donors. Ag specificity was assessed by IFN-γ ELISPOT, the T cells being skewed to a Th1 phenotype by addition of IL-12 at the beginning of the cell culture. As shown in Fig. 1A and 1B, 7 and 5 of the 20 seeded culture wells contained T cell lines specific for RLN2 from the donors no. 329 and no. 321, respectively. They specifically reacted to RLN2, whereas KLH-specific T cell lines were not stimulated by RLN2. A total of 70 RLN2-specific T cell lines were isolated from the 18 responders out of 23 donors with diverse HLA (Supplemental Table I). As demonstrated previously (17), the distribution of the Ag-specific CD4 T cells at the initiation of our assay follows a Poisson distribution. This allowed us to calculate a mean frequency of RLN2-specific CD4 T cells of 0.61 cells per million with a maximum response of 2.23 cells per million, as shown Fig. 1C.

RLN2 T cell epitopes were found in both α- and β-chains

Peptide specificity of CD4 T cell lines from 14 donors was investigated using overlapping peptides (Table I). T cell lines were submitted to a first ELISPOT assay using peptide pools, and their specificity was confirmed in a second, independent ELISPOT with individual peptides (Fig. 2). The T cell lines reacted strongly to either a single peptide or to adjacent peptides. As examples, T cell lines 85.2 (donor 85, well 2), 85.21, 321.25, 390.8, and 356.20 specifically reacted with peptides A1–15. T cell line 379.18 was specific for the peptides B1–15, B4–18, and B7–21. T cell line 398.16 was specific for peptide B1–15, whereas T cell line 356.18 was specific for peptides B1–15 and B4–18. Together, T cell lines specific for RLN2 peptides were isolated from 12 donors (Fig. 3A, Supplemental Table II). Half of the T cell lines reacted with the peptide A1–15, and half of the donors responded to this peptide. Almost all the remaining RLN2-specific T cells were specific for peptides included in the sequence B1–21. Therefore, the T cell response to RLN2 relies mainly on the four peptides A1–15, B1–15, B4–18, and B7–21.

RLN2 peptides of both α- and β-chains bound to multiple HLA class II molecules

All the peptides were submitted to binding assays specific for 10 common HLA class II molecules, including six HLA-DR molecules encoded by the HLA-DRB1 gene, two second HLA-DR molecules, and 2 HLA-DP4 molecules (Fig. 3B, Table II). A1-15 bound with high affinity to HLA-DRB1*0101 and HLA-DRB1*1501 and with moderate affinity to DRB1*0701. All other peptides from the α-chain appeared to be scarcely active. In contrast, all the peptides of the β-chain bound with high or moderate affinity to at least one HLA class II molecule. Peptides B4–18, B7–21, B10–24, and B13–27 bound to at least four molecules, the peptide B4–18 being a ligand for six different HLA class II molecules, including the HLA-DPB1*04:02 molecule. Peptides B1–15 and B1–29 bound to one of the selected HLA class II molecules only. The relatively broad specificity of peptides A1–15 and B4–18 for HLA class II molecules appeared to coincide with the multiple donors responding to these peptides (Fig. 3B).

RLN2 peptide-specific T cell lines are almost restricted to HLA-DR molecules

We finally identified the HLA class II molecules (HLA-DR, -DQ, or -DP) involved in the T cell response to RLN2, as multiple peptides bound to HLA-DR and one to HLA-DPB1*04:02. We therefore selected four supplementary donors with frequent HLA-DRB1 typing, two of them being HLA-DPB1*04:02, and derived from these donors 36 independent T cell lines specific for RLN2 peptides. Restriction was assessed by blocking the peptide recognition with anti–HLA-DP, -DR, or -DQ mAbs in the ELISPOT assay (Fig. 4). From the five T cell lines specific for A1–15, four T cell lines were restricted to the HLA-DR molecule, as their activation was inhibited by the anti–HLA-DR mAb but not by the anti–HLA-DQ or -DP mAbs. Only one T cell line specific for A1–15 was restricted to HLA-DQ molecules of donor 864 (HLA-DQB1*04:02 or 06:02). All other T cell lines specific for the peptides B1–15, B4–18, or B7–21 were restricted to HLA-DR, including six T cell lines specific for B4–18 from the two donors HLA-DPB1*0402 (820 and 848). We concluded from this study that the vast majority of T cells specific to RLN2 peptides are raised against HLA-DR molecules.

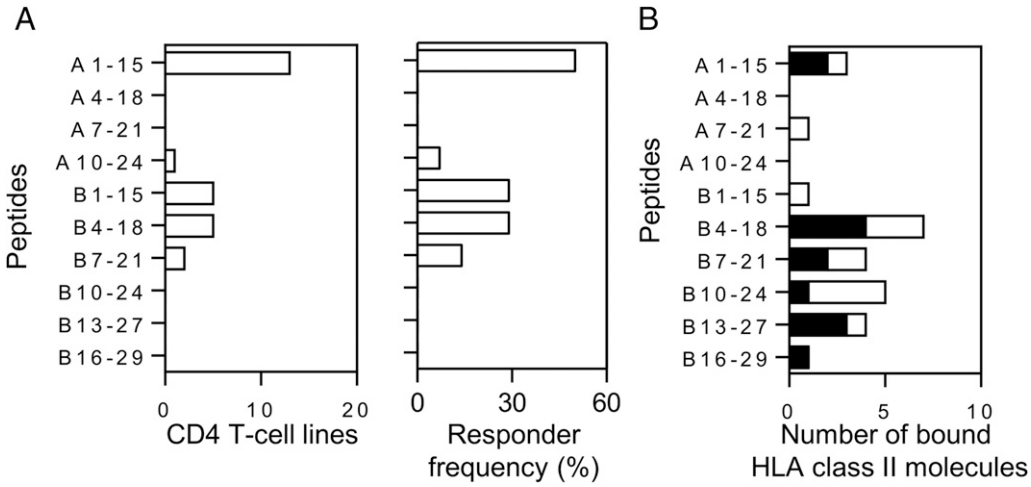

FIGURE 3. Comparison of T cell–stimulating properties and HLA class II binding of RLN2 peptides. (**A**) Number of specific CD4 T cell lines and frequency of responding donors were reported for each peptide. (**B**) Number of HLA class II molecules bound by the individual RLN2 peptides. Relative affinities ≤100 but >20 correspond to good affinities (empty), and those ≤20 correspond to high affinities (filled).

Table II. Relative affinities of the overlapping peptides of the sequence of RLN2

RLN2 Peptide	HLA Class II Molecules										Bound HLA Class II	
	DRB1 0101	DRB1 0301	DRB1 0401	DRB1 0701	DRB1 1101	DRB1 1501	DRB4 0101	DRB5 0101	DPB1 0401	DPB1 0402	High	Moderate
A1–15	**0.7**	>626	135	82	392	**2**	>114	153	>1667	800	2	1
A4–18	>3144	>626	>238	>373	>539	1250	>114	>813	1500	>1000	0	0
A7–21	3000	>626	>238	>373	78	10,000	>114	>813	1333	>1000	0	1
A10–24	>3411	>626	>238	>373	207	1028	>114	824	>1667	>1000	0	0
B1–15	42	>626	>238	211	120	298	>114	731	>1667	900	0	1
B4–18	**0.4**	33	>238	>373	**14**	**3**	**0.3**	75	>1667	100	4	2
B7–21	**7**	157	>238	>373	82	1527	**14**	755	>1667	84	2	2
B10–24	30	>626	88	>373	45	486	**3.5**	35	1000	700	1	4
B13–27	**0.4**	>626	**11**	>373	>539	44	>114	**4**	167	>1000	3	1
B16–29	**7.5**	>626	>238	>373	>539	>5059	>114	369	1000	>1000	1	0

Ten overlapping peptides covering the sequence of human RLN2 were submitted to competitive ELISA specific for 10 HLA class II molecules. Data are expressed as relative activity (ratio of the IC_{50} of the peptide to the IC_{50} of the reference peptide, which is a high binder to the HLA class II molecule). Values result from at least two independent experiments. Relative affinities ≤100 but >20 correspond to good affinities (underlined), and those ≤20 correspond to high affinities (bold).

Discussion

Because of the clinical immunogenicity observed for RLN2, a human hormone with clinical potential, we looked for RLN2-specific T cells in the blood of healthy donors with the perspective to understand the unexpected immune response to this self-protein.

We derived T cell lines by three weekly rounds of stimulation with RLN2 and estimated the frequency of RLN2-specific cells in the blood of the donors by counting the proportion of RLN2-specific T cell lines. We have already applied this long-term T cell assay to various therapeutic proteins (11, 16, 17, 19) and found a very good concordance with their immunogenicity in humans (15–17). This assay has been designed to measure the size of the Ag-specific CD4 T cell repertoire, owing to the role of the naive T cell repertoire to shape the Ag-specific memory T cell response (12, 14, 15). Alternative approaches to evaluate the frequency of specific T cells in healthy donors have also been developed by others, relying on polyclonal expansion of naive T cells (13) or on the use of HLA class II tetramers (14). They have been used with highly immunogenic proteins such as KLH

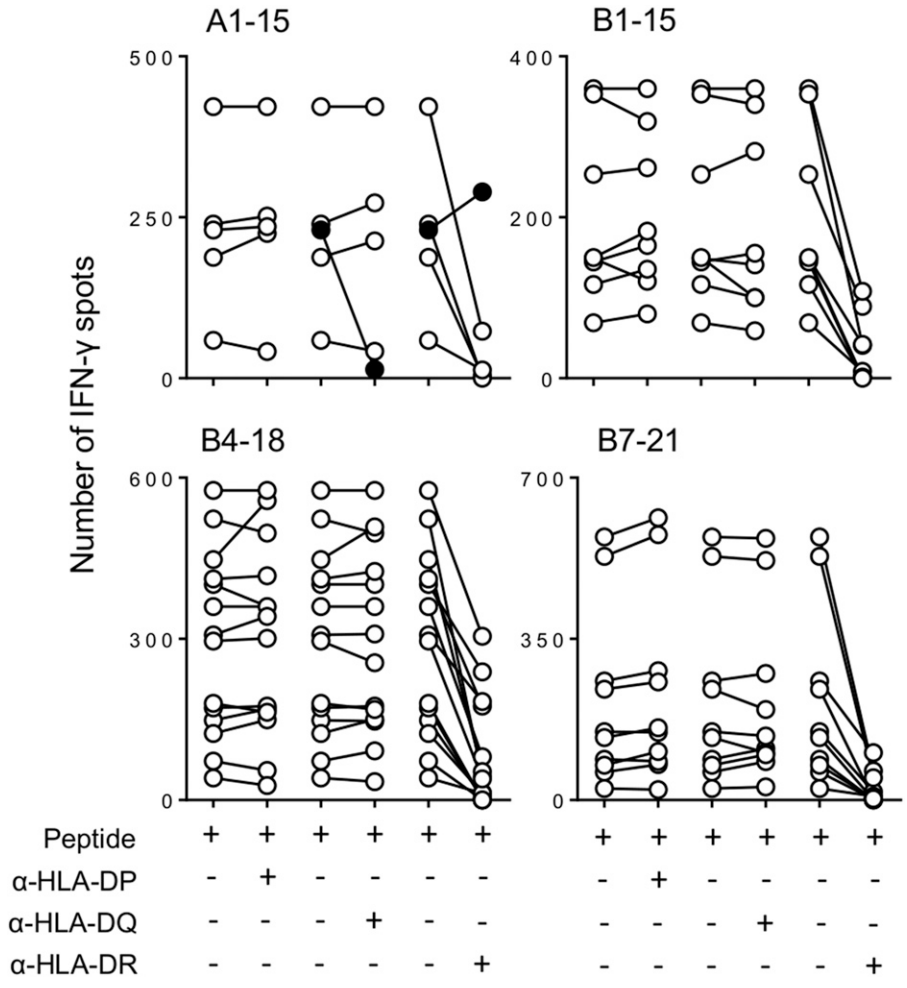

FIGURE 4. HLA class II restriction of T cells specific for RLN2 peptides. T cell lines were generated by three weekly rounds of stimulation with RLN2 T cell epitopes (A1–15, A10–24, B1–15, B4–18, and B7–21) from four HLA-typed healthy donors (820: DRB1*11:01, 15:02/DPB1*04:02; 848: DRB1*01:01, 13:01/DPB1*04:02; 859: DRB1*04:01, 07:01; and 864: DRB1*08:01, 15:01), and their specificity and HLA restriction were tested by IFN-γ ELISPOT. Briefly, T cell lines were incubated for 2 h with autologous PBMCs with or without peptide (10 g/ml) in the presence or absence of mAb anti–HLA-DP (B7–21), anti-DQ (SPVL3), or anti–HLA-DR (L243) (10 μg/ml). Results were expressed as mean number of IFN-γ spots for each T cell line corrected by the control condition (PBMCs alone). Filled circles represent the HLA-DQ restricted T cell line.

or protective Ag of *Bacillus anthracis* (13, 14). By our approach, we detected RLN2-specific CD4 T cells in 18 responders out of 23 donors and estimated that there were 0.61 pre-existing RLN2-specific CD4 T cells per million CD4 T cells in the blood of healthy donors. The frequency of RLN2-specific CD4 T cells is in the range of immunogenic therapeutic Abs such as infliximab, rituximab, and adalimumab (17) and above that of the nonimmunogenic Ab trastuzumab or the fusion protein etanercept (16). This frequency is also comparable to that of human EPO (16) but above that of rIFN-β (data not shown), insulin (16), and the nonimmunogenic anti-trypsin and anti-thrombin proteins (16).

In the absence of any external stimulation by RLN2 injection, the presence of circulating RLN2-specific T cells appears to be the result of an escape from the thymic negative selection. As an endogenous hormone, RLN2 circulates at very low concentration in the blood and probably at an insufficient level to be detected by immune cells. Although mRNA of RLN2 has been detected in the thymus (https://www.ncbi.nlm.nih.gov/gene/6019) (20), it is unknown whether RLN2 is sufficiently expressed by medullary thymic epithelial cells, in contrast to human insulin (21), to contribute to the negative selection of self-reactive T cells (22). As we already proposed for human EPO (16) and Factor VIII (19), we suggest that RLN2 is at least partially ignored by the immune cells during T cell ontogeny (23). RLN2-specific T cells are, therefore, poorly counterselected in the thymus and are potentially functional but remain inactive owing to insufficient signals of T cell activation. However, repeated injections of recombinant RLN2 might provide sufficient local concentrations of RLN2 and inflammatory signals resulting from tissue damage provoked by the injection (24) or provided by RLN2 itself (25) to initiate a T cell response and the subsequent anti-drug Ab response, in agreement with the clinical immunogenicity of RLN2 (4, 5).

We also characterized the T cell epitope content of RLN2 by assessing the reactivity of T cell lines raised against RLN2 to overlapping peptides encompassing its whole sequence. Two regions appeared to host most of the CD4 T cell epitopes, namely A1–15 and B1–21. As already shown for EPO (26) or therapeutic Abs (11), T cell epitopes of RLN2 mainly corresponded to peptides with a good to moderate affinity to HLA class II molecules. As the T cell epitopes contribute to the immunogenic properties of the proteins by stimulating the CD4 T cells, their localization would permit their removal from the RLN2 sequence and the engineering of nonimmunogenic but active RLN2-like molecules. This deimmunization strategy has been already applied to EPO (26), Factor VIII (27), and *Pseudomonas* exotoxin A (28), but their immunogenicity has not been assessed in clinic yet. Our data also illustrate the limit of sequence humanization to circumvent immunogenicity and highlight the risk of immunogenicity for therapeutic proteins whose endogenous and self-counterparts circulate at low levels in the blood of patients.

By quantifying and characterizing RLN2-specific CD4 T cells isolated from the blood of healthy donors, we provided an explanatory model to account for the clinical immunogenicity of RLN2. Our data also highlight the relevance of investigating the naive T cell repertoire to anticipate immunogenicity of therapeutic proteins in humans.

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Disclosures

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Donor	HLA DRB1 typing		RLN2-specific CD4 T-cell lines
85	01:01	15:108	3
297	04:04	07:01	2
315	04:05	07:01	0
321	04:01	13:01	5
325	07:01	07:01	10
328	01:01	01:04	6
329	03:01	11:01	7
350	11:01	13:03	0
351	01:02	11:04	1
352	04:04	08:01	0
354	07:01	08:01	1
356	01:01	01:02	2
376	03:01	11:01	3
378	10:01	13:01	3
379	04:01	09:01	4
387	04:04	11:04	4
389	04:07	14:54	2
390	01:01	13:02	5
395	01:01	14:54	2
398	11:04	15:01	9
399	04:04	07:01	0
400	07:01	13:01	0
402	03:57	15:01	1

Table S1. Specificity of T-cell lines *in vitro* induced with RLN2.

$CD4^+$ T-cell lines were obtained as described in Fig. 1. CD4 T-cell lines were considered as specific when a spot count was two-fold higher in the presence of RLN2 than in its absence, with a minimal difference of 25 spots.

HLA-DR typing			RLN2 peptides					
Donor	DRB1	DRB1	A1-15	A10-24	B1-15	B4-18	B7-21	Total
85	01:01	15:108	2					2
321	04:01	13:01	2					2
356	01:01	01:02	1		1	1		3
378	10:01	13:01	1					1
379	04:01	09:01			1	1	1	3
387	04:04	11:04			2	1		3
389	04:07	14:54	1					1
390	01:01	13:02	4					4
395	01:01	14:54	2					2
398	11:04	15:01		1	1	2	1	5
Number of specific T-cell lines			13	1	5	5	2	26
Total responder frequency (%)			50	7	29	29	14	71

Table 2. Peptide specificity of T-cell lines induced *in vitro* with RLN2.

RLN2-specific CD4 T-cell lines were tested for recognition of the 10 overlapping RLN2 peptides by IFN-γ ELISpot. Number of peptides were reported for each HLA-Typed responding donor.