

Humoral Immune Responses against the Immature Laminin Receptor Protein Show Prognostic Significance in Patients with Chronic Lymphocytic Leukemia^{1,2}

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Chronic lymphocytic leukemia (CLL) is characterized by a highly variable clinical course. The role of an autologous tumor-specific immune control contributing to the variable length of survival in CLL is poorly understood. We investigated whether humoral immunity specific for the CLL-associated Ag oncofetal Ag/immature laminin receptor (OFA/iLR) has a prognostic value in CLL. Among sera of 67 untreated patients with CLL, 23 (34.3%) had detectable OFA/iLR Abs that were reactive for at least one specific OFA/iLR epitope. Patients with humoral responses compared with patients with nonreactive sera had a longer progression-free survival ($p = 0.029$). IgG subclass analyses showed a predominant IgG1 and IgG3 response. OFA/iLR Abs were capable of recognizing and selectively killing OFA/iLR-expressing CLL cells in complement-mediated and Ab-dependent cellular cytotoxicity assays. In the analysis of 11 CLL patients after allogeneic hematopoietic stem cell transplantation, 8 showed high values for OFA/iLR Abs that specifically recognized the extracellular domain of the protein, suggesting a potential role of anti-OFA/iLR-directed immune responses to the graft-vs-leukemia effect in CLL. Our data suggest that spontaneous tumor-specific humoral immune responses against OFA/iLR exist in a significant proportion of CLL patients and that superior progression-free survival in those patients could reflect autologous immune control. *The Journal of Immunology*, 2008, 180: 6374–6384.

Chronic lymphocytic leukemia (CLL)⁵ remains an incurable disease involving clonal mature B lymphocytes and is associated with a heterogeneous clinical course in terms of prognosis and necessity of treatment (1). Some patients remain almost asymptomatic and have a nearly unaltered life expectancy, while others show rapidly progressive disease with early need for intensive treatment (2). A variety of prognostic markers, including CD38, ZAP70, and distinct cytogenetic abnormalities, have been described that, alone or in combination, are being used

to define treatment strategies according to the patient's individual risk profile (3–8). The mutational status of the Ig H chain genes (V_H status) is considered to have the strongest prognostic value, with unmutated Ig H chain (IgV_H) genes being associated with a more rapidly progressive course of disease and shorter overall survival as compared with cases with mutated IgV_H genes (9, 10).

There is growing evidence that interactions between the host immune system and the tumor may have an impact on clinical outcome (11–13). Ansell et al. demonstrated that the presence of high Th cell numbers in diffuse large B cell lymphoma was associated with a better prognosis (14). Although CTLs directed against tumor Ags directly mediate killing of tumor cells, the role of humoral immune responses controlling malignant disease is less understood. In patients with follicular lymphoma, Weng et al. found humoral immune responses after Id vaccination to be associated with prolonged progression-free survival (15).

The target Ag in this study, the tumor-associated oncofetal Ag/immature laminin receptor (OFA/iLR) is widely expressed in many types of human tumors, including hematological malignancies, while it is absent in normal adult differentiated tissues (16–22). Functional properties of OFA/iLR have not completely been elucidated although it is confirmed to be the precursor molecule of the nonimmunogenic mature 67-kDa protein that plays a role in cell adhesion-associated processes by laminin binding (23, 24). The strong overexpression of OFA/iLR on the cell surface of CLL cells made it a valuable candidate to investigate the role of tumor-specific Ab immunity in CLL patients (22).

The immunogenicity of OFA/iLR has previously been shown in two other human tumor systems, namely breast cancer and renal cell carcinoma. In breast cancer patients, Rohrer et al. established several OFA/iLR-specific CD4⁺ and cytotoxic as well as regulatory CD8⁺ T cell clones (19). In renal cell carcinoma, Höltl et al. showed that patients receiving dendritic cells with lysate of OFA/

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⁵ Abbreviations used in this paper: CLL, chronic lymphocytic leukemia; OFA/iLR, oncofetal Ag/immature laminin receptor; FISH, fluorescence in situ hybridization; ADCC, Ab-dependent cellular cytotoxicity; PFS, progression-free survival; allo-SCT, allogeneic stem cell transplantation; CDC, complement-dependent cytotoxicity.

iLR-expressing tumor cells developed enhanced immune responses against OFA/iLR (25).

In this study, we measured humoral anti-OFA/iLR reactivity in untreated patients with CLL and in patients after allogeneic transplantation of blood stem cells. We further determined the specificity and functionality of anti-OFA/iLR Abs and finally investigated progression-free survival in CLL patients in relation to the presence of tumor-specific humoral immune responses.

Materials and Methods

Patients

Sera and PBMC were obtained from a total of 67 patients with untreated CLL, 11 patients with CLL who had undergone allogeneic stem cell transplantation, and 50 healthy donors serving as negative controls. Diagnosis of CLL was based on morphologic and immunophenotypic features according to the classification of the World Health Organization (WHO) (26). Sample collection took place from 2003 to 2006. Written informed consent was provided according to the Declaration of Helsinki. The study was approved by the review board of the local ethics committee.

The prognostic parameters were performed at time of diagnosis or shortly thereafter. If analysis of somatic hypermutations or fluorescence in situ hybridization (FISH) had not been performed at diagnosis, they were performed at the same time of the immune studies. For all patients except four, information on IgV_H gene mutational status and FISH analysis for genetic abnormalities was available. Patient characteristics are summarized in Table I. Healthy donor samples were obtained from blood donors of the institutional department of transfusion medicine ($n = 50$, male/female ratio 3:1, mean age 55 years).

CLL cells and nonmalignant subsets

Peripheral blood was drawn from healthy donors or CLL patients, and PBMC were obtained by Ficoll density gradient centrifugation and cryopreserved in liquid nitrogen. Isolation of CD19⁺ B cells, as well as CD4⁺, CD8⁺, CD14⁺, CD56⁺ and CD34⁺ nonmalignant subsets from healthy donors, was conducted using MACS technology (Miltenyi Biotec) following the manufacturer's instructions. FACS analysis of the different cell population revealed cell purity of >90%. Sera were stored at -20°C until use.

OFA Ab and peptides

Mouse anti-OFA/iLR mAb was used as previously described (21). A set of 42 synthetic peptides (OFA₁₋₄₂) with a length of 17–20 aa overlapping by 5 aa and representing the entire sequence of OFA/iLR were obtained from Peptides & Elephants. Peptides were provided with >70% purity.

Flow cytometry for CD38, ZAP70, and OFA/iLR expression

Staining of OFA/iLR on CLL cells and nonmalignant subsets ($n = 3$ for each subset) was performed as previously described (21). FITC-labeled goat anti-mouse Ig (Dako) was used as a secondary reagent. Assessment of intracellular ZAP70 and cell-surface expression of CD38 was conducted as described earlier using the following Ab conjugates: anti-ZAP70 (clone 1E7.2, Caltag Laboratories), anti-CD19-PerCP/Cy5.5 (clone SJ25C1, Beckman Coulter/Immunotech), and anti-CD38-PE (clone HB-7, Caltag Laboratories), or respective isotype control (7, 27). At least 10,000 cells were acquired for all FACS analysis in the CellQuest program on a BD FACScan flow cytometer (BD Biosciences).

Cytogenetic analysis

Based on conventional cytogenetic studies and data from comparative genomic hybridization, a comprehensive set of FISH DNA

Table I. Characteristics and risk factors of patients

	<i>n</i>	%
Healthy donors	50	
Patients	67	
Sex		
Male	50	74.6
Female	17	25.4
Male/female ratio	2.9:1	
Mean age, years (range)	64.7 (38–85)	
Clinical stage at diagnosis		
Binet A	60	89.5
Binet B	6	9.0
Binet C	1	1.5
Mutational Status		
Mutated (<98% homology)	30	44.8
Biallelic	1	
Unmutated (>98% homology)	34	49.3
Biallelic	1	
Not available	3	4.5
Cytogenetics		
Normal	26	38.8
Deletion 13q14	27	40.2
Deletion 11q	12	17.9
Deletion 17p	1	1.5
Trisomie 12	7	10.4
CD38 Expression		
>30%	29	43.3
≤30%	38	56.7
ZAP70 Expression		
>20%	39	58.2
≤20%	28	41.8

probes (Abbott Diagnostics) was developed to diagnose the most frequent genomic aberrations in CLL. Our probe set allowed screening for the following partial deletions, partial trisomies, and translocations: del(6q23), del(11q22), tri12, del(13q14), and del(17p13), as previously described (27).

IgV_H mutational analysis

The sequences were compared with published germline V_H, D, and J_H genes using DNAPLOT software and IMGT database (IMGT, the international ImMunoGeneTics database, <http://imgt.cines.fr>). Mutational status was calculated as percentage deviation from the closest matching germline V_H segment. As previously decided, a cutoff of ≥98% germline homology defined the group with unmutated IgV_H (28).

Western blot analysis for OFA/iLR expression and for Abs to recombinant OFA/iLR protein

Standard Western blot analysis for OFA/iLR expression was performed as described previously using the monoclonal anti-OFA/iLR Ab at a concentration of 0.75 μg/ml (21).

ELISA for Abs to recombinant OFA/iLR protein

For the analysis of specific anti-OFA/iLR Abs, a standard ELISA was performed as described elsewhere (29). Purified human OFA/iLR (6 μg/ml) and HIV p24 (1 μg/ml) (Aalto Bio Reagents) were bound to ELISA plates (Greiner Bio-One). Sera were used in a dilution of 1/50. OD was read at 450 nm. Responses against HIV p24 (all patients negative) were used as a negative control and nonspecific reactivity was subtracted from each OFA/iLR OD value. Epitope mapping was performed using 42 overlapping OFA/iLR peptides. For IgG subtype analysis peroxidase-conjugated mouse anti-human IgG1, IgG2, IgG3, or IgG4 were used (all from Zymed Laboratories). All analyses were performed in duplicate.

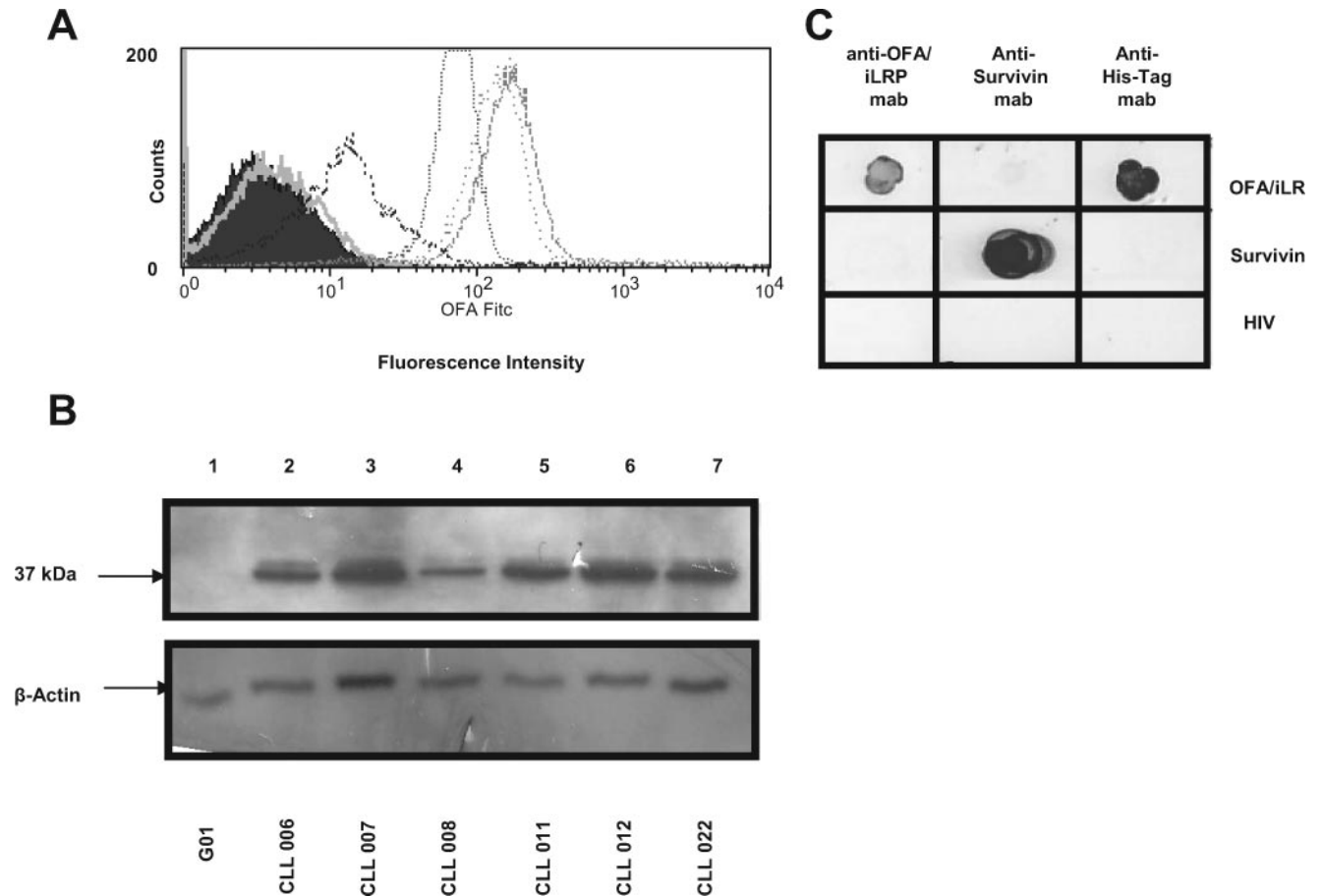


FIGURE 1. Expression and antigenicity of OFA/iLR in CLL patients and healthy donors. *A*, One representative experiment of FACS analysis of CD19⁺ lymphocytes. The filled profile indicates isotype control; nonfilled gray profile, healthy donor; nonfilled dotted profiles, CLL patient nos. 1–4. *B*, Detection of OFA/iLR using Western blot analysis. Total protein from a healthy donor (*lane 1*) and different CLL patients (*lanes 2–7*) were separated by SDS-PAGE and blotted to nitrocellulose filter membranes. Monoclonal anti-OFA/iLR Ab was used as a primary and goat anti-mouse as a secondary Ab. *C*, Dot blot showing specific recognition of OFA/iLR protein by anti-OFA/iLR Abs. Recombinant His-tagged OFA/iLR protein and recombinant non-His-tagged surviving protein were blotted on a nitrocellulose membrane. Truncated, non-His-tagged HIV protein was used as a negative control. Proteins were incubated with anti-OFA/iLR mAb, anti-survivin polyclonal Ab, and anti-His-tagged Ab.

Complement-mediated cytotoxicity and Ab-dependent cellular cytotoxicity (ADCC)

Specific cell lysis with Ab and complement was measured using a 2-h ⁵¹Cr-release assay as previously described by Bellucci et al. (30). For complement-dependent cytotoxicity, a 1/4 dilution of rabbit complement (Calbiochem) was chosen. For ADCC, PBMC from healthy donors were used as effector cells at an E:T ratio of 10:1. OFA/iLR-expressing CLL cells (autologous or allogeneic), cells from patients with CLL in Richter transformation that were negative for OFA/iLR expression, and CD19⁺ purified B cells from healthy donors were used as target cells and incubated with different dilutions of patient serum. All CLL samples used as targets were analyzed by FACS for CD19⁺ surface expression and contained >90% CD19⁺ cells, either for high leukemic burden or after CD19⁺ isolation using MACS as described above.

Statistical methods

To establish cut-off points for OFA/iLR OD values that distinguished between negative and positive cases, we used different approaches including the mean plus two SDs of the healthy donor samples and the receiver operator characteristic curve (31, 32). Using these approaches, the suggested cut-off points lie between 0.06 and 0.13 OD. As for the exploratory nature of the study and to avoid false-positives, an OD of 0.11 for IgG and 0.13 for IgM

were chosen as initial cut-off values for determination of positive Ab reactivity for OFA/iLR and were subsequently applied to healthy donors and CLL patients. Student's *t* test was used to determine differences in anti-OFA/iLR Abs between healthy donors and CLL patients. Following Dohner et al.'s hierarchical classification, high-risk cytogenetics was defined as the presence of del17p and/or del11q (3). As six of seven patients with tri12 concomitantly had a del11q, the remaining patient with isolated tri12 was also included in the group of high-risk cytogenetics. Survival data were obtained from clinical records. Progression-free survival (PFS) was defined as the time from diagnosis to either disease progression or death from any cause (events) or last follow-up (censored) and was estimated using the method of Kaplan and Meier. In a univariate analysis we compared the Kaplan-Meier curves by the log-rank test and additionally by the Breslow test, which gives more emphasis to early events and is preferred in situations when the proportional hazard assumption may be violated by the data (33). Since CLL is an incurable disease, all patients will eventually experience disease progression, and early differences in events are important. Multivariate Cox regression models, adjusted for established risk factors, were fitted to evaluate the impact of the humoral immune response as a novel prognostic factor on PFS. Relative risks and the corresponding 95% CIs were calculated. *p* values ≤ 0.05 were considered to be significant. All

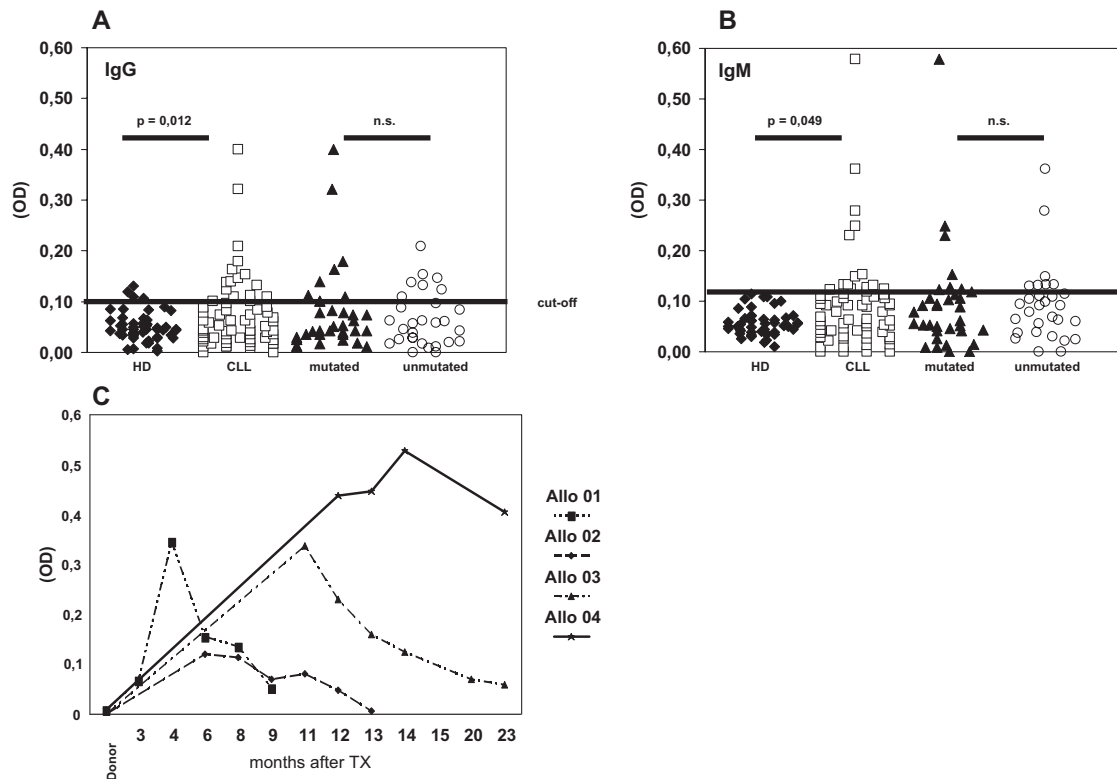


FIGURE 2. Titers of IgG and IgM anti-OFA/iLR Abs in untreated CLL patients, healthy donors, and CLL patients after allogeneic transplantation. *A* and *B*, Results from anti-OFA/iLR ELISA are shown for healthy donors (HD) (■), untreated CLL patients (CLL) (□), mutated CLL patients (▲), and unmutated CLL patients (○) for (A) IgG and (B) IgM, respectively. Black horizontal bars indicate cut-off values for IgG (0, 11) and IgM (0, 12). Ab response is displayed as OD. *C*, OD levels from an ELISA analysis for four CLL patients after allogeneic transplantation. Time point –1 represents pretransplant donor sera.

statistical analyses were performed using SPSS/PC+ software (version 12.0) for Windows.

Results

Expression of OFA/iLR in patients with CLL and normal B cells

Expression of OFA/iLR protein on CLL cells and B lymphocytes purified from the blood of healthy individuals was determined by flow cytometry and Western blot analysis using the previously described monoclonal OFA/iLR-specific Ab (21). OFA/iLR expression was detectable in all of the 67 samples of CLL patients, although the range of OFA/iLR expression levels was relatively broad (10–98%, mean 63.3%). As described earlier, no OFA/iLR expression was found by FACS on blood cells, including both CD3⁺/CD4⁺ and CD3⁺/CD8⁺ T lymphocytes, CD 14⁺ monocytes, CD19⁺ B cells, CD34⁺ progenitor cells, and CD56⁺/CD16⁺ NK cells (21). Interestingly, cells from patients with Richter's syndrome ($n = 5$), a secondary high-grade lymphoma developing in patients with CLL, did not show OFA/iLR expression. Fig. 1*A* shows flow cytometric analyses of purified CD19⁺ B lymphocytes from one healthy donor and the expression profile of OFA/iLR on malignant B cells of four CLL patients. OFA/iLR expression seen by flow cytometry was confirmed by Western blot analysis for healthy donors and CLL patients (Fig. 1*B*).

OFA/iLR Abs are present in sera from untreated patients with CLL as well as in patient sera after allogeneic stem cell transplantation

First, specificity of the monoclonal anti-OFA/iLR Ab used for all subsequent ELISA analysis was confirmed by dot blot shown in Fig. 1*C*. Anti-OFA/iLR Abs specifically detected the recombinant

protein but not the nonrelated protein survivin and, conversely, OFA/iLR protein was not recognized by the nonrelated anti-survivin Ab. Given the frequent expression of OFA/iLR on the surface of CLL cells, we next analyzed whether patients developed spontaneous humoral immune responses toward this Ag. The presence of anti-OFA/iLR IgG and IgM Abs was evaluated by means of an ELISA assay using the recombinant OFA/iLR protein as a coating agent. Sixty-seven untreated patients with CLL and 50 healthy volunteers were analyzed. In 22 of 67 patients (32.8%), IgG OFA/iLR Abs were detected (Fig. 2*A*). In 12 of 67 patients (17.9%), IgM OFA/iLR Abs were identified (Fig. 2*B*), whereas 5 (7.4%) patients had both IgG and IgM Abs detected. Four of 50 healthy donors (8%) had IgG or IgM OD levels above the cut-off point. Untreated patients with advanced stage of disease (Binet C) did not show any detectable levels of IgG or IgM Abs against OFA/iLR ($n = 10$, data not shown). CLL patients had statistically significant higher levels of anti-OFA/iLR-IgG and -IgM ($p = 0.012$ and $p = 0.049$, respectively) while there was no statistically significant difference between the humoral response of mutated and unmutated patients (Fig. 2, *A* and *B*).

We also looked for anti-OFA/iLR Ab levels in 11 patients with CLL who had undergone allogeneic stem cell transplantation (allo-SCT). The time from allo-SCT until first analysis of sera varied from 4 to 44 mo (median 14.8 mo). Eight of 11 sera showed detectable IgM and/or IgG anti-OFA/iLR Abs after allo-SCT. Interestingly, six of eight patients who achieved a complete remission after transplantation with complete donor chimerism also showed significant anti-OFA/iLR responses. Characteristics of allotransplanted patients and Ab responses are shown in Table II. For four patients, donor sera were available, which all tested negative for

Table II. Characteristics of patients after transplantation of allogeneic blood stem cells^a

Patient	Stage after SCT	Time from SCT to Analysis (mo)	Humoral Response (Epitope No.)	Treatment (Donor)	GVHD	Immunosuppression ^b
allo01	CR	7	Yes (IgG 34/35; IgM 34/35)	Flu/Cy (MUD)	No	No
allo02	CR	13 [†]	Yes (IgG 30/34)	Flu/Cy (MUD)/DLI	aGVHD (skin I°)	No
allo03	CR	9	Yes (IgM 30)	Flu/Bu/Cy (MUD)	cGVHD (skin II°)	No
allo04	CR	44	Yes (IgM 41)	Flu/Cy (Sib)	No	No
allo05	PD	24	Yes (IgG 36)	Flu/Cy (MUD)	cGVHD (skin II–III°)	No
allo06	CR	16	No	Cla/Cy (Sib)	No	No
allo07	PR	8	No	Flu/Cy (Sib)	No	No
allo08	CR	21	Yes (IgM 38)	Flu/Cy (MUD)	No	No
allo09	CR	4	Yes (IgG 25)	Flu/Cy (MUD)	No	No
allo10	PR	4	Yes (IgG 33)	Flu/Cy (MUD)	No	No
allo11	CR	8	No	Flu/Cy (MUD)	No	CyA

^a Bu indicates busulfan; CyA, cyclosporin A; Cy, cyclophosphamide; CR, complete remission; DLI, donor lymphocyte infusion; Flu, fludarabin; a/cGVHD, acute/chronic graft-vs-host disease; MUD, matched unrelated donor; PD, progressive disease; PR, partial remission; Sib, sibling donor; SCT, stem cell transplantation.

^b At time of analysis.

^c Analysis after donor lymphocyte infusion.

anti-OFA/iLR Abs. Immune responses against OFA/iLR developed after allo-SCT sustained for several months, but decreased over time without signs of relapse or loss of full donor chimerism (Fig. 2C).

Specific recognition of OFA/iLR epitopes differs between untreated patients and patients after allogeneic SCT

All patient sera with significant anti-OFA Ab levels (IgG, $n = 20$; IgM, $n = 9$; IgG + IgM, $n = 5$) were included in an epitope mapping assay to confirm epitope specificity. A panel of 42 overlapping peptides representing the entire sequence of the OFA/iLR protein was used in an IgG and IgM ELISA. Twenty-three patients recognized at least one distinct epitope of the OFA/iLR sequence. To exclude nonspecific reactivity, only those patients with specific epitope recognition were considered to have reactive sera. Fifteen patients with reactive sera recognized more than one epitope. Fig. 3A shows the distribution of all recognized peptide epitopes from untreated CLL patients in relation to the localization of functional domains of OFA/iLR. The distribution of specific IgG and IgM responses includes association with intracellular as well as extracellular located epitopes. CLL patients after allogeneic stem cell transplantation who mounted Ab responses against OFA/iLR ($n = 8$) exclusively recognized peptide epitopes from aa 159–290 located toward the extracellular C-terminal end of the OFA/iLR protein sequence. In contrast to the untreated patient group, intracellular domains were not recognized (Fig. 3B).

Binding of anti-OFA/iLR Abs can be specifically inhibited

To validate the specificity of the detected anti-OFA/iLR Abs, an inhibition assay was performed in the presence of recombinant OFA/iLR protein, which was formerly used as a coating Ag in the ELISA. As demonstrated in Fig. 4A, reactivity to OFA/iLR of respective patient sera was inhibited by addition of the OFA/iLR protein in a dose-dependent manner. No inhibitory effect was observed when HIV p24 protein or β_2 -microglobulin was added. Using reactive serum as the source of primary Ab and a FITC-conjugated secondary Ab in a FACS analysis, CD19⁺ purified malignant B lymphocytes (CLL cells) from an OFA/iLR positive patient were recognized but not OFA/iLR-negative CLL cells (Fig. 4B). Serial dilutions of serum at 1/25, 1/50, and 1/100 showed dose-dependent recognition.

Predominant subclasses of OFA/iLR Abs are IgG1 and IgG3 and implicate a Th1-biased immune response

For further characterization of OFA/iLR-reactive sera, IgG subclass analysis was performed in an ELISA assay. Sera from pa-

tients recognizing at least one specific OFA/iLR epitope were subclassified. Sera from healthy donors were also analyzed for control purposes although they were not reactive in the screening of ELISA for OFA/iLR reactivity. Fig. 5A shows values of OD for

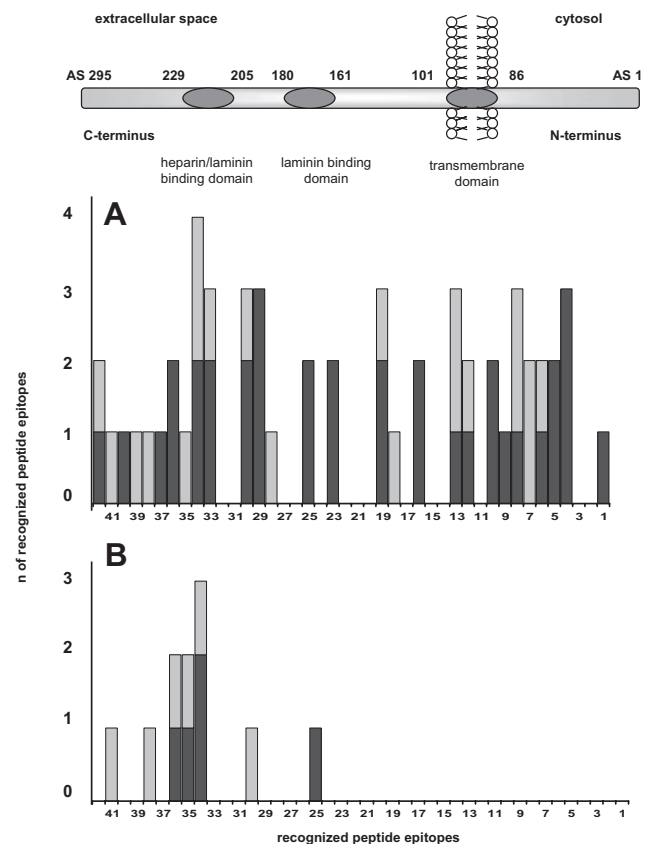


FIGURE 3. Distribution of recognized epitopes by IgG and IgM anti-OFA/iLR Abs in relation to the OFA/iLR sequence in untreated CLL patients and postallogeneic stem cell transplantation. Schematic view of the OFA/iLR protein and its identified functional domains. OFA/iLR spans the plasma membrane once from aa 86–101 with its C terminus directed toward the extracellular space. Laminin binding sites are identified at aa 161–180 and 205–229. A, Distribution of recognized IgG and IgM OFA/iLR epitopes in relation to the functional OFA/iLR peptide sequence. Results from OFA/iLR ELISA using 42 overlapping peptides. B, Post-allo-SCT sera recognize extracellular located epitopes of the OFA/iLR sequence. OD for IgG and IgM is displayed for each of the 42 epitopes. A and B, Black bars represent IgM, gray bars represent IgG.

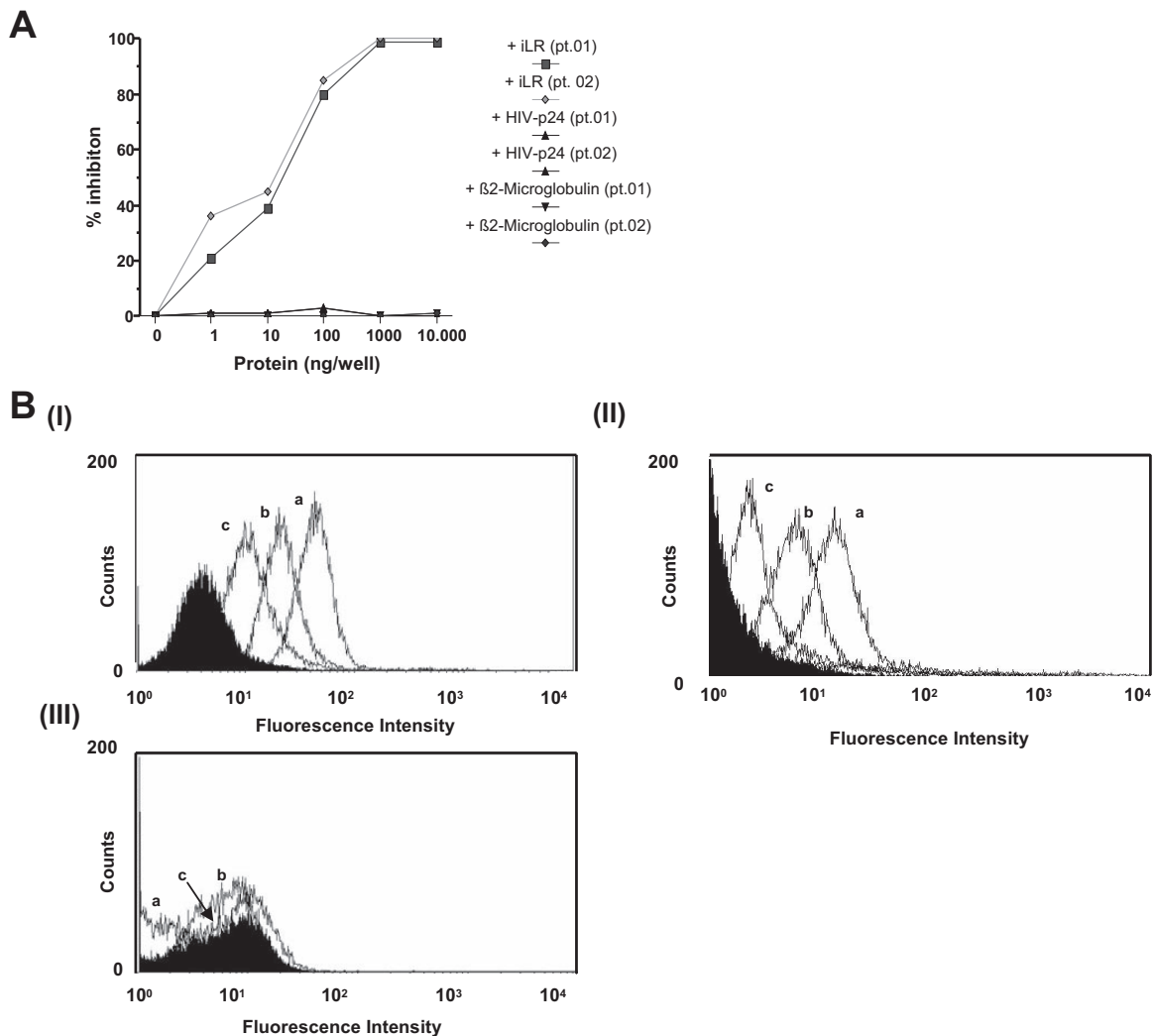


FIGURE 4. Anti-OFA/iLR Abs are specifically inhibited by addition of OFA/iLR protein and bind to OFA/iLR-expressing cells. *A*, Reactive sera from two CLL patients were incubated with either recombinant OFA/iLR-protein, HIV p24-truncated protein, or β_2 -microglobulin and analyzed in an ELISA. Results are shown as percentage of inhibition in regard to initial OD values for OFA/iLR ELISA. *B*, Reactive serum recognizes OFA/iLR-positive CLL cells but not OFA/iLR-negative CLL cells. CD19⁺ purified B lymphocytes from two OFA/iLR-positive CLL patients (*I* and *II*) and cells from a patient with CLL in Richter transformation (*III*) were incubated with OFA/iLR-reactive serum in serial dilutions of 1/25, 1/50, and 1/100 as a source of primary Ab and a rabbit anti-human IgG as a secondary Ab.

IgG1, IgG2, IgG3, and IgG4 Abs. The distribution of subclasses is shown in Fig. 5*B*, which defines the highest value for each patient as predominant IgG subclass. IgG1 was the predominant subclass in 14 of 18 patients, while IgG3 anti-OFA/iLR Ab was the major IgG subclass for 8 patients. IgG4 was found to be predominant in serum from only one patient. In five patients, high values for both IgG1 and IgG3 Abs were detectable. No correlation was found between the detection of predominant IgG subclass and the mutational status or OFA/iLR expression of CLL cells (data not shown).

Anti-OFA/iLR Abs induce ADCC and complement activation

We next determined the functional properties of the reactive sera. The ability of OFA/iLR Abs to lyse OFA/iLR-expressing CLL cells was determined in an ADCC assay and complement-dependent cytotoxicity (CDC) assay. Sera from patients that showed strong responses in the initial screening ELISA as being responsive to one specific OFA/iLR epitope in the epitope mapping were able to induce lysis of autologous OFA/iLR-positive CLL cells (Fig. 6, *A* and *E*) in a dose-dependent manner. Fur-

thermore, reactive sera were able to induce lysis of allogeneic, OFA/iLR-expressing CLL (Fig. 6, *B* and *F*) cells but not normal CD19⁺ B cells (Fig. 6, *C* and *G*) or CLL cells from patients with Richter transformation, which are negative for OFA/iLR expression (Fig. 6, *D* and *H*). In contrast, no lytic effect in ADCC or CDC was observed using healthy donor sera against OFA/iLR expressing CLL cells (Fig. 6, *A*, *B*, *E*, and *F*). In ADCC, a constant background lysis of 10% of the CLL cells was observed for all serum dilutions owing to spontaneous NK cell-mediated lysis.

PFS in patients with humoral reactivity to OFA/iLR

To determine whether the presence of specific anti-OFA/iLR Abs influenced PFS, patients were grouped according to the presence/absence of a humoral immune response, which specifically recognized at least one OFA/iLR epitope, and were then analyzed using Kaplan-Meier plots. The median duration of follow-up was 72 mo. In a univariate analysis, the group of patients with reactive sera ($n = 23$) showed a significantly lower probability of disease progression compared with patients

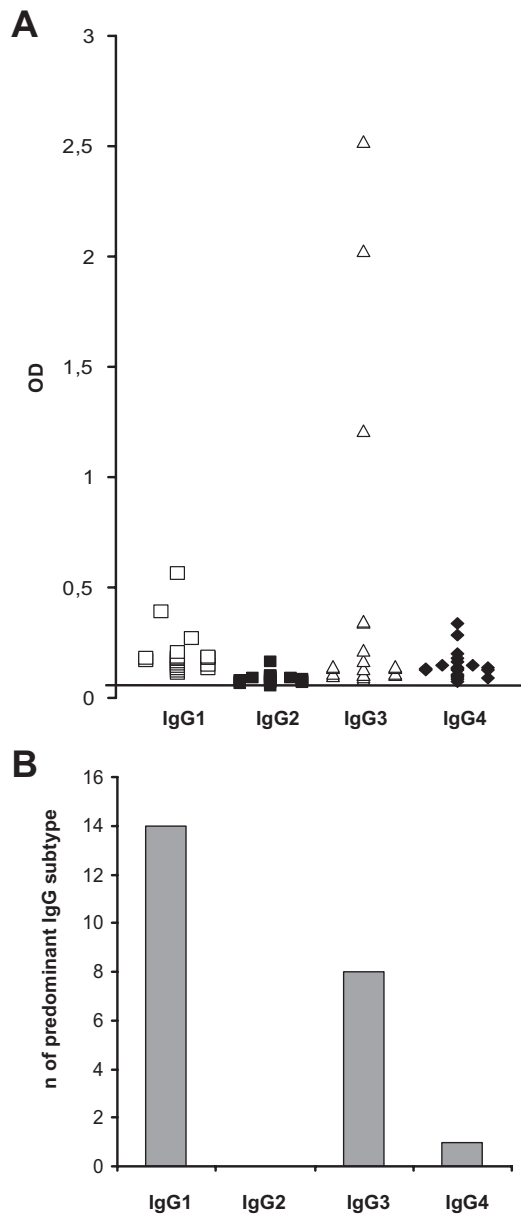


FIGURE 5. IgG1 and IgG3 are the predominant subclasses of OFA/iLR-specific Abs. *A*, All patients recognizing at least one specific OFA/iLR epitope were analyzed for IgG subclasses. Values of OD for IgG1, IgG2, IgG3, and IgG4 anti-OFA/iLR Abs are shown. *B*, Summary of predominant IgG subclass of each patient.

with nonreactive sera (Fig. 7A). Median PFS for patients with reactive sera was 114 mo compared with 66 mo for the nonreactive group ($p = 0.029$). Patients with unmutated V_H genes had a PFS of 70 mo compared with 96 mo for mutated V_H genes ($p = 0.128$) (Fig. 7B). A PFS of 36 vs 96 mo was found for patients with presence or absence of high-risk cytogenetics, respectively ($p = 0.005$) (Fig. 7C). Comparing the reactive and nonreactive groups of patients, the distribution of V_H status did not vary significantly due to the low number of patients. The number of patients with high-risk cytogenetics differed significantly (reactive 8.6% vs nonreactive 40.9%) between the two subgroups. Exact characteristics of the two groups are displayed in Table III. Keeping in mind the possible limitations, we investigated whether the presence of humoral immune responses influenced the time to progression in a multivariate analysis. In the final model with adjustment for V_H mutational status and

high-risk cytogenetics, the presence of humoral immune responses against OFA/iLR remained significant and revealed a relative risk of 0.36 (95% CI 0.14–0.94; $p = 0.038$). Results from the statistical analysis are summarized in Table IV.

Discussion

The role of spontaneous tumor-specific immune responses in controlling CLL is largely unknown. Furthermore, the number of characterized tumor Ags expressed on the cell surface of CLL cells is limited. As an example, the individual B cell receptor and its complementary determining region (CDR3) represent an individual-specific tumor Ag that elicits immune responses in a number of B cell malignancies including CLL. The advantage of a highly selective specificity of the Id is counterbalanced by the enormous technical difficulties to generate Id-derived epitopes (34–37). The OFA/iLR is expressed intra- and extracellularly in various tumor entities (17, 25, 38–40). Recently, our group identified HLA-A*0201-presented T cell epitopes derived from OFA/iLR protein in patients with hematological malignancies including B-CLL (22). The wide expression on CLL cells and its known immunogenicity made OFA/iLR a suitable target structure to determine tumor-specific humoral immunity in CLL patients (19, 21, 41).

In the current study, we were able to detect specific IgG and IgM Abs against OFA/iLR in 23 of 67 untreated patients with CLL, significantly more than those present in healthy donor sera. Moreover, 8 of 11 patients after allogeneic SCT showed significant levels of anti-OFA/iLR Abs. Only few data exist documenting the presence and significance of humoral immune responses against defined tumor Ags. In acute myeloid leukemia and myelodysplastic syndrome, Elisseeva et al. described humoral immune responses against Wilms tumor gene products and suggested a possible correlation between leukemic tumor burden and production of Wilms tumor Abs (42). In the present study, anti-OFA/iLR Abs are detectable both in untreated patients with potentially ongoing antigenic stimulation and in patients after allotransplantation in the absence of malignant B cells. Analysis of serial samples of patients after allo-SCT showed a decline of Ab responses after transplantation in the absence of relapse or loss of chimerism, which might be explained by decreasing tumor burden with concomitantly decreasing OFA/iLR expression.

IgG subclass analysis of the detected anti-OFA/iLR Abs revealed a predominant IgG1 and IgG3 Ab response in both untreated and transplanted patients, which is in agreement with previous data on autologous Ab responses in acute myeloid leukemia and myelodysplastic syndrome. Some experimental evidence exists that this reactivity pattern is associated with a Th1-driven immune response, also necessary for CTL induction (43–45). In agreement with our previous data, the current findings support the rationale for the use of OFA/iLR-derived epitopes to elicit OFA/iLR-specific CTL responses (21). The presence of both IgG and IgM isotypes suggests the involvement of a class switch promoting $CD4^+$ Th cells as evidence of a coordinated immune response against OFA/iLR.

The graft-vs-leukemia (GVL) effect after allogeneic stem cell transplantation can result in complete remission of CLL and indicates the usefulness of immunotherapeutic approaches for this disease (28, 46). Although T cells clearly remain the critical components of the GVL effect, some studies also emphasize the presence of a tumor-specific humoral immune response (29, 47–50). We were able to detect significant levels of Ab titers against OFA/iLR in CLL patients receiving an allotransplant, although further investigation is needed to clarify the functional

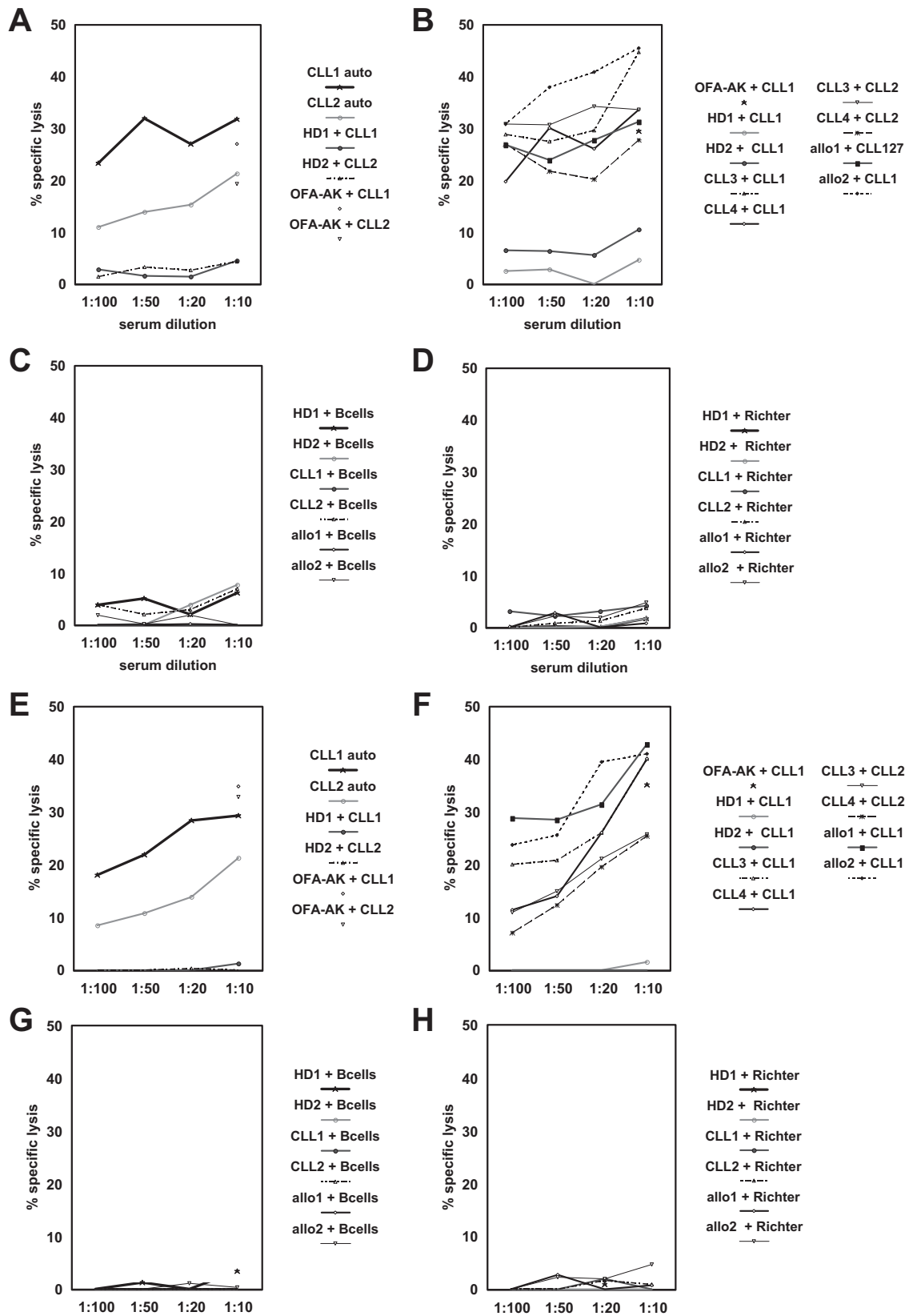


FIGURE 6. OFA/iLR-reactive patient serum mediates specific CDC and ADCC. *A–D*, Ab-mediated cellular cytotoxicity; healthy donor PBMC were added in an E:T ratio of 1:10. *E–H*, Complement-dependent cytotoxicity. *A* and *E*, OFA/iLR-reactive patient sera (CLL1 and CLL2) mediate lysis of autologous CLL cells via ADCC (*A*) and CDC (*E*). *B* and *F*, OFA/iLR-reactive sera from untreated patients (CLL3 and CLL4) and patients after allogeneic SCT (allo1 and allo2) lyse OFA/iLR-expressing cells (CLL1 and CLL2). *A*, *B*, *E*, and *F*, Anti-OFA/iLR Ab was used as a positive control. Healthy donor sera (HD1 and HD2) were used as negative controls. *C* and *G*, Negative control with normal B cells as target cells. *D* and *H*, Negative control with OFA/iLR-negative CLL cells in Richter transformation as target cells.

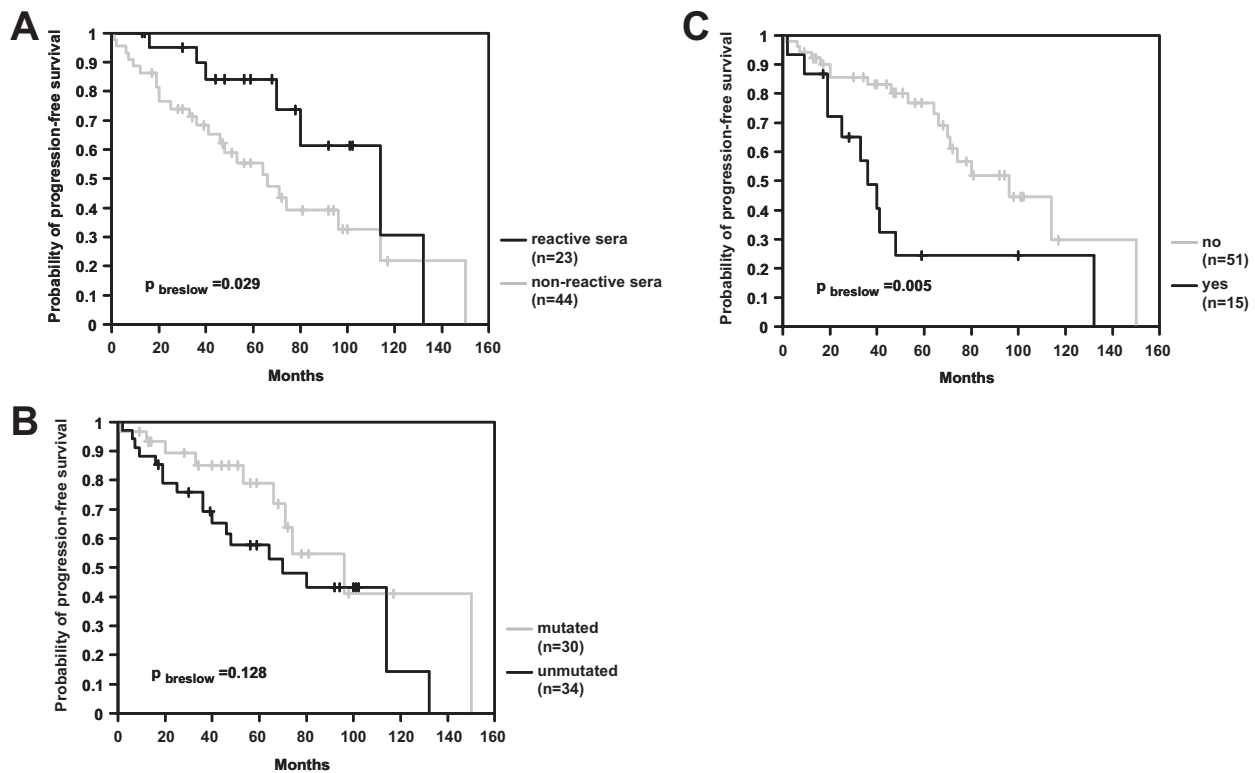


FIGURE 7. Superior PFS for patients with OFA/iLR-reactive sera. PFS of 67 untreated CLL patients grouped according to (A) their ability to specifically recognize at least one OFA/iLR ELISA epitope, (B) mutated or unmutated status of the V_H genes, and (C) presence or absence of either del17p, tri12, and/or del11q.

role of these Abs with regard to GVL in CLL. The detected Abs from untreated as well as transplanted patients were capable of recognizing and efficiently killing OFA/iLR-expressing CLL cells while sparing OFA/iLR-negative cells. It remains unclear at this point why the killing of CLL cells is inefficient in vivo. It is possible that deficient killing in vivo may be linked to Fc receptor polymorphisms or NK cell defects, which remains to be investigated.

Interestingly, Ab responses observed in patients after allogeneic transplantation only recognized peptides of the extracellular part toward the C-terminal end of the OFA/iLR sequence while Abs from untreated CLL patients recognized epitopes from the entire sequence. Although it has to be investigated whether this phenomenon is an expression of a specific immune recognition pattern, it

provides valuable information for the generation of therapeutic Abs directed against OFA/iLR-expressing tumor cells.

Untreated CLL patients mounting an Ab response against OFA/iLR showed a significantly longer period of time to progression compared with those patients with nonreactive sera, which was also confirmed in a multivariate analysis. Prognostic relevance of humoral immune responses was recently demonstrated for several other malignancies including ovarian cancer and follicular lymphoma (15, 51). Our data presented here suggest that humoral immunity to OFA/iLR is a new indicator of prolonged PFS in untreated CLL patients. Considering the retrospective design of the study, the rather small cohort, and a median follow-up time of only 72 mo, the presence of OFA/iLR Abs must be assessed prospectively along with other markers to confirm the prognostic value seen in this study.

Table III. Characteristics of CLL patients with reactive and nonreactive sera against OFA/iLR

	Positive Anti-OFA/iLR Response		Negative Anti-OFA/iLR Response	
	n = 23	%	n = 44	%
Cytogenetics (FISH)				
Normal	9	39.1	17	38.6
Deletion 13q14	12	52.2	15	34.1
Trisomie 12	1	4.3	6	13.6
Deletion 11q	1	4.3	11	25.0
Deletion 17p	0		1	2.3
V_H status				
Mutated (>98% homology)	15	65.2	19	43.2
Unmutated (>98% homology)	8	34.8	22	50.0
Not available	0		3	6.8

Table IV. Univariate and multivariate statistical analysis of risk factors including anti-OFA/iLR humoral immune response, V_H mutational status, and cytogenetic aberrations

Risk Factor (n)	Median PFS (mo)	Univariate Analysis		Multivariate Analysis		
		95% CI	p	Relative Risk	95% CI	p
Humoral response			0.029			
Yes (23)	114	64–164		0.36	0.14–0.94	0.038
No (44)	66	40–92				
V_H status			0.128			
Unmutated (34)	70	27–113		2.26	0.88–5.77	0.089
Mutated (30)	96	63–129				
Unfavorable FISH			0.005			
Yes (15)	36	24–48		1.88	0.78–4.53	0.159
No (51)	96	58–134				

In summary, we for the first time describe tumor-specific anti-OFA/iLR Abs in sera of CLL patients with prognostic relevance, which is suggestive of a humoral immune control in CLL. The functionality of these Abs and the recognition of extracellular epitopes of the OFA/iLR sequence have significant implications for the development of immunotherapeutic approaches targeting OFA/iLR⁺ tumor cells.

Disclosures

The authors have no financial conflicts of interest.

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