Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Pescovitz MD, Greenbaum CJ, Krause-Steinrauf H, et al. Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. N Engl J Med 2009;361:2143-52.

ONLINE APPENDIX. Type 1 Diabetes TrialNet Anti-CD20 Study Group

Steering Committee: Jay S. Skyler (University of Miami Diabetes Research Institute), Chairman; Mark Anderson (University of California San Francisco), Dorothy Becker (University of Pittsburgh), Christophe Benoist (Joslin Diabetes Center), Penelope Bingley (University of Bristol), Emanuele Bosi (San Raffaele Hospital), H. Peter Chase (University of Colorado Barbara Davis Center for Childhood Diabetes), Michael Clare-Salzler (University of Florida), Peter Colman (Walter and Eliza Hall Institute of Medical Research), George S. Eisenbarth (University of Colorado Barbara Davis Center for Childhood Diabetes), C. Garrison Fathman (Stanford University), Stephen Gitelman (University of California San Francisco), Robin Goland (Columbia University), Peter Gottlieb (University of Colorado Barbara Davis Center for Childhood Diabetes), Gilman Grave (NICHD), Carla Greenbaum (Benaroya Research Institute), Leonard Harrison (Walter and Eliza Hall Institute of Medical Research), Kevan Herold (Yale University), Richard Insel (Juvenile Diabetes Research Foundation), Francine Kaufman (Childrens Hospital Los Angeles), Jeffrey P. Krischer (University of South Florida), Ellen Leschek (NIDDK), Jeffrey Mahon (University of Western Ontario), Jennifer Marks (University of Miami Diabetes Research Institute), Antoinette Moran (University of Minnesota), Kirsti Nanto-Salonen (Hospital District of Southwest Finland), Gerald Nepom (Benaroya Research Institute), Tihamer Orban (Joslin Diabetes Center), Jerry P. Palmer (University of Washington), Robertson Parkman (Childrens Hospital Los Angeles), Mark Peakman (Guy's, King's, and St. Thomas' School of Medicine), Mark Pescovitz (Indiana University), John Peyman (NIAID), Alberto Pugliese (University of Miami Diabetes Research Institute), Philip Raskin (University of Texas Southwestern Medical School), Henry Rodriguez (Indiana University), Desmond Schatz (University of Florida), Robert Sherwin (Yale University), Mark Siegelman (University of Texas Southwestern Medical School), Olli Simell (Hospital District of Southwest Finland), Massimo Trucco (University of Pittsburgh), John Wagner (University of Minnesota), Diane Wherrett (University of Toronto), Darrell Wilson (Stanford University), William Winter (University of Florida), Anette Ziegler (Institut für Diabetesforschung), Judith Fradkin (NIDDK, ex-officio). Past Members: Jeffrey Bluestone (University of California San Francisco), David Brown (University of Minnesota), Catherine Cowie (NIDDK), Bernard Hering (University of Minnesota), Stanley Jordan (Cedars-Sinai Medical Center), John M. Lachin (George Washington University), John Ridge (NIAID).

Executive Committee: Jay S. Skyler, Carla J. Greenbaum, Jeffrey P. Krischer, Ellen Leschek, John Peyman, Lisa Rafkin-Mervis (University of Miami Diabetes Research Institute), Peter Savage (NIDDK), Lisa Spain (NIDDK). Past Members: Catherine Cowie, Mary Foulkes (George Washington University), Heidi Krause-Steinrauf (George Washington University), John M. Lachin, Saul Malozowski (NIDDK), John Ridge, Stephanie J. Zafonte (George Washington University).

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Rituximab, B-lymphocyte Depletion and Preservation of Beta-cell Function

ONLINE APPENDIX

Additional Methods

C-peptide levels were measured from frozen plasma using a two-site immunoenzymometeric assay (Tosoh Bioscience, South San Francisco, CA). Reliability coefficients for the assay were above 0.99 from split duplicate samples. C-peptide AUC was computed using the trapezoidal rule from the timed measurements of C-peptide during the MMTT (including the basal). The AUC-mean equals the AUC divided by the interval of time, e.g. 120 minutes for a 2-hour MMTT. Groups were compared using an analysis of covariance model (ANCOVA) adjusting for baseline C-peptide, age and gender. The *log(mean C-peptide+1)* transformation of the baseline and follow-up AUC-mean was used to allow for mean C-peptide values close to zero and to normalize the distribution of the residuals. The geometric means and 95% confidence limits for the 2-hour AUC-mean stimulated C-peptide levels over time for all subjects in the ITT cohort within each group were obtained from a separate ANCOVA at each visit time (rituximab, 49 subjects and placebo control, 29 subjects at 12 months).

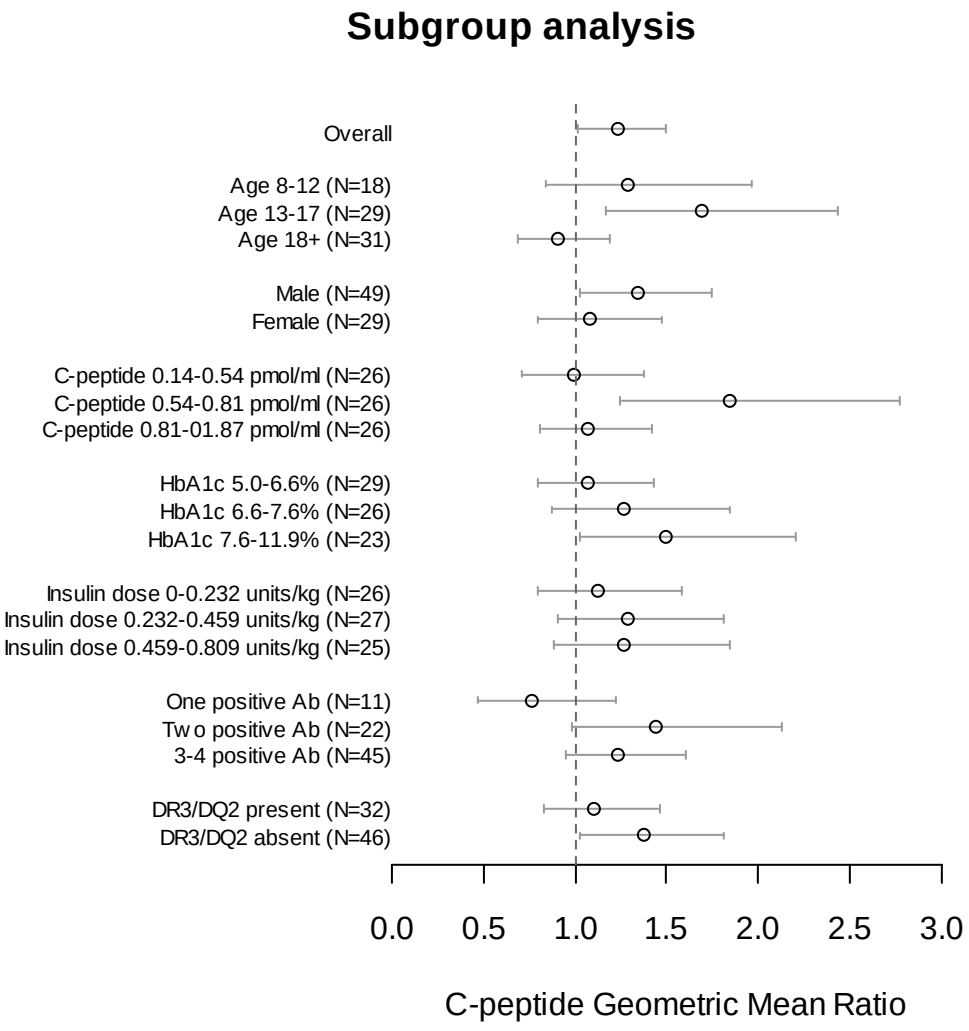
HbA1c was measured quarterly using ion-exchange high performance liquid chromatography (Variant II, Bio-Rad Diagnostics). Reliability coefficients for the assay were above 0.99 from split duplicate samples.

Multiparameter flow cytometry was performed by the Immune Tolerance Network (at Roswell Park Cancer Institute, Buffalo, NY) from fresh blood.

Measurement of immunoglobulins was performed on a Hitachi 917 with reagents from Roche Diagnostics.

A subset of 36 patients (20 rituximab, 16 control) participated in a sub-study to determine de novo immunization response to a neoantigen, the phage PhiX174. They received four immunizations at 2 (primary), 8 (secondary), 48 (tertiary) and 54 (quaternary) weeks after the fourth study drug infusion. Serum samples were obtained before and 1, 2, and 4 wks after each immunization and stored frozen until analyzed. Anti-phage antibody titers were measured using a phage neutralization assay and calculated and expressed as the first order kinetic constant, Kv. Using a repeated measures model adjusting for age and sex, the log(kv+1) was used in the model and the results transformed back to the original scale, such that data are presented as the geometric means of log-transformed Kv. The % IgM of the total Kv was calculated after measuring the amount of reduction in Kv activity after treatment with dithiothreitol.

Appendix Figure A. Subgroup Plot of Ratios for Effect of Treatment on Mean AUC C-Peptide at 1 Year. Ratio of geometric means for rituximab versus placebo control group, with 95% confidence intervals, within subgroups of patients defined at baseline. When adjusted for multiple subgroup analyses, there was no significant heterogeneity (test of treatment by subgroup interaction) among subgroups. The 69% improvement with rituximab versus control in the adolescent age (13-17 years) sub-group was nominally significant (p=0.002, not adjusted for multiple tests) while not significant in the other two subgroups; but this difference among age subgroups was within the realm of chance variation. The upper HbA1c tertile (7.6-11.9%) had a 51% improvement in C-peptide mean AUC at one year but the difference among HbA1c tertiles was not significant (p = 0.15).



Results of immunization response to phage PhiX174 (Appendix Table A below).

The rituximab treated patients did not respond to phage at the time of the primary nor the secondary (recall) immunization at which time B-lymphocytes were depleted. At the time of the tertiary immunization (when the effect of rituximab was largely gone and B-lymphocytes had recovered), the treated patients showed a IgM response, similar to the primary response. At the time of the quaternary immunization, the total Kv in the rituximab treated patients had increased to the normal range and was predominantly IgM.

Appendix Table A. Kv and Percent IgM antibody response to PhiX174 Immunizations immediately after rituximab treatment and at one year at time of B cell recovery

	Kv (95% mean confidence limits)			%IgM (95% mean confidence limits)		
Time point:	Rituximab	Control	p-value	Rituximab	Control	p-value
2 weeks post-Rx (secondary)	0.4 (-0.2, 1.6)	213.9 (106.7, 427.8)	< 0.0001	97 (89, 104) (n=21)	54 (45, 63) (n=14)	< 0.0001
2 weeks post-Rx (tertiary)	64.5 (35.6, 116.1)	1077.2 (571.7, 2028.9)	< 0.0001	93 (82, 104) (n=18)	13 (1.0, 24) (n=15)	< 0.0001
2 weeks post-Rx (quaternary)	778.1 (525.0, 1152.8)	708.5 (486.6, 1031.6)	0.7318	67 (55, 79) (n=14)	11 (-1.3, 22) (n=16)	< 0.0001

All Adverse Events by Treatment Group

Adverse events were reported per protocol by the clinical sites and centrally adjudicated by the TrialNet Medical Monitor. Clinical laboratory data for each subject were also centrally reviewed by the Medical Monitor. The monitor was masked to treatment assignment of each subject.

The medical monitor classified and graded each adverse event (AE) for severity according to the NCI Common Terminology Criteria for Adverse Events version 3.0 (CTCAE). The general guideline for severity is as follows: grade 1= mild, grade 2 = moderate, grade 3= severe, grade 4 = life-threatening or disabling AE, grade 5 = death related to AE.

TrialNet Safety Monitoring Committee also reviewed all serious adverse events (masked to treatment assignment) for concurrence regarding the grading of the severity of the event and its relationship to study treatment, and as the follow-up monitoring of the subject for resolution of the event.

Appendix Table B summarizes adverse events during infusions divided by which infusion the event occurred.

Appendix Table C summarizes all Grade 2 or 3 adverse events by treatment group.

Appendix Table D summarizes all adjudicated adverse events by treatment group and by event severity (grade 1, 2 or 3) reported through 12 months of follow-up. There were no grade 4 or 5 events. This table also includes the events associated with the infusion process that were directly reported by clinical sites.

Appendix Table B – Infusion Problems by Infusion Number

	Rituximab (N=87)		Control (N=30)	
Infusion #	**# Receiving**	**# (%) with Events**	**# Receiving**	**# (%) with Events**
1	56	52 (92.9)	30	7 (23.3)
2	51	10 (19.6)	29	3 (10.3)
3	49	6 (12.2)	29	5 (17.2)
4	49	7 (14.3)	28	6 (21.4)

Appendix Table C. Summary of Grade 2+ Adverse Events, and Serious Adverse Events by Treatment Group

	Rituximab (53.1 PY*)		**Control (30 PY*)**		**Rate Ratio***			**Ritux. (N=57)**	**Control (N=30)**	**Odds Ratio†**		
	# Events	Rate*	# Events	Rate*	Value	95% CI	p <	# (%) Subjects w/ Event	# (%) Subjects w/ Event	Value	95% CI	p <
Infection	24	45.19	10	33.33	1.36	(0.63, 3.2)	0.27	16 (28)	7 (23)	1.28	(0.4, 4.2)	0.42
Conjunctivitis	2	3.77	0	0.00	--	(0.11, ∞)	0.41	2 (3.5)	0 (0)	--	(0.2, ∞)	0.43
Lyme disease	0	0.00	1	3.33	--	(0, 22.0)	0.37	0 (0)	1 (3.3)	0.00	(0, 10)	1.0
Otitis media	2	3.77	1	3.33	1.13	(0.06, 67)	0.71	2 (3.5)	1 (3.3)	1.05	(0.05, 64)	0.73
Scarlet fever	1	1.88	0	0.00	--	(0.014, ∞)	0.64	1 (1.8)	0 (0)	--	(0.03, ∞)	0.66
Sinusitis	2	3.77	1	3.33	1.13	(0.06, 67)	0.71	1 (1.8)	1 (3.3)	0.52	(0.01, 42)	0.89
Sore throat	3	5.65	2	6.67	0.85	(0.1, 10.2)	0.60	2 (3.5)	1 (3.3)	1.05	(0.05, 64)	0.73
Strep throat	2	3.77	0	0.00	--	(0.11, ∞)	0.41	2 (3.5)	0 (0)	--	(0.15, ∞)	0.43
Stye	1	1.88	0	0.00	--	(0.014, ∞)	0.64	1 (1.8)	0 (0)	--	(0.03, ∞)	0.66
Upper respiratory tract	7	13.18	5	16.67	0.79	(0.22, 3.2)	0.45	5 (8.8)	4 (13)	0.63	(0.12, 3.5)	0.85
Urinary tract	2	3.77	0	0.00	--	(0.11, ∞)	0.41	1 (1.8)	0 (0)	--	(0.03, ∞)	0.66
Varicella	1	1.88	0	0.00	--	(0.014, ∞)	0.64	1 (1.8)	0 (0)	--	(0.03, ∞)	0.66
Wart	1	1.88	0	0.00	--	(0.014, ∞)	0.64	1 (1.8)	0 (0)	--	(0.03, ∞)	0.66
Hematologic	28	52.73	11	36.67	1.44	(0.65, 2.9)	0.27	12 (21)	7 (23)	0.88	(0.27, 3)	0.71
Leucopenia	8	15.06	1	3.33	4.52	(0.6, 201)	0.11	6 (11)	1 (3.3)	3.41	(0.4, 162)	0.24
Lymphopenia	4	7.53	0	0.00	--	(0.373, ∞)	0.17	3 (5.3)	0 (0)	--	(0.31, ∞)	0.28
Mononucleosis	0	0.00	1	3.33	--	(0, 22.0)	0.37	0 (0)	1 (3.3)	--	(0, 10)	1.0
Neutropenia	16	30.13	9	30.00	1.00	(0.42, 2.9)	0.59	11 (19)	6 (20)	0.96	(0.28, 3.6)	0.65
Serious Adverse Event	6	11.30	3	10.00	1.13	(0.9, 7.0)	0.59	6 (10.5)	2 (6.7)	1.65	(0.27, 18)	0.44
Allergic reaction	1	1.88	0	0.00	--	(0.014, ∞)	0.64	1 (1.8)	0 (0)	--	(0.03, ∞)	0.66
Diabetic ketoacidosis	0	0.00	1	3.33	--	(0, 22.0)	0.37	0 (0)	1 (3.3)	--	(0, 10)	1.0
Fracture, thumb	1	1.88	0	0.00	--	(0.014, ∞)	0.64	1 (1.8)	0 (0)	--	(0.03, ∞)	0.66
Neutropenia	3	5.65	2	6.67	0.85	(0.1, 10.2)	0.60	3 (5.3)	1 (3.3)	1.61	(0.12, 87)	0.58
Sinusitis	1	1.88	0	0.00	--	(0.014, ∞)	0.64	1 (1.8)	0 (0)	--	(0.03, ∞)	0.66

* PY = patient years of follow-up among all patients randomized. Rate presented per 100 PY. Exact Poisson 95% confidence limits and p-value for the rate ratio, one-sided for harm.

† Exact binomial 95% confidence limits for the odds ratio and Fisher's exact p-value, one-sided for harm.

Ritux., rituximab

Appendix Table D – Classification of All Adverse Events Reported in Anti-CD20 Study by Treatment Group Through 12 Months of Follow-up

Appendix Table D (a). Grade 1 Severity Events

	Rituximab (N=57)		**Control (N=30)**	
Event	# Events	# Subjects w/ Event (%)	# Events	# Subjects w/ Event (%)
Allergy/Immunology	8	7 (12.3)	3	3 (10.0)
Gastrointestinal	45	32 (56.1)	7	6 (20.0)
Infection	30	14 (24.6)	26	12 (40.0)
Metabolic/Laboratory	2	1 (1.8)	0	0 (0)
Musculoskeletal/Soft Tissue	7	7 (12.3)	7	7 (23.3)
Neurology	6	6 (10.5)	1	1 (3.3)
Auditory/Ear	1	1 (1.8)	0	0 (0)
Ocular/Visual	0	0 (0)	1	1 (3.3)
Pain	16	12 (21.1)	6	6 (20.0)
Pulmonary/Upper Respiratory	16	12 (21.1)	5	4 (13.3)
Renal/Genitourinary	0	0 (0)	1	1 (3.3)
Sexual/Reproductive Function	1	1 (1.8)	0	0 (0)
Syndromes	1	1 (1.8)	0	0 (0)
Vascular	5	3 (5.3)	1	1 (3.3)
Blood/Bone Marrow	10	6 (10.5)	12	3 (10.0)
Cardiac Arrhythmia	12	11 (19.3)	2	1 (3.3)
Cardiac General	19	12 (21.1)	9	5 (16.7)
Constitutional Symptoms	27	22 (38.6)	5	3 (10.0)
Dermatology/Skin	47	29 (50.9)	6	6 (20.0)
Total	253	53 (71.6)	92	21 (28.4)

Appendix Table D (b). Grade 2 Severity Events

	Rituximab (N=57)		Control (N=30)	
Event	# Events	# Subjects w/ Event (%)	# Events	# Subjects w/ Event (%)
Allergy/Immunology	1	1 (1.8)	1	1 (3.3)
Endocrine	1	1 (1.8)	0	0 (0)
Gastrointestinal	21	16 (28.1)	4	3 (10.0)
Infection	23	16 (28.1)	10	7 (23.3)
Metabolic/Laboratory	5	5 (8.8)	5	4 (13.3)
Musculoskeletal/Soft Tissue	6	5 (8.8)	4	4 (13.3)
Neurology	0	0 (0)	2	2 (6.7)
Pain	5	3 (5.3)	7	3 (10.0)
Pulmonary/Upper Respiratory	1	1 (1.8)	1	1 (3.3)
Renal/Genitourinary	0	0 (0)	0	0 (0)
Sexual/Reproductive Function	2	1 (1.8)	0	0 (0)
Syndromes	4	3 (5.3)	2	2 (6.7)
Vascular	0	0 (0)	0	0 (0)
Blood/Bone Marrow	25	12 (21.1)	9	7 (23.3)
Cardiac Arrhythmia	8	5 (8.8)	2	2 (6.7)
Cardiac General	12	12 (21.1)	0	0 (0)
Constitutional Symptoms	7	5 (8.8)	3	3 (10.0)
Dermatology/Skin	12	8 (14.4)	3	2 (6.7)
Total	133	42 (68.9)	53	19 (31.2)

Appendix Table D (c). Grade 3 Severity Events – Severe Adverse Events

	Rituximab (N=57)		**Control (N=30)**	
Event	# Events	# (%) Subjects w/ Event	# Events	# (%) Subjects w/ Event
Allergy/Immunology	1	1 (1.8)	0	0 (0)
Blood/Bone Marrow	3	3 (5.3)	2	1 (3.3)
Infection	1	1 (1.8)	0	0 (0)
Metabolic/Laboratory	0	0 (0)	1	1 (3.3)
Musculoskeletal/Soft Tissue	1	1 (1.8)	0	0 (0)
Total	6	6 (10.5)	3	2 (6.7)