CLINICAL TRIALS AND OBSERVATIONS

Upfront immunization with autologous recombinant idiotype Fab fragment without prior cytoreduction in indolent B-cell lymphoma

Marcelo A. Navarrete,[1] Kristina Heining-Mikesch,[1] Frank Schüler,[2] Cristina Bertinetti-Lapatki,[1] Gabriele Ihorst,[3] Andrea Keppler-Hafkemeyer,[1] Gottfried Dölken,[2] and Hendrik Veelken[1,4]

[1]Department of Hematology/Oncology, University Medical Center Freiburg, Freiburg, Germany; [2]Department of Hematology and Oncology, Ernst-Moritz-Arndt-University Greifswald, Greifswald, Germany; [3]Department of Medical Biometry and Statistics, University Medical Center Freiburg, Freiburg, Germany; and [4]Department of Hematology, Leiden University Medical Center, Leiden, The Netherlands

Idiotype vaccination for follicular lymphoma is primarily being developed as remission consolidation after chemotherapy. We investigated idiotype vaccination as primary intervention for treatment-naive indolent B-cell lymphoma and in a separate cohort as remission consolidation after chemotherapy to assess immunization-induced immune responses in relation to progression-free survival (German Clinical Trials Register, DRKS00000227). Twenty-one patients in each cohort received 6 intradermal injections of adjuvanted recombinant idiotype Fab fragment (Fab[Id]); 76% of patients in both groups developed anti-idiotype antibodies and/or cellular immunity as measured by enzyme-linked immunosorbent assay and interferon-γ ELISpot. In treatment-naive patients, only cellular responses correlated with superior progression-free survival ($P < .002$) and durable objective remissions ($P = .04$). Immunization-induced T cells recognized hypermutated or complementarity-determining region 3 epitopes. After remission consolidation immunization, induction of anti-idiotype antibodies correlated with progression-free survival. Low B-cell counts after rituximab therapy predicted for failure to develop anti-idiotype antibodies. These results are similar to published trials showing an association of humoral immunity with control of residual lymphoma. In contrast, effective immunity against untreated lymphoma appears to be dependent on idiotype-specific T cells. Sustained remissions in patients with vaccination-induced cellular immunity suggest clinical benefit and warrant a randomized comparison of this vaccine with expectant management for asymptomatic follicular lymphoma. (*Blood*. 2011;117(5): 1483-1491)

Introduction

Indolent B-cell lymphomas in advanced stages remain incurable with available therapies.[1-3] The initiation of cytoreductive therapy immediately after diagnosis does not confer a survival advantage for asymptomatic patients compared with deferral of treatment until symptomatic progression.[4,5] Given the unavoidable risk of undesirable side effects of conventional chemotherapy and antibody-based cytoreductive regimens, observation remains the recommended approach in this patient population despite improved survival rates with rituximab-based chemoimmunotherapy for follicular lymphoma (FL) patients who require treatment.[2,4-6]

The individual B-cell receptor, commonly referred to as the lymphoma "idiotype," constitutes a unique structure and hence a tumor-specific antigen of a malignant B-cell clone. Immunization of indolent B-lymphoma patients against their tumor's idiotype can induce specific antitumor immune responses in vivo.[7-11] Based on the assumption that active tumor immunotherapy has the best chance of providing clinical benefit in the clinical setting of minimal residual disease, when only residual tumor cells must be suppressed or eliminated, most clinical trials have investigated idiotype vaccination as remission consolidation after successful prior cytoreductive therapy. In this setting, FL patients who develop an anti-idiotype antibody response experience improved progression-free survival compared with patients who fail to develop a response to the vaccine.[8,12,13] Retrospective analyses suggest that this benefit may translate into improved overall survival.[14]

Recently reported controlled randomized trials of idiotype vaccination for FL in clinical remission have, however, yielded conflicting results in intention-to-treat analyses: When patients were immunized after rituximab monotherapy or after conventional chemotherapy, progression-free survival was not significantly different in vaccination and control groups.[12,15] In contrast, when immunization was restricted to patients in complete remission, patients who received an otherwise similar idiotype vaccine formulation had superior relapse-free survival compared with control immunization.[16]

The potential benefit of idiotype vaccination for lymphoma patients who have not received prior cytoreductive therapy has not yet been addressed systematically. Induction of an efficacious antitumor immune response before other interventions may represent an attractive approach to delay or prevent the necessity for conventional cytoreductive therapy in advanced-stage but asymptomatic indolent B-lymphoma patients. We have recently presented indirect, "reverse immunology" evidence for T cell–mediated

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The online version of this article contains a data supplement.

Table 1. Clinical characteristics of patients: group A (watch and wait)

		All patients	FL	MCL	nMZL
n		21	15	3	3
Median (range) age	years	53 (30-78)	44 (30-60)	77 (56-78)	68 (57-75)
Stage	III A	10	9	0	1
	IV A	11	6	3	2
Risk score, n		IPI 1/5: 13	FLIPI 1/5: 5	MIPI 5-6: 1	IPI 1/5: 1
		IPI 2-3/5: 8	FLIPI 2/5: 8	MIPI 6-7: 2	IPI 2/5: 2
			FLIPI 3/5: 2		
Median (range) time from diagnosis to first vaccination	months	13 (5-105)	13 (5-105)	11 (7-15)	18 (8-27)
Remission status	SD	17	11	3	3
	PD	4	4	0	0
FcγRIIIa genotype	158FF	9	6	2	1
	158FV	8	6	1	1
	158VV	3	2	0	1

IPI indicates International Prognostic Index; FLIPI, Follicular Lymphoma International Prognostic Index; and MIPI, Mantle Cell Lymphoma International Prognostic Index.

immunosurveillance against the idiotype of follicular lymphomas.[17] These results permit to speculate that subclinical T-cell reactivity that was insufficient to control lymphoma growth could be enhanced by active immunotherapy to exert clinically meaningful antilymphoma activity.

We report here the results of a phase II vaccination trial designed to demonstrate the immunologic efficacy of recombinant idiotype vaccination as upfront intervention for asymptomatic patients and for remission consolidation. To ease the technical challenge of manufacturing idiotype vaccines on a patient-individual basis, we have developed the expression of idiotype protein as Fab fragments (Fab[Id]) in *Escherichia coli* based on validated immunoglobulin clonality criteria.[18,19] In a phase I trial, we have previously demonstrated the feasibility and tolerability of intradermal injection of Fab[Id] with the lipid-based adjuvant MF59 in conjunction with subcutaneous granulocyte-macrophage colony-stimulating factor.[12]

Methods

Patients

Adult patients with nonsecretory indolent B-cell lymphoma in Ann Arbor stage III or IV were eligible if fresh lymphoma tissue could be obtained without general anesthesia. Active infections and severe concomitant illness, active central nervous system involvement, and systemic antineoplastic therapy, including corticosteroids within 4 weeks before the first vaccination were exclusion criteria. Pregnant or lactating women and women with reproductive capacity not using contraception were ineligible. The trial enrolled patients at the University Medical Centers of Freiburg and Greifswald from 2003 to 2009 into 2 separate cohorts: group A consisted of patients who had not received any type of antineoplastic therapy and had no indications for cytoreductive therapy.[20] Vaccinations were withheld for patients with evidence for spontaneous lymphoma regression. Patients in objective clinical remission according to Cheson criteria[21] after cytoreductive therapy were eligible for vaccination as group B.

The trial protocol was approved by the Institutional Review Board at University Medical Center Freiburg and registered in the German Clinical Trials Register (no. DRKS00000227). All participants gave informed consent in accordance with the Declaration of Helsinki.

Vaccine production and vaccination schedule

Recombinant Fab[Id] vaccines were formulated with the adjuvant MF59 and purchased from CellGenix GmbH.[10] Fab[Id] fragments were produced by anchored PCR cloning of consensus immunoglobulin (Ig) heavy and light chain transcripts from unfixed lymphoma tissue and expression in *E coli*.[18]

One vaccination consisted of subcutaneous injections of 250 μg sargramostim (Immunex) per day on 5 consecutive days at the same location. On day 2, 0.5 mg Fab[Id]/MF59 was injected intradermally at the sargramostim-conditioned site. Six vaccinations were given in 4-weekly intervals. Patients without lymphoma progression were offered additional maintenance vaccinations at 3-month intervals starting at week 36. Anti–HBs-negative patients received hepatitis B immunizations (Gen-HB-Vax; Aventis Pasteur) with the first, third, and sixth idiotype vaccination. Anti-HBs titers were measured at week 24.

A complete restaging was performed before the first vaccination.[21] Disease sites were reassessed on weeks 8, 16, 24, 36, and 52. Subsequently, disease status was reevaluated every 3 months or at suspected relapse. Vaccinations were discontinued if clinical progression was documented. Adverse events were reported according to World Health Organization criteria.[22]

Immunologic studies

Serum IgG concentration and circulating CD4^{+}, CD8^{+}, and CD19^{+} lymphocytes were measured before vaccination by flow cytometry according to the institutional diagnostic standards. AntiFab[Id] immune responses of freshly isolated peripheral blood mononuclear cells (PBMCs) were assessed by interferon-γ (IFN-γ) enzyme-linked immunospot (ELISpot) with a protocol validated by blinded interlaboratory testing (www.cimt.eu)[23] and adapted for presentation of polypeptides by autologous dendritic cells.[10] Only assays with 200 to 400 spots/2×10^4 cells in lectin controls were evaluated. Background spots obtained with dendritic cells without addition of antigen were subtracted. A cellular response to the vaccine idiotype was defined as a significant increase ($P < .05$ by 2-sided t test) of spots at week 24 and at least one additional postvaccination time point compared with prevaccination samples. The specificity of idiotype-reactive T cells was subsequently tested by ELISpot with control dendritic cells pulsed with allogeneic idiotype that were chosen to share the same VH family with the vaccine idiotype. For epitope mapping studies, idiotype peptides potentially presented by a patient's major histocompatibility complex class I alleles were identified by the SYFPEITHI algorithm (www.syfpeithi.de) and synthesized. Peptide-loaded (1 μg/mL) dendritic cells were used as stimulators in ELISpot assays with unexpanded PBMCs.

To measure Fab[Id]-induced proliferation, overnight-rested PBMCs were labeled with 0.5μM carboxyfluorescein succinimidyl ester and cultured with antigen-pulsed or unpulsed dendritic cells. Anti-CD3/CD28–coated beads (Dynal) or phytohemagglutinin (ICN Biomedicals) served as positive controls. After 6 days of stimulation, cells were stained with anti-CD4–allophycocyanin and anti-CD8–Pacific blue antibodies or appropriate isotype controls (all from eBioscience). Loss of carboxyfluorescein succinimidyl ester was analyzed for the lymphocyte gate and in CD4^{+} and CD8^{+} cells by flow cytometry.[24]

To distinguish T helper type 1 (Th1)- and Th2-dominated immune responses, secretion of interleukin-4, interleukin-5, IFN-γ, and tumor necrosis factor-α by Fab[Id]-stimulated peripheral blood cells was measured

Figure 1. Immune status and immune responses. (A) Prevaccination lymphocyte subsets in the peripheral blood of untreated lymphoma patients (group A) and patients in remission (group B). Dashed lines indicate normal ranges. R indicates rituximab therapy. (B) Lymphocyte subsets of patients according to detection or absence of cellular (left and middle panels) or humoral immune responses (right panel). n.s. indicates not significant.
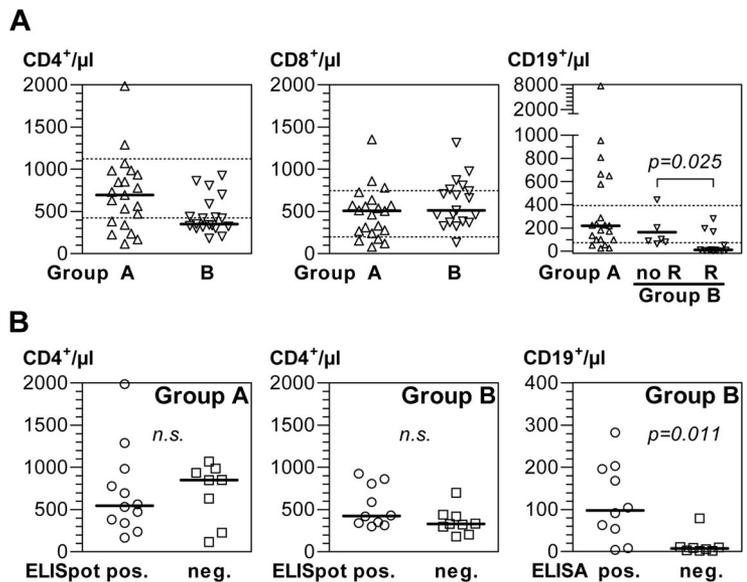
by cytokine bead arrays (BD Biosciences). More detailed information on T-cell assays according to the MIATA framework (www.miataproject.org; Version 1.0, modules 1-5) is available on request.[25]

Antibody responses to the vaccine were detected by enzyme-linked immunosorbent assay and were defined as a 4-fold titer increase compared with prevaccination serum[10] at week 24 and at least one additional time point. To demonstrate binding of immune serum to tumor cells, IgG was isolated by negative selection (Melon Gel IgG purification kit; Thermo Fisher Scientific) from prevaccination and postvaccination serum and incubated with viable cells from tumor biopsies. After fixation, cells were stained with Vectashield/DAPI (Vector Laboratories), anti-CD19–Alexa 647, and anti-IgG–biotin/streptavidin–fluorescein isothiocyanate (both Invitrogen). The fraction of fluorescein isothiocyanate$^+$ cells in the CD19$^+$ population were analyzed by automated digital fluorescence microscopy (ScanŘ High Content Screening Station; Olympus) with 20× UPLSAPO NA 0.75 air objective and an ORCA-AG camera (Hamamatsu). Images were acquired with Olympus ScanŘ Acquisition Software Version 2.1.0.16 and analyzed with Olympus ScanŘ Analysis Software Version 1.2.06).

Fc receptor polymorphism analysis

Sequence variations at position 158 of FcγRIIIa (CD16) were assessed by PCR-based restriction fragment length polymorphism analysis of genomic DNA.[26]

Statistical methods

The trial was designed according to the optimal 2-stage design[27] to detect as primary endpoint an immune response rate of $P_1 \geq 30\%$ at week 24 against a spontaneous response rate of $P_0 = 5\%$ with α and β errors of 10%. An immune response was defined as a cellular response, antibody response, or both. These criteria translate into patient number $n_1 = 7$ and n = 21. Both patient groups (upfront immunization and remission consolidation) were analyzed independently.

Secondary endpoints were objective lymphoma responses, progression-free survival, treatment-free survival, and overall survival from the start of vaccination and from diagnosis. Progression-free survival was defined as the interval between start of vaccination and death from any cause or objective lymphoma progression according to Cheson criteria, whichever came first.[21] Treatment-free survival was defined as the interval between start of vaccination and next cytoreductive therapy or death. Progression-free and treatment-free survival was estimated by the Kaplan-Meier method and compared from start of vaccination and in a landmark analysis[28] from week 24 by log-rank test (Prism Version 5.0; GraphPad Software).

Comparisons of laboratory data between patient groups were performed with the 2-tailed Mann-Whitney test. Analyses of experimental results from different time points were performed with Wilcoxon 2-tailed matched-pair test.

Results

Patient population and toxicity

In both groups, FL was the most common diagnosis. The majority of patients had additional disease-related risk factors beyond the advanced clinical stage and fell into intermediate-risk categories according to their disease-adapted prognostic scores (Tables 1 and 2). Four FL patients had objectively progressing disease at the start of vaccination. Two additional FL patients in "watch and wait" situation who had been scheduled for group A had evidence for spontaneous tumor regression and were not treated.

Grade 1 or 2 injection site reactions occurred in the majority of patients. The only case of grade 3 toxicity was a generalized erythema. After the sargramostim dose was reduced to 100 μg/day, the patient tolerated all subsequent vaccinations well. All patients completed the planned 6 vaccinations. Eight patients in group A and 9 patients in group B received between 1 and 29 additional maintenance immunizations (supplemental Table 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Immune status before vaccination

Six (29%) group A patients had reduced CD4$^+$ and 4 (19%) reduced CD8$^+$ T cells (Figure 1A). Ten of 12 (83%) anti–HBs-negative group A patients failed hepatitis B vaccination.

In the remission group, 12 of 19 patients (63%) with complete immune status had reduced CD4$^+$ and one (5%) reduced CD8$^+$ T cells. In association with prior rituximab therapy, 11 patients (58%) had reduced B-cell counts (Figure 1A). Fourteen of 15 (93%) anti–HBs-negative remission patients failed hepatitis B vaccination.

Table 2. Clinical characteristics of patients: group B (remission consolidation)

		All patients	FL	MCL	LL
n		21	14	6	1
Median (range) age	years	54 (29-70)	51 (29-70)	56 (44-64)	37
Stage	IIA*	1*	1*	0	0
	IIIA	6	5	1	0
	IIIB	2	2	0	0
	IVA	10	5	5	1
	IVB	2	1	1	0
Risk score: n		IPI 1/5: 10	FLIPI 1/5: 4	MIPI 5-6: 3	IPI 2/5: 1
		IPI 2-3/5: 9	FLIPI 2/5: 7	MIPI 6-7: 3	
		IPI 4-5/5: 2	FLIPI 3-4/5: 3		
Last systemic therapy	CHOP	2	2	0	0
	R-CHOP	6	6	0	0
	F	1	0	1	0
	FC	2	1	0	1
	FC-R	2	2	0	0
	Auto-SCT	5	0	5	0
	R mono	2	2	0	0
	Chloramb	1	1	0	0
Remission status	First CR	11	6	5	0
	First PR	3	1	1	1
	Second CR	2	2	0	0
	Second PR	4	4	0	0
	Fifth PR	1	1	0	0
Median (range) time end chemotherapy to first vaccination	months	5 (1-21)	6 {1-21)	3 (3-15)	1
FcγRIIIa genotype	158FF	11	8	2	1
	158FV	8	5	3	0
	158VV	2	1	1	0

IPI indicates International Prognostic Index; FLIPI, Follicular Lymphoma International Prognostic Index; MIPI, Mantle Cell Lymphoma International Prognostic Index; CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone; R-CHOP, CHOP and rituximab; F, fludarabine; FC, fludarabine and cyclophosphamide; FC-R, FC and rituximab; Auto-SCT, high-dose chemotherapy and autologous stem cell transplantation; R mono, rituximab monotherapy; and Chloramb, chlorambucil.

*One FL patient with stage IIA was deemed unsuitable for radiation therapy because of bulky intra-abdominal disease. She was treated as a stage IIIA patient with chemotherapy and was therefore included into this trial.

Primary endpoint: induction of immune responses

In group A, 5 of the first 7 patients (n_1) developed an immune response, and the trial was continued to the second stage. In the final analysis, 12 of evaluable 20 patients (60%) developed a cellular immune response to idiotype. Nine of 21 patients (43%) developed anti-idiotype IgM or IgG antibodies (5 IgM; 7 IgG). Sixteen patients (76%; 95% confidence interval, 58%-94%) of group A had at least one type of immune response. Both anti-HBs responders also developed anti-idiotype antibodies but remained negative by ELISpot.

In the first stage of group B, all patients had an immune response. In the final analysis, circulating idiotype-reactive cells were induced in 11 (55%) and antibody responses in 10 (50%) of 20 evaluable group B patients (6 IgM, 9 IgG). At least one type of response was detected in 16 patients (76%; 95% confidence interval, 58%-94%).

In both cohorts, the development of cellular or humoral immune responses was not correlated to prevaccination numbers of $CD4^+$ and $CD8^+$ T cells (Figure 1B; and data not shown). Lack of antibody responses was associated in group B with reduced B cells (Figure 1B). Serum immunoglobulin levels were not associated with any type of immune response (data not shown).

Kinetics and qualities of immune responses

Additional experiments were performed in patients with sufficient available PBMCs to gain more information on the vaccination-induced immune responses. The frequency of idiotype-reactive T cells increased during the initial 6 vaccinations, had a tendency to decline in the following 3-month interval, but could be boosted by maintenance immunization (Figure 2A). AntiFab^{Id} antibody titers peaked at the end of the 6 monthly vaccinations and persisted for at least additional 6 months (Figure 2B). In vitro stimulation experiments showed proliferation of PBMCs in response to Fab^{Id} (Figure 2C). The proliferating cells were composed of 8% to 68% $CD4^+$ and 8% to 62% $CD8^+$ cells (representative example in Figure 2D; raw data in supplemental Table 1). The fraction of cells with a regulatory phenotype within the $CD4^+$ PBMC population declined during the immunization period in all analyzed patients and was significantly decreased at the time point of the primary endpoint after completion of the 6 planned vaccinations (Figure 2E). Cytokine profiling of Fab^{Id}-stimulated blood cells at the same time point showed increased release of typical Th1 cytokines but not of Th2 cytokines after Fab^{Id} immunization (Figure 2F).

Target specificity and tumor cell recognition of anti-idiotype immune responses

In group A, the ELISpot assays indicated an increase of T cells with reactivity to the vaccine Fab^{Id} during the vaccination but also an initial response to control Fab^{Id} with the same VH family (Figure 3A). Analysis of the induced vaccine-reactive T-cell response against the background of the nonspecific ELISpot counts by means of an objective Poisson regression analysis[10] confirmed that the vaccination-induced T-cell reactivity was indeed nonspecific
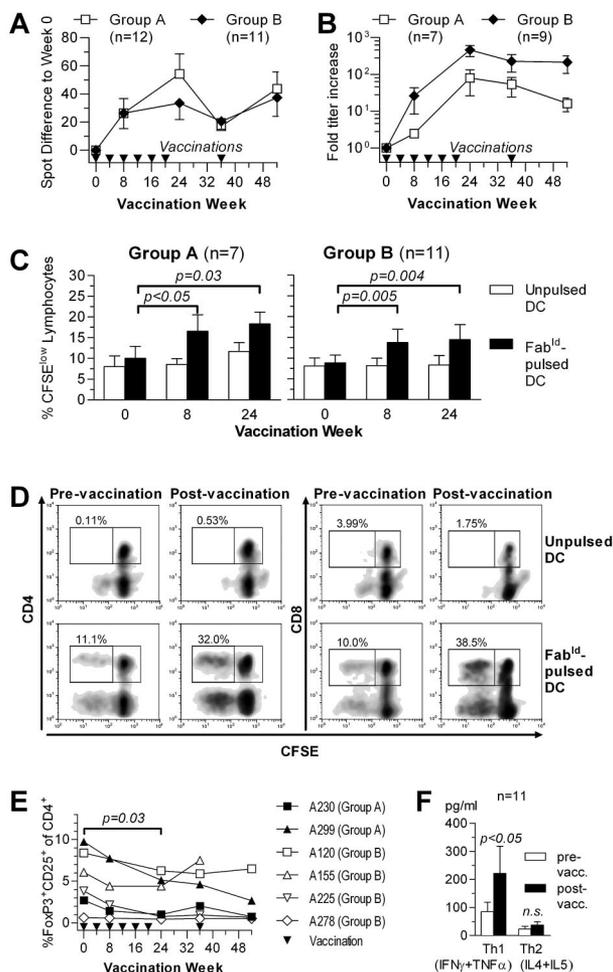
Figure 2. Kinetics of immune responses. (A) Kinetics of circulating idiotype-reactive cells in responding patients as measured by IFN-γ ELISpot. (B) Kinetics of anti-idiotype IgG titers in responding patients as measured by enzyme-linked immunosorbent assay. (C) PBMC proliferation in groups A (left) and B (right) induced by autologous dendritic cells as determined by flow cytometry measurement of carboxyfluorescein succinimidyl ester loss. (D) Analysis of CD4$^+$ and CD8$^+$ lymphocyte subsets in proliferating cells after Fab^{Id} stimulation by flow cytometry in a representative patient. (E) Kinetics of the proportion of CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells in peripheral blood lymphocytes during the vaccination period. (F) Prevaccination and postvaccination release of T helper 1 (IFN-γ and tumor necrosis factor-α) and T helper 2 cytokines (interleukin-4 [IL4] and interleukin-5 [IL5]) by peripheral blood lymphocytes on Fab^{Id} stimulation.

after 2 vaccinations (at week 8) but developed into an antigen-specific response to the patient's idiotype after 6 vaccinations in group A. In group B, the induced cellular immune response was specific throughout the immunization period.

Functional epitope mapping experiments demonstrated T-cell activation by peptides derived from complementarity-determining regions (Figure 3B). Framework epitopes did not elicit IFN-γ release, except a framework peptide of patient A119 (AIFRD-NAKN) that contained 2 mutations to the germline sequence (TISRDNAKN).

Humoral responses had also predominant specificity for the autologous idiotype as evident from testing for cross-reactivity to a fixed panel of 3 control Fabs (Figure 4A). Binding of vaccination-induced antiFab^{Id} antibodies to viable tumor cells was demonstrated for IgG purified from prevaccination and postvaccination sera by automated digital fluorescence microscopy (Figure 4B).

Clinical outcome and immune responses in patients without prior therapy (group A)

After a median follow-up of 54 months (range, 20-78 months), the median progression-free and treatment-free survival after the first vaccination were 18 and 30 months, respectively, and 47 and 48 months after diagnosis (Figure 5A). Fifteen patients (71%) required cytoreductive therapy between 6 and 45 months after vaccination start. Two patients with mantle cell lymphoma died 45 and 46 months after their first vaccination, and a patient with marginal zone lymphoma after 33 months. Six patients (29%), 4 with FL and one with nodal marginal zone lymphoma, remain free of therapy 38 to 64 months after vaccination start and have an excellent quality of life.

At the prespecified time point for the analysis of immune responses (24 weeks), the tumor burden appeared to be minimally to moderately reduced in some patients, especially in patients with cellular immune responses (Figure 5B). Tumor regressions continued and also began to occur beyond this time point. In 6 of 15 (40%) FL patients, including 2 patients with progressive disease at the time of their first vaccination, these regressions reached the criteria for objective partial remissions between 6 and 29 months after the start of idiotype vaccination (Figure 5C; patients marked with "R" in Figure 5B). Four patients are in continuing remission at 57 to 64 months; 2 responding lymphoma eventually progressed at 34 and 39 months, respectively.

Cellular immune responses, particularly in FL patients, were associated with freedom from progression (Figure 6A). Because
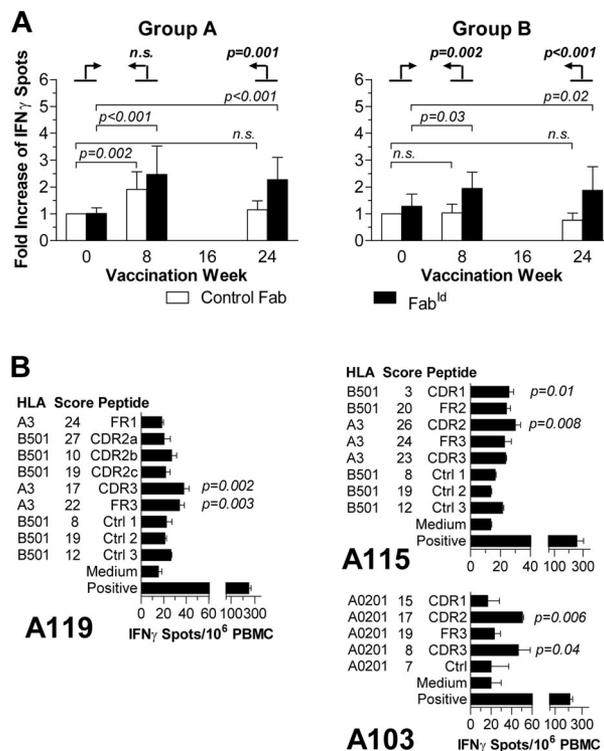

Figure 3. Specificity of cellular anti-idiotype immune response. (A) Comparison of the normalized relative frequency of IFN-γ-releasing cells induced by Fab^{Id} from all ELISpot responders at various time points against the actual frequency of control Fab-reactive cells. Prevaccination reactivity to control Fab was defined as 1. Error bars represent 95% confidence intervals. The significances of relative differences of Fab^{Id}-induced ELISpots to week 0 against the background change of control-Fab-induced differences are calculated by a Poisson regression model[10] and indicated in boldface type. (B) Epitope mapping analysis of postvaccination peripheral blood mononuclear cells by ELISpot with synthetic HLA-restricted peptides identified by the SYFPEITHI algorithm in the patient's lymphoma idiotype IgH chain.
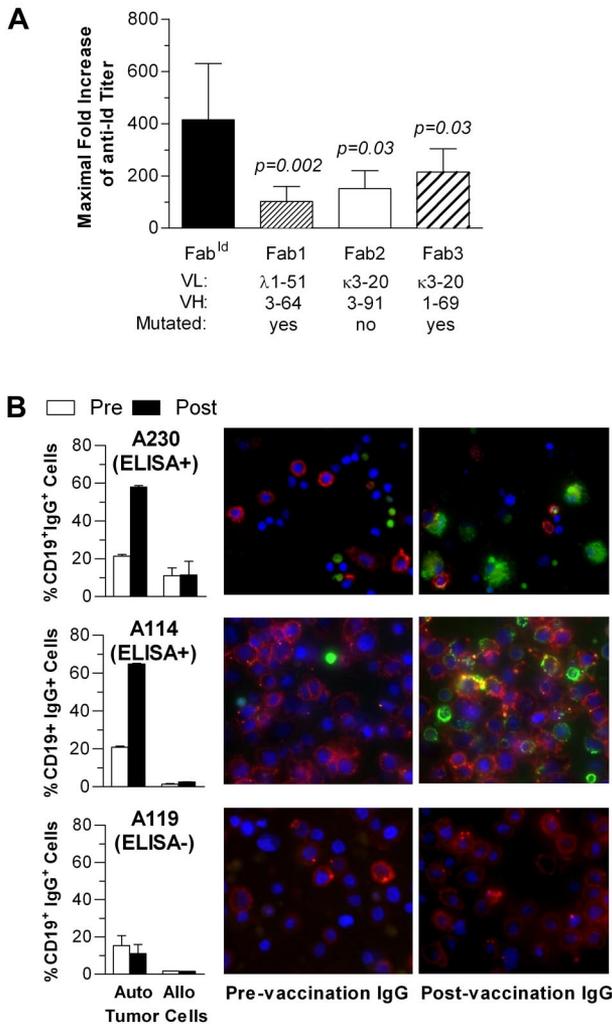
Figure 4. Specificity of humoral anti-idiotype immune response. (A) Maximal anti-Fab^{Id} IgG titer increase after idiotype vaccination in all enzyme-linked immunosorbent assay responders compared with a fixed panel of 3 control Fab. (B) Binding of Fab^{Id} vaccination-induced antibodies to autologous and allogeneic tumor cells assessed by automated digital fluorescence microscopy. Bar graphs represent the percentage of tumor cells with surface-bound IgG. Photographs show autologous tumor cells incubated with prevaccination or postvaccination serum IgG. Red represents anti-CD19; green, anti-IgG; and blue, nuclear counterstain with DAPI.

there were no events before the time point to assess the primary endpoint at 24 weeks, equivalent results were observed in a landmark outcome analysis at this time point. Humoral immune responses and the FcγRIIIa genotype were not correlated with outcome. All objective responders developed a cellular immune response ($P = .04$, Fisher exact test). Anti-idiotype antibodies were induced in 3 of the 6 objectively responding patients.

Immune responses and outcome in remission consolidation

At a median follow-up of 46 months (range, 10-69 months) after the first vaccination, the estimated median progression-free and treatment-free survival of group B are 30 and 53 months, respectively. Ten patients in this group, including 9 of 14 patients (64%) with FL, remain in remission. Ten patients have actuarially required additional cytoreductive therapy 8 to 53 months after vaccination start and 15 to 70 months after the last chemotherapy, respectively. Two patients with mantle cell lymphoma have died resulting from lymphoma relapse 27 and

52 months after vaccination start. The patient with lymphocytic lymphoma, carrying the prognostically poor del11q22, died 41 months after the first vaccination. Progression-free survival in FL patients was correlated with humoral but not cellular immune responses (Figure 6). There was no correlation of the FcγRIIIa genotype alone or in combination with humoral immune responses and outcome.

Discussion

This trial demonstrates a high immune response rate to intradermal administration of the recombinant Fab^{Id} vaccine in indolent B-cell lymphoma patients. Both patient cohorts enrolled in this trial had a high incidence of numerical and functional immunodeficiency as indicated by high failure rates of simultaneous hepatitis B vaccination. Despite this impairment, the primary endpoint of a 30% anti-idiotype immune response rate was surpassed for both indications studied.
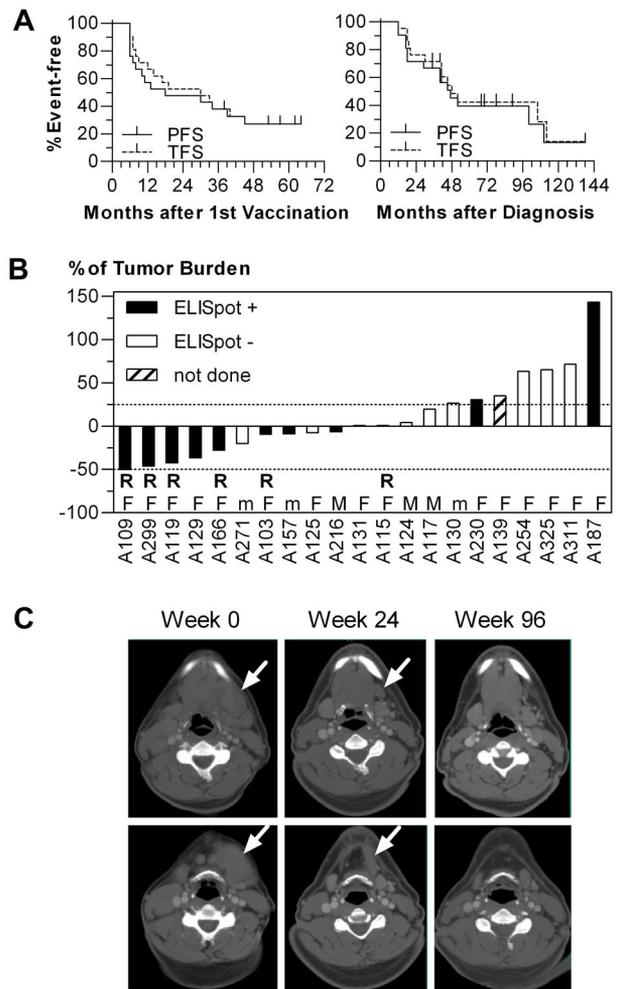

Figure 5. Clinical outcome in upfront vaccination (group A). (A) Kaplan-Meier curves of progression-free survival (PFS) and treatment-free survival (TFS) after vaccination start (left panel) and lymphoma diagnosis (right panel). (B) Evolution of total lymphoma burden at 24 weeks after the first vaccination in relationship to cellular immune responses and lymphoma entity. R indicates patients who eventually achieved an objective partial remission; F, follicular lymphoma; M, mantle cell lymphoma; and m, marginal zone lymphoma. (C) Computed tomography scans of patient A119 with a near-complete disappearance of a disfiguring submandibular lymphoma manifestation.
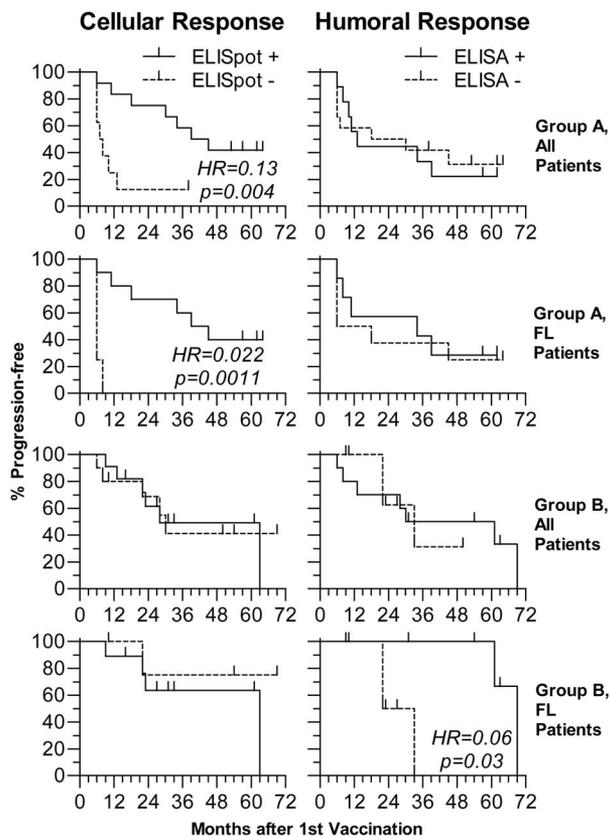
Figure 6. Progression-free survival and anti-idiotype immune responses. Progression-free survival in patients with upfront idiotype vaccination (group A) and idiotype vaccination as remission consolidation (group B) according to the development of cellular (left) or humoral immune responses (right). HR indicates hazard ratio.

The immune response rate in the remission consolidation cohort appears to be similar to published idiotype vaccination studies in FL.[9,11,13,14,16,29] Most of these reported trials have used idiotype vaccines conjugated to the immunologic carrier keyhole limpet hemocyanin.[8,9,11-13] The satisfactory immunogenicity of our unconjugated Fab[Id] vaccine may be attributable to a combination of several factors, including the combination with the adjuvant MF59 and extended conditioning of the injection site with sargramostim. We think that the intradermal injection route, which has been recognized to be highly efficacious,[30] represents perhaps the single most important aspect for achieving immune responses in immunologically impaired persons. Finally, the Fab[Id] vaccine antigen lacks the Fc region of immunoglobulin. The Fc regions are known to harbor Tregitopes (ie, human leukocyte antigen [HLA] class II-presented epitopes that are recognized by regulatory T cells) and induce immunosuppressive activity.[31] The existence of Tregitopes may provide a structural basis for immunomodulatory activity of polyclonal immunoglobulin. Therefore, Tregitopes could dampen immune responses to an entire lymphoma-derived idiotype immunoglobulin, and their absence from a vaccine, as is the case in our Fab[Id] format, may result in a superior immune response rate. To support this hypothesis, we are currently conducting in vitro stimulation experiments comparing entire monoclonal immunoglobulin with their corresponding recombinant Fab[Id].

In the most commonly studied indication of remission consolidation, the induction of anti-idiotype antibodies has consistently been associated with superior progression-free survival, and possibly with better overall survival.[12-14] The clinical outcome of our group B matches these results and suggests similar biologic activity

for the Fab[Id] vaccine. Importantly, we demonstrate that the vaccination-induced antiFab[Id] antibodies indeed target autologous tumor cells. Furthermore, the absence of the prognostically favorable antibody response in patients with low counts of circulating B cell may explain the failure of an randomized trial testing idiotype vaccination shortly after rituximab monotherapy.[15] An association of B-cell depletion through prior administration of rituximab and subsequent failure to develop humoral immune responses strongly suggests that idiotype vaccination should precede a B cell–depleting antibody therapy if active and passive immunotherapy components were to be integrated in a combined regimen.

Most importantly, this report describes the first trial of idiotype vaccination monotherapy in untreated lymphoma patients who carry a considerable tumor burden. Regression of measurable tumor manifestations after idiotype vaccination has been described before[8-10,32] but has not been analyzed systematically in a prospective trial.

In prospective studies of watch and wait management of FL, the median time to requirement of cytoreductive therapy has been 23 to 31 months.[4] In our FL patients, the median time to therapy was 48 months after diagnosis. These values, however, cannot be compared because of considerable delays between diagnosis and vaccination in our trial. These delays, mostly attributable to the referral of previously diagnosed patients from outside institutions, were not correlated to immune response rate or outcome (data not shown). On the other hand, the presence of additional risk factors beyond advanced stage indicates that our trial did not selectively include a patient population with minimal risk of tumor progression. Nevertheless, the intervals from diagnosis to vaccination may represent a selection bias that calls for a prospective randomized trial.

The occurrence of durable remissions in FL patients may therefore represent a better indicator for potential clinical efficacy of Fab[Id] vaccination. The spontaneous remission rate of FL was 4.6% in the only prospective trial reported in the literature.[4] We exerted meticulous scrutiny in excluding patients who showed any evidence for spontaneous tumor regression, and indeed withheld therapy in 2 such persons. The occurrence of durable objective remissions in otherwise untreated FL patients, which occurred even in progressing lymphomas and were associated with measurable immune responses in every case, suggest clinical benefit.

In contrast to patients vaccinated in clinical remission after conventional cytoreductive therapy, where long-term suppression of residual lymphoma cells was consistently associated with the induction of anti-idiotype antibodies,[12-14] superior progression-free survival was associated in our trial with cellular rather than humoral immune responses. Control or even reduction of large tumor burdens may therefore depend on the induction of tumor-specific cellular immune activity.

Of note, both patients who developed anti-HBs responses failed to develop cellular anti-idiotype responses. Together with the independence of immune responses from the numerical immune status, these results indicate that the potential favorable effect of Fab[Id] vaccination is not merely attributable to a superior general state of the immune system.

Cellular idiotype-specific immune responses have been identified previously as indispensable in several animal vaccination models.[33-36] In these models, idiotype-specific T cells recognize preferentially unique epitopes from complementarity-determining region or generated through somatic hypermutation, whereas the natural T-cell receptor repertoire is considered tolerant toward germline-encoded immunoglobulin sequences.[37] We have recently

demonstrated that T regulatory cells (Tregs) appear to play an important role in the suppression of effector T cells with specificity for framework region epitopes.[38]

In accordance with these animals studies, the Poisson regression analyses of the ELISpot results in our consecutive trials,[10] and our in vitro experiments with idiotype-transfected surrogate target cells[19] suggest that vaccination-induced T-cell activity is directed predominantly against individual rather than shared idiotype peptides. This conclusion was confirmed by the determination of the precise specificity of the observed T-cell responses in epitope mapping experiments: Together with a case in our previous trial[10] and the identification of an individual HLA class II–restricted antigen by others,[39] these experiments consistently show that anti-idiotype cellular immunity targets predominantly complementarity-determining region-derived and/or hypermutated idiotype peptides. Cytotoxic T cells with specificity for framework-derived peptides are known to circulate in B-lymphoma patients,[40] but these T cells apparently fail to respond to idiotype vaccination in vivo.

In contrast to the question of specificity and regulation of idiotype-directed T-cell immunity, the cellular effector mechanisms that may lead to lymphoma regression are more difficult to identify. Dependent on the particular model and immunization strategy, efficient antilymphoma immunity in mouse studies depended either on both $CD4^+$ and $CD8^+$ effector cells,[33,35] or on $CD4^+$ T cells alone as the single dominant effector cell population.[34,41] In the $CD4^+$ T cell–dependent models, idiotype vaccination was efficacious even when the effector cells did not directly bind to idiotype-derived peptides on the lymphoma cells. These animal studies suggest that idiotype-specific cellular effector mechanisms may range from classic, perforin- and granzyme-mediated cytotoxicity to humoral mechanisms that do not require direct contacts between T cells and tumor cells. Likewise, the crucial cellular effector mechanisms in humans might vary from patient to patient. T cells with classic cytotoxicity toward lymphoma cells can be stimulated by idiotype vaccination in vivo and in vitro.[9,19] Nevertheless, we chose quantitative measurement of IFN-γ secretion as our most important surrogate endpoint because a broad range of studies indicate that IFN-γ is a central and pleiotropic mediator of efficient antitumor immunity.[42] Important effects of IFN-γ include the suppression of Treg activity,[43] inhibition of angiogenesis,[44] and the polarization of T helper cells toward a Th1 milieu.[42] Furthermore, IFN-γ appears to play a special role in the immunosurveillance against naturally occurring lymphomas in mice.[45]

We have recently presented bioinformatic evidence for selection of a given FL idiotype for relatively low immunogenicity by the affected patient's HLA type.[17] Although these findings may be taken as evidence for potential spontaneous antilymphoma T-cell activity against HLA class I–presented idiotype epitopes, our current data suggest that active idiotype immunization may convert an apparently inefficient immune recognition of intermediately immunogenic individual idiotypes into a clinically efficacious

T-cell response. In this context, it may be a potentially important observation that the fraction of circulating Tregs, the most important cell population to maintain peripheral tolerance,[46] decreased in all analyzed patients. In contrast, a vaccination-induced increase of Tregs was strongly associated with failure of idiotype vaccination to induce an effector T-cell response in myeloma patients.[47] The cytokine milieu of the cellular immune response may represent another potentially important mechanism for the vaccination effects: We observed conversion of a T helper 2–dominated T-cell milieu into predominance of Th1 cells, but not the opposite phenomenon. Because Th2-biased, idiotype-specific T helper cells can even induce B-cell lymphomagenesis in a transgenic mouse model,[48] the induction of idiotype-specific Th2 cells should be avoided in clinical idiotype vaccination, and a Th1-dominated immune response appears to be desirable with respect to both safety and efficacy.

In conclusion, our results demonstrate the induction of specific immunity and associated durable clinical remissions in FL patients receiving idiotype vaccination as first-line therapy. Concomitant immunologic studies identify crucial candidate effector mechanisms and potentially important surrogate markers for the in vivo induced immune responses. This vaccination strategy has the potential to fulfill the requirements for a biologic, well-tolerated intervention to delay or prevent the necessity for cytoreductive therapy in asymptomatic indolent lymphoma patients.

Authorship

Contribution: H.V. and G.I. designed the study; K.H.-M., F.S., A.K.-H., G.D., and H.V. recruited patients and collected clinical data; C.B.-L. and M.A.N. performed immunologic analyses; M.A.N., K.H.-M., G.I., A.K.-H., and H.V. analyzed the data and take responsibility for the accuracy of the data analysis; H.V. and M.A.N. wrote the paper; and all authors approved the final version of the manuscript.

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Correspondence: Hendrik Veelken, Department of Hematology, Leiden University Medical Center, PO Box 9600, C2-R-140, 2300 RC Leiden, The Netherlands; e-mail: j.h.veelken@lumc.nl.

References

1. Liu Q, Fayad L, Cabanillas F, et al. Improvement of overall and failure-free survival in stage IV follicular lymphoma: 25 years of treatment experience at the University of Texas M. D. Anderson Cancer Center. *J Clin Oncol.* 2006;24(10):1582-1589.

2. Fisher RI, LeBlanc M, Press OW, Maloney DG, Unger JM, Miller TP. New treatment options have changed the survival of patients with follicular lymphoma. *J Clin Oncol.* 2005;23(33):8447-8452.

3. Pulte D, Gondos A, Brenner H. Ongoing improvement in outcomes for patients diagnosed as having non-Hodgkin lymphoma from the 1990s to the early 21st century. *Arch Intern Med.* 2008;168(5):469-476.

4. Ardeshna KM, Smith P, Norton A, et al. Long-term effect of a watch and wait policy versus immediate systemic treatment for asymptomatic advanced-stage non-Hodgkin lymphoma: a randomised controlled trial. *Lancet.* 2003;362(9383):516-522.

5. Brice P, Bastion Y, Lepage E, et al. Comparison in low-tumor-burden follicular lymphomas between an initial no-treatment policy, prednimustine, or interferon alfa: a randomized study from the Groupe d'Etude des Lymphomes Folliculaires. Groupe d'Etude des Lymphomes de l'Adulte. *J Clin Oncol.* 1997;15(3):1110-1117.

6. Gribben JG. How I treat indolent lymphoma. *Blood.* 2007;109(11):4617-4626.

7. Kwak LW, Campbell MJ, Czerwinski DK, Hart S,

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Miller RA, Levy R. Induction of immune responses in patients with B-cell lymphoma against the surface-immunoglobulin idiotype expressed by their tumors. *N Engl J Med.* 1992;327(17):1209-1215.

8. Hsu FJ, Caspar CB, Czerwinski D, et al. Tumor-specific idiotype vaccines in the treatment of patients with B-cell lymphoma: long-term results of a clinical trial. *Blood.* 1997;89(9):3129-3135.

9. Bendandi M, Gocke CD, Kobrin CB, et al. Complete molecular remissions induced by patient-specific vaccination plus granulocyte-monocyte colony-stimulating factor against lymphoma. *Nat Med.* 1999;5(10):1171-1177.

10. Bertinetti C, Zirlik K, Heining-Mikesch K, et al. Phase I trial of a novel intradermal idiotype vaccine in patients with advanced B-cell lymphoma: specific immune responses despite profound immunosuppression. *Cancer Res.* 2006;66(8):4496-4502.

11. Inoges S, Rodriguez-Calvillo M, Zabalegui N, et al. Clinical benefit associated with idiotypic vaccination in patients with follicular lymphoma. *J Natl Cancer Inst.* 2006;98(18):1292-1301.

12. Levy R, Robertson M, Leonard J, Vose J, Denney D. Results of a phase 3 trial evaluating safety and efficacy of specific immunotherapy, recombinant idiotype (ID) conjugated to KLH (ID-KLH) with GM-CSF, compared to non-specific immunotherapy, KLH with GM-CSF, in patients with follicular non-Hodgkin's lymphoma (FNHL). *Ann Oncol.* 2008;19[suppl 4]:iv101.

13. Weng WK, Czerwinski D, Timmerman J, Hsu FJ, Levy R. Clinical outcome of lymphoma patients after idiotype vaccination is correlated with humoral immune response and immunoglobulin GFc receptor genotype. *J Clin Oncol.* 2004;22(23):4717-4724.

14. Ai WZ, Tibshirani R, Taidi B, Czerwinski D, Levy R. Anti-idiotype antibody response after vaccination correlates with better overall survival in follicular lymphoma. *Blood.* 2009;113(23):5743-5746.

15. Freedman A, Neelapu SS, Nichols C, et al. Placebo-controlled phase III trial of patient-specific immunotherapy with mitumprotimut-T and granulocyte-macrophage colony-stimulating factor after rituximab in patients with follicular lymphoma. *J Clin Oncol.* 2009;27(18):3036-3043.

16. Schuster SJ, Neelapu SS, Gause BL, et al. Idiotype vaccine therapy (BiovaxID) in follicular lymphoma in first complete remission: phase III clinical trial results [abstract]. *J Clin Oncol.* 2009;27(18 suppl):2.

17. Strothmeyer AM, Papaioannou D, Duhren-von Minden M, et al. Comparative analysis of predicted HLA binding of immunoglobulin idiotype sequences indicates T cell-mediated immunosurveillance in follicular lymphoma. *Blood.* 2010;116(10):1734-1736.

18. Bertinetti C, Simon F, Zirlik K, et al. Cloning of idiotype immunoglobulin genes in B cell lymphomas by anchored PCR and production of individual recombinant idiotype vaccines in Escherichia coli. *Eur J Haematol.* 2006;77(5):395-402.

19. Osterroth F, Garbe A, Fisch P, Veelken H. Stimulation of cytotoxic T cells against idiotype immunoglobulin of malignant lymphoma with protein-pulsed or idiotype-transduced dendritic cells. *Blood.* 2000;95(4):1342-1349.

20. Hiddemann W, Kneba M, Dreyling M, et al. Frontline therapy with rituximab added to the combination of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) significantly improves the outcome for patients with advanced-stage follicular lymphoma compared with therapy with CHOP alone: results of a prospective randomized study of the German Low-Grade Lymphoma Study Group. *Blood.* 2005;106(12):3725-3732.

21. Cheson BD, Horning SJ, Coiffier B, et al. Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas: NCI Sponsored International Working Group. *J Clin Oncol.* 1999;17(4):1244.

22. Miller AB, Hoogstraten B, Staquet M, Winkler A. Reporting results of cancer treatment. *Cancer.* 1981;47(1):207-214.

23. Britten CM, Gouttefangeas C, Welters MJ, et al. The CIMT-monitoring panel: a two-step approach to harmonize the enumeration of antigen-specific CD8+ T lymphocytes by structural and functional assays. *Cancer Immunol Immunother.* 2008;57(3):289-302.

24. Putz T, Ramoner R, Gander H, et al. Monitoring of CD4+ and CD8+ T-cell responses after dendritic cell-based immunotherapy using CFSE dye dilution analysis. *J Clin Immunol.* 2004;24(6):653-663.

25. Janetzki S, Britten CM, Kalos M, et al. "MIATA"-minimal information about T cell assays. *Immunity.* 2009;31(4):527-528.

26. Koene HR, Kleijer M, Algra J, Roos D, von dem Borne AE, de Haas M. Fc gammaRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRIIIa, independently of the Fc gammaRIIIa-48L/R/H phenotype. *Blood.* 1997;90(3):1109-1114.

27. Simon R. Optimal two-stage designs for phase II clinical trials. *Control Clin Trials.* 1989;10(1):1-10.

28. Anderson JR, Cain KC, Gelber RD. Analysis of survival by tumor response. *J Clin Oncol.* 1983;1(11):710-719.

29. Redfern CH, Guthrie TH, Bessudo A, et al. Phase II trial of idiotype vaccination in previously treated patients with indolent non-Hodgkin's lymphoma resulting in durable clinical responses. *J Clin Oncol.* 2006;24(19):3107-3112.

30. Belshe RB, Newman FK, Cannon J, et al. Serum antibody responses after intradermal vaccination against influenza. *N Engl J Med.* 2004;351(22):2286-2294.

31. De Groot AS, Moise L, McMurry JA, et al. Activation of natural regulatory T cells by IgG Fc-derived peptide "Tregitopes." *Blood.* 2008;112(8):3303-3311.

32. Timmerman JM, Czerwinski DK, Davis TA, et al. Idiotype-pulsed dendritic cell vaccination for B-cell lymphoma: clinical and immune responses in 35 patients. *Blood.* 2002;99(5):1517-1526.

33. Kwak LW, Young HA, Pennington RW, Weeks SD. Vaccination with syngeneic, lymphoma-derived immunoglobulin idiotype combined with granulocyte/macrophage colony-stimulating factor primes mice for a protective T-cell response.

Proc Natl Acad Sci U S A. 1996;93(20):10972-10977.

34. Lauritzsen GF, Weiss S, Dembic Z, Bogen B. Naive idiotype-specific CD4+ T cells and immunosurveillance of B-cell tumors. *Proc Natl Acad Sci U S A.* 1994;91(12):5700-5704.

35. Levitsky HI, Montgomery J, Ahmadzadeh M, et al. Immunization with granulocyte-macrophage colony-stimulating factor-transduced, but not B7–1-transduced, lymphoma cells primes idiotype-specific T cells and generates potent systemic antitumor immunity. *J Immunol.* 1996;156(10):3858-3865.

36. Qin H, Cha SC, Neelapu SS, et al. Vaccine site inflammation potentiates idiotype DNA vaccine-induced therapeutic T cell-, and not B cell-, dependent antilymphoma immunity. *Blood.* 2009;114(19):4142-4149.

37. Bogen B, Ruffini P. Review: to what extent are T cells tolerant to immunoglobulin variable regions? *Scand J Immunol.* 2009;70(6):526-530.

38. Warncke M, Buchner M, Thaller G, et al. Control of the specificity of T cell-mediated anti-idiotype immunity by natural regulatory T cells [published online ahead of print September 17, 2010]. *Cancer Immunol Immunother.* doi:10.1007/s00262-010-0918-x.

39. Baskar S, Kobrin CB, Kwak LW. Autologous lymphoma vaccines induce human T cell responses against multiple, unique epitopes. *J Clin Invest.* 2004;113(10):1498-1510.

40. Trojan A, Schultze JL, Witzens M, et al. Immunoglobulin framework-derived peptides function as cytotoxic T-cell epitopes commonly expressed in B-cell malignancies. *Nat Med.* 2000;6(6):667-672.

41. King CA, Spellerberg MB, Zhu D, et al. DNA vaccines with single-chain Fv fused to fragment C of tetanus toxin induce protective immunity against lymphoma and myeloma. *Nat Med.* 1998;4(11):1281-1286.

42. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol.* 2006;6(11):836-848.

43. Nishikawa H, Kato T, Tawara I, et al. IFN-γ controls the generation/activation of CD4+ CD25+ regulatory T cells in antitumor immune response. *J Immunol.* 2005;175(7):4433-4440.

44. Qin Z, Schwartzkopff J, Pradera F, et al. A critical requirement of interferon gamma-mediated angiostasis for tumor rejection by CD8+ T cells. *Cancer Res.* 2003;63(14):4095-4100.

45. Street SE, Trapani JA, MacGregor D, Smyth MJ. Suppression of lymphoma and epithelial malignancies effected by interferon gamma. *J Exp Med.* 2002;196(1):129-134.

46. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell.* 2008;133(5):775-787.

47. Hansson L, Abdalla AO, Moshfegh A, et al. Long-term idiotype vaccination combined with interleukin-12 (IL-12), or IL-12 and granulocyte macrophage colony-stimulating factor, in early-stage myeloma patients. *Clin Cancer Res.* 2007;13(5):1353-1355.

48. Zangani MM, Froyland M, Qiu GY, et al. Lymphomas can develop from B cells chronically helped by idiotype-specific T cells. *J Exp Med.* 2007;204(5):1181-1191.

Upfront immunization with autologous recombinant idiotype Fab fragment without prior cytoreduction in indolent B-cell lymphoma

Marcelo A. Navarrete, Kristina Heining-Mikesch, Frank Schüler, Cristina Bertinetti-Lapatki, Gabriele Ihorst, Andrea Keppler-Hafkemeyer, Gottfried Dölken and Hendrik Veelken

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