CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Regulatory T Cells Improve Nephrocalcinosis but Not Dystrophic Cardiac Calcinosis in DBA/2 Mice

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Accepted for publication
April 24, 2013.

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Nephrocalcinosis is characterized by aberrant deposition of calcium in the kidneys and is seen in phosphate nephropathy, primary hyperparathyroidism, and distal renal tubular acidosis. To further evaluate the specific pathophysiologic role of T cells in ectopic calcification, we used DBA/2 mice that are prone to develop nephrocalcinosis and dystrophic cardiac calcinosis. Female DBA/2 mice were depleted of T cells ($n = 10$) or regulatory T cells (Tregs) ($n = 15$) using either an anti-CD3 ϵ or an anti-CD25 monoclonal antibody and compared with isotype-treated controls ($n = 9$; $n = 15$), respectively. After this immunomodulation, the DBA/2 mice were given a high-phosphate diet for 9 days and the degree of calcification was assessed by microcomputed tomography. Successful depletion was confirmed by flow cytometry of splenocytes. In DBA/2 mice, the high-phosphate diet induced a phenotype of nephrocalcinosis and dystrophic cardiac calcinosis. T-cell depletion significantly increased renal calcification in microcomputed tomography ($P = 0.022$). Concordantly, Treg depletion significantly deteriorated acute phosphate nephropathy ($P = 0.039$) and was associated with a significantly increased mortality rate ($P = 0.004$). Immunomodulation had no impact on the amount of cardiac calcification. Semiquantitative histopathologic evaluations with Alizarin Red staining independently confirmed the respective radiologic measurements. In summary, our data suggest a pivotal role of T cells, particularly Tregs, in the progression of nephrocalcinosis and emphasize the fact that inflammation deteriorates the outcome in acute phosphate nephropathy. (*Am J Pathol* 2013, 183: 382–390; <http://dx.doi.org/10.1016/j.ajpath.2013.04.012>)

Nephrocalcinosis is characterized by aberrant deposition of calcium in the kidney parenchyma and tubules, it is associated with tubular atrophy and interstitial inflammation, and it is seen in a multitude of clinical settings, such as acute phosphate nephropathy,¹ primary hyperparathyroidism,² and distal renal tubular acidosis.³ Unlike symptomatic urolithiasis, nephrocalcinosis is often asymptomatic and can lead to progressive renal failure and end-stage renal disease.⁴

Nephrocalcinosis is not only the mere consequence of mechanical intrarenal or extrarenal urinary outflow obstruction but also seen in a variety of both systemic and renal metabolic disorders. Furthermore, it is also actively modulated by diverse inflammatory repair mechanisms that lead to tissue remodeling, aberrant calcium deposition, and ultimately the loss of functioning renal parenchyma. The acute

inflammatory response to calcium crystal precipitations in crystallopathies, such as gout and pseudogout, and in calcium oxalate nephropathy are generally initiated by the NLRP3 inflammasome via activation of caspase-1 and secretion of IL-1 β .⁵ Intrarenal dendritic cells and macrophages are essential regulators of these initial responses of innate immunity. They recruit further inflammatory cells for clearance of dead tissue and initiate reparative responses by inducing interstitial fibrosis.⁵

Supported by the Austrian Research Funds (P-21402) (A.R.R.) and by the Paracelsus and Joseph Skoda Award of Austrian Society of Internal Medicine (E.P.).

A.H.K. is a recipient of a DOC Fellowship of the Austrian Academy of Sciences and enrolled in the PhD program in molecular medicine at the Medical University of Graz.

Adaptive immunity, T lymphocytes, and especially regulatory T cells (Tregs) play a central role in the modulation of renal inflammation. CD4⁺CD25⁺Foxp3⁺ Tregs counteract proinflammatory cell populations, such as Th1 and Th17 cells, orchestrate prolonged soft tissue remodeling, and can thus control chronic inflammation.^{6,7} However, the role of Tregs in the pathogenesis of ectopic calcification has not been systematically investigated. In the setting of nephrocalcinosis, which is accompanied by a strong and pathogenic innate inflammatory response, it is conceivable that on the one hand proinflammatory effector T cells and on the other hand anti-inflammatory Tregs significantly modulate the immune response. To shed light on this aspect of acute phosphate nephropathy, we used DBA/2 mice that are prone to develop nephrocalcinosis.⁸ DBA/2 mice have an alternative splice variant of the *Abcc6* gene, which encodes for an orphan transporter primarily located in the basolateral plasma membranes of hepatocytes and basal membranes of renal proximal tubules.^{9–13} *Abcc6* mutations are associated with dysfunctional mitochondria and a decreased maximal respiratory capacity.¹⁴ These mice are thereby naturally susceptible to develop acute phosphate nephropathy and aberrant tissue calcification and ideally suited to study the effects of immunomodulation in these settings.

Materials and Methods

Study Design

Female 8-week-old DBA/2Ncr1 (hereafter referred to as DBA/2) mice were obtained from Charles River (Sulzfeld, Germany) and housed in a virus- and antibody-free environment in the laboratory animal facility of the Medical University of Graz. DBA/2 mice develop nephrocalcinosis.^{8,11} We chose to study female DBA/2 mice because they are more prone to develop calcification when compared with their male littermates.^{15,16} Mice were treated with either 10 µg of anti-CD3ε monoclonal antibody (mAb; clone 145-2C11; Biolegends, San Diego, CA) or isotype control antibodies administered intraperitoneally on 5 consecutive days.¹⁷ After this pretreatment, DBA/2 mice were given a high-phosphate diet *ad libitum* (Altromin, Lage, Germany). In a parallel set of experiments, mice were treated with either 200 µg of an anti-CD25 mAb (clone PC61; BD Biosciences, San Diego, CA) or an isotype control antibody administered intraperitoneally before giving them on the high-phosphate diet.^{6,18–20} Injection of the anti-CD25 mAb leads to functional inactivation of Tregs.¹⁹ The high-phosphate diet contained 20.2 g of phosphorus, 9.4 g of calcium, 0.7 g of magnesium, and 500 IU/kg of vitamin D₃. The standard chow contained 7.0 g of phosphorus, 10.0 g of calcium, 2.2 g of magnesium, and 1000 IU/kg of vitamin D₃. Mice were then followed up for 9 days. Urine collection was performed during a fasting overnight stay in metabolic cages. All animal experiments were approved by Austrian veterinary authorities (BMWF-66.010/0047-II/3b/2012) and corresponded to the European Commission Directive 86/609/EEC.

Flow Cytometry

To prove the effect and duration of T-cell and Treg depletion, we performed flow cytometry experiments of splenocytes 9 days after the initial immunomodulation. Single-cell suspensions from spleen were stained for CD4 (clone RM4-5; BD Biosciences) and CD25 (clone PC61.5.3; Immunotools, Friesoythe, Germany) according to the manufacturer instructions. Data collection and analysis were performed with a FACS Calibur (BD Biosciences).

Telemetry

We implanted ECG transmitters (model ETA-F20; Data Science International, St. Paul, MN) s.c. in the back space in Avertin-anesthetized (0.025 mg/kg body weight), female, 8-week-old DBA/2 mice. Body temperature was maintained at 37°C by a thermostatically controlled heating pad until the recovery from anesthesia. Five days were allowed for recovery before putting mice either on a high-phosphate diet or standard chow diet and water *ad libitum* (*n* = 5 mice for each treatment group). ECGs were recorded continuously (24 hours) after the surgical procedure in conscious, freely moving mice kept in single cages with either normal chow or high-phosphate diet (starting on day 6 after surgery). ECG signals were acquired at a sampling rate of 1 kHz.

µ-CT Quantification of Aberrant Calcification

Ectopic calcifications were quantified at the end of each experiment *ex vivo* by scanning explanted organs using Inveon microcomputed tomography (Siemens, Munich, Germany), as recently described by Le Corre et al.²¹ Briefly, the source was set at 60 keV and 500 µA. The spatial resolution was 26.3 µm on-a-side cubic voxel. After acquisition on an Inveon Acquisition Workplace 1.5.0.28 (Siemens), each raw data set was reconstructed using Inveon Research Workplace version 3.0 software package for two- and three-dimensional models (Siemens). The measurements of aberrant calcification were representative of the entire organs and covered the whole heart and kidney, respectively. Thus, we can definitely exclude any sampling error. For calcification quantification, total organ volume was defined as the volume of tissue with voxel intensity between −444 and +3000. The amount of radiopaque tissue was determined as the volume of tissue with voxel intensity value between −244 and +3000. The absolute amount of radiopaque tissue was defined as volume of calcified renal or cardiac tissue, whereas the relative amount of radiopaque tissue was defined as the proportion of calcified volume from the whole kidney or heart and was given in percentages, respectively.

Inductively Coupled Plasma Mass Spectrometry

Freeze-dried kidney samples (10 to 30 µg thick) were digested with nitric acid in a microwave-heated autoclave

(UltraCLAVE III; EMLS, Leutkirch, Germany). The temperature was ramped in 45 minutes to 250°C and maintained for 45 minutes. After cooling, the samples were transferred to 50-mL polypropylene tubes. The calcium and phosphorus concentrations were determined with an inductively coupled plasma mass spectrometry (Agilent 7500ce; Agilent Technologies, Waldbronn, Germany) at a mass-to-charge ratio of 43 for calcium and 31 for phosphorus. The accuracy of the results was validated with the reference material bovine muscle (RM 8414; NIST, Gaithersburg, MD).

Histopathologic Evaluations

Frozen sections of kidneys and hearts were evaluated for calcification by performing von Kossa and Alizarin Red stains using standard protocols. For von Kossa staining, paraffin sections were incubated with 0.1% silver nitrate solution under UV light and then rinsed in 5% sodium-thiosulfate solution (both Merck, Darmstadt, Germany). Alizarin Red staining was performed by incubating rehydrated paraffin sections in 2% Alizarin Red S solution (Sigma, St. Louis, MO) followed by rinsing in acetone and acetone xylene. Furthermore, organs were evaluated for T-cell and macrophage infiltration. Formalin-fixed hearts were embedded in paraffin, cut in 4-μm sections, and stained with PAS using standard staining techniques. Macrophages were stained using rat anti-mouse CD68 antibodies. For the detection of CD4⁺ T cells, rat anti-mouse CD4 mAb (Serotec, Oxford, UK) was used. Biotin-conjugated goat anti-rat IgG antibody (Jackson Immuno Research Laboratories, West Grove, PA) was used as a secondary antibody, followed by incubation with a streptavidin-biotin complex and subsequent development with 0.4% 3-amino-9-ethylcarbazole for 6 minutes and counterstaining with Gill's Hematoxylin No. 3 (Vector, Burlingame, CA).

Biochemical and Molecular Biological Evaluations

Biochemical analysis included serum electrolytes and urea (Roche Diagnostics, Mannheim, Germany), serum fibroblast growth factor 23 (FGF-23; Immotopics, San Clemente, CA), and parathyroid hormone (ALPCO, Salem, NH). Urinary neutrophil gelatinase-associated lipocalin (Lcn2) was quantified using a commercially available enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) and normalized for urinary creatinine concentration using a spectrophotometric assay (Sigma) based on the Jaffe reaction. Both were measured on a FLUOstar Omega photometer (BMG Labtech, Ortenberg, Germany). Real-time PCR for *Foxp3*, *Il10*, *Tgfb1*, *Il6*, *Tnf*, *Tbx21*, *Rorc2*, and *Gata3* was performed to evaluate cytokine changes and master regulator expression of various Th populations. Total RNA was isolated from the kidney using Trizol (Invitrogen, Carlsbad, CA) according to a standard protocol. Thereafter, 2 μg of total RNA was reverse transcribed using Superscript III Transcription Kit (Invitrogen) and random primers (Roche, Basel, Switzerland).

Real-time PCR was performed in duplicate on a CF96 real-time detection system (BioRad, Vienna, Austria). For quantification of *Foxp3*, *Tnf*, *Il10*, *Tgfb1*, *Il6*, *Tbx21*, *Rorc2*, and *Gata3*, the gene expression assays Mm00475162_m1, Mm00443258_m1, Mm00439616_m1, Mm03024053_m1, Mm00446190_m1, Mm00450960_m1, Mm01261022_m1, and Mm00484683_m1 were used, respectively. *Hprt* gene served as the housekeeping control and was assessed using SYBR green (Invitrogen) and the following primers: forward 5'-GCTTCCTCCTCAGACCGGTTTTC-3' and reverse 5'-ATCGCTAATCACGACGCTGGGACTG-3'.

Statistical Analysis

Results from animal experiments are presented as means ± SEM. Normal distribution of the data were assessed by the Kolmogorov-Smirnov test with Lilliefors correction. Differences between groups were compared with either the nonparametric *U*-test or the unpaired Student's *t*-test as appropriate, depending on the distribution of the tested variable. *Gata3* expression was correlated with the relative amount of renal calcification by computing the Pearson correlation coefficient. Differences in mortality rates were assessed by the log rank test. *P* < 0.05 was considered significant. Statistical analysis was performed with GraphPad Prism version 6.0 for Macintosh (GraphPad Software, La Jolla, CA).

Results

Acute Phosphate Nephropathy in DBA/2 Mice

DBA/2 mice on standard chow revealed no aberrant calcification (Figure 1). On the other hand, feeding a high-phosphate diet to 8-week-old DBA/2 mice induced a remarkable phenotype of cortical nephrocalcinosis within 9 days (Figure 1). The histopathologic evaluations with von Kossa (Figure 1A) and Alizarin Red (Figure 1B) staining and PAS reaction (Figure 1C) of the kidneys revealed a radial anatomical distribution of the renal precipitations that followed the renal tubular architecture and were most pronounced beneath the renal fascia and within the renal cortex. Immunohistochemical (IHC) staining for CD68-positive macrophages and CD4-positive T cells revealed considerable infiltration by both cell types, which colocalized with precipitations (Figure 1, D and E). Microcomputed tomography confirmed the localization and distribution of renal precipitations (Figure 2, A–D). Thus, compared with previous data by our group on acute phosphate nephropathy in *Lepr^{db}* mice, DBA/2 mice on a high-phosphate diet developed a similar phenotype in a considerably shorter period.¹⁸

CD3-Depletion Deteriorates Nephrocalcinosis

To investigate the role of T cells in inflammation and progression of nephrocalcinosis, we treated female DBA/2

Figure 1 Nephrocalcinosis in DBA/2 mice fed with a high-phosphate diet (HPD). DBA/2 mice fed with an HPD developed a clear phenotype of nephrocalcinosis within 9 days, whereas controls on a standard chow diet (SCD) had no histopathologic signs of renal calcifications. The histopathologic evaluation with von Kossa (**A**), Alizarin Red staining (**B**), and PAS reaction (**C**) revealed a radial distribution of the renal calcifications that were most pronounced beneath the renal fascia and within the renal cortex. IHC for CD68-positive macrophages (**D**) and CD4-positive T cells (**E**) revealed colocalization of inflammatory infiltrates with renal precipitations.

mice with either 10 μg of anti-CD3 ϵ mAB or isotype control for 5 consecutive days. After this pretreatment, both CD3-depleted DBA/2 mice and their respective isotype-treated controls were set on the identical high-phosphate diet for 9 days, thus excluding a possible impact of the dietary composition on the amount of nephrocalcinosis.^{22,23} The mice were sacrificed after this short period, and the kidneys and hearts were evaluated for precipitation of calcium hydroxyapatite crystals. Flow cytometry experiments from splenocyte single-cell suspensions demonstrated the effect and duration of T-cell depletion (Figure 3A). The two- and three-dimensional images obtained with microcomputed tomography of the explanted kidneys allowed us to quantify the severity of the soft tissue calcification by measuring the absolute and relative amount of radiopaque tissue in single

organs (Figure 2 and Supplemental Video S1). Interestingly, the systemic depletion of T cells by anti-CD3 ϵ antibody pretreatment resulted in a significantly higher amount of renal radio-opaque calcium hydroxyapatite precipitations when compared with isotype-treated controls ($44.3\% \pm 7.5\%$ versus $61.3\% \pm 5.9\%$; $P = 0.022$) (Figure 3B). Semiquantitative histopathologic evaluations with Alizarin Red staining independently confirmed these radiologic measurements (Figure 3C). Chemical analyses of renal calcifications by mass spectrometry revealed significantly higher renal phosphorus contents in CD3-depleted DBA/2 mice when compared with isotype-treated controls (66.7 ± 5.9 versus 49.7 ± 5.4 $\mu\text{g}/\text{mg}$ dry weight; $n = 10$ per group; $P = 0.048$), whereas the renal calcium contents were not statistically different between the two treatment groups (105.1 ± 11.3 versus 73.0 ± 10.6 $\mu\text{g}/\text{mg}$ dry weight; $P = 0.055$). Collectively, the systemic absence of T cells resulted

Figure 2 Computed tomographic assessment of nephrocalcinosis in DBA/2 mice. Microcomputed tomography confirmed the histopathologic assessment of nephrocalcinosis in DBA/2 mice on a high-phosphate diet. **A**: Whole-body imaging and three-dimensional reconstruction with false color display of aberrant calcifications (yellow) shows the prominent renal calcification. Representative parasagittal (**B**) and coronal (**C**) whole-body imaging confirms the radial distribution of renal calcifications *in situ*. **D**: To perform high-resolution microcomputed tomography of calcified organs, both kidneys of a DBA/2 mouse on a high-phosphate diet were explanted and imaged *ex vivo*, which confirmed the radial distribution of intrarenal calcifications within the cortex following the tubular architecture.

Figure 3 Nephrocalcinosis in T-cell-depleted DBA/2 mice. **A:** Flow cytometry of splenic single-cell suspensions were stained for CD4 to prove the effect and duration of T-cell depletion ($n = 4$; white bars) when compared with controls ($n = 3$; black bars). The percentages of CD4-positive T cells are shown as means $\pm$ SEM. **B:** Voxel intensities of the native μ -computed tomography images of the explanted kidneys were used to calculate the absolute and relative amount of renal calcification. Female DBA/2 mice that were depleted of T cells by anti-CD3 ϵ pretreatment had a significantly higher amount of calcified renal tissue when compared with isotype-treated controls (means $\pm$ SEM; $n = 9$ in the isotype group and 10 in the CD3-depletion group; $P = 0.022$). **C:** Semiquantitative Alizarin Red staining of kidney sections independently confirmed deterioration of nephrocalcinosis in CD3-depleted animals. Representative sections are shown. Two independent experiments were performed. **D:** Renal expression of mRNA in T-cell-depleted animals ($n = 10$; white bars) and isotype-treated controls ($n = 9$; black bars). * $P < 0.05$, **** $P < 0.0001$. Original magnification: $\times 40$ (C, upper row); $\times 200$ (C, bottom row).

in an increased severity of renal calcification. Therefore, we hypothesized that the anti-inflammatory subpopulation of regulatory T cells might be involved in the progression of acute phosphate nephropathy.

Tregs Blunt Calcium Deposition in Nephrocalcinosis

After having demonstrated that the mere presence of T cells was a prerequisite for controlling the acute phase of ectopic

calcification, we next studied the role of anti-inflammatory Tregs in our animal model of nephrocalcinosis. We treated the female DBA/2 mice with one intraperitoneal injection of a Treg-depleting anti-CD25 antibody or an isotype control. After pretreatment, DBA/2 mice were again given the same high-phosphate diet for 9 days. The mice were sacrificed after this short period, and the renal and cardiovascular tissues were evaluated for precipitation of calcium hydroxyapatite crystals. To prove the effect and duration of Treg depletion, we performed flow cytometry experiments at the end of the experiment (Figure 4A). The depletion of

Figure 4 Nephrocalcinosis in Treg-depleted DBA/2 mice. **A:** Flow cytometry of splenic single cell suspensions were stained for CD25 to prove the effect and duration of Treg depletion ($n = 9$; gray bars) when compared with controls ($n = 9$; black bars). The percentages of CD4⁺CD25⁺ T cells are shown as means $\pm$ SEM. **B:** Voxel intensities of the native μ -computed tomography images of the explanted kidneys were used to calculate the amount of renal calcification. Female DBA/2 mice that were depleted of Tregs had a significantly higher relative amount of calcified renal tissue when compared with isotype-treated controls ($n = 15$ per group; $P = 0.039$). **C:** Semiquantitative Alizarin Red staining of kidney sections independently confirmed deterioration of nephrocalcinosis in CD25-depleted animals. Representative sections are shown. These more severe renal precipitations in Treg-depleted animals were associated with a significantly lower expression of *Foxp3* and *Gata3* ($n = 15$ per group; $P = 0.020$) (D) and a significant increase in serum FGF-23 levels ($n = 15$ per group; $P = 0.005$) (E), respectively. Three independent experiments were performed. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Original magnification: $\times 40$ (C, upper row); $\times 200$ (C, bottom row).

Table 1 Serum Biochemical Parameters of DBA/2 Mice on a High-Phosphate Diet

Parameter	Control (n = 9)	CD3 depletion (n = 10)	Control (n = 15)	CD25 depletion (n = 15)
Urea (mg/dL)	190 ± 24	199 ± 14	265 ± 31	292 ± 35
Calcium (mmol/L)	1.6 ± 0.1	1.5 ± 0.1	2.9 ± 0.2	3.3 ± 0.3
Phosphorus (mmol/L)	5.4 ± 0.5	4.4 ± 0.4	6.3 ± 0.6	6.7 ± 0.8
Ca × P (mmol ² /L ²)	8.1 ± 1.4	5.8 ± 0.9	19.5 ± 3.0	25.3 ± 5.0
FGF-23 (ng/L)	5141 ± 806	5426 ± 1017	6736 ± 1068*	12,617 ± 1545*
Parathyroid hormone (ng/L)	563 ± 72	1015 ± 275	984 ± 225	1059 ± 162

**P* = 0.005.

Tregs resulted in a significantly higher amount of renal radio-opaque calcium hydroxyapatite precipitations compared with isotype-treated controls ($47.3\% \pm 4.6\%$ versus $59.9\% \pm 3.2\%$; $n = 15$ mice per group; $P = 0.039$) (Figure 4B). Semiquantitative histopathologic evaluations with Alizarin Red staining independently confirmed these radiologic measurements (Figure 4C). Chemical analyses of renal calcifications by mass spectrometry displayed significantly higher renal phosphorus contents in CD25-depleted DBA/2 mice when compared with isotype-treated controls (72.1 ± 4.6 versus 57.1 ± 3.7 $\mu\text{g}/\text{mg}$ dry weight; $n = 5$ per group; $P = 0.045$), whereas the renal calcium contents were not statistically different between the two treatment groups (128.4 ± 11.4 versus 94.5 ± 8.8 $\mu\text{g}/\text{mg}$ dry weight; $P = 0.060$). The systemic depletion of Tregs was paralleled by a significantly lower expression rate of *Foxp3* and *Gata3* in renal tissue (Figure 4D) and by significantly higher serum Fgf23 levels (Figure 4E). *Foxp3* is the master regulator in the development and function of Tregs, whereas *Gata3* is the master regulator of Th2 differentiation.²⁴ Thus, the lower renal expression of *Foxp3* in Treg-depleted animals confirms the effect and duration of Treg depletion in the kidney, whereas the lower expression of *Gata3* in Treg-depleted animals indicates a shift from Th2 toward Th1 response. Furthermore, we found a significant inverse correlation of *Gata3* levels with the amount of renal mineral deposits throughout the entire sample of mice studied in our experiments ($r = -0.3$; $P = 0.037$; $n = 49$). No significant differences were found in serum parameters, such as urea, parathyroid hormone, calcium, and phosphate levels, between Treg-depleted animals and isotype-treated controls, respectively (Table 1). Because renal retention parameters were highly elevated after 9 days on a high-phosphate diet, we speculated that more sensitive parameters of renal damage might more accurately reflect the increase in renal calcification in Treg-depleted mice. To this end, we determined urinary neutrophil gelatinase-associated Lcn2. Lcn2 is a marker for acute kidney injury, which has repeatedly been reported to sensitively reflect renal damage through a wide spectrum of clinical and experimental settings.^{25,26} DBA/2 mice were partly anuric after 9 days of a high-phosphate diet. Therefore, we collected urine samples 3 days after putting Treg-depleted DBA/2 mice and respective isotype-treated controls on a high-phosphate diet ($n = 4$ per group)

and measured the Lcn2/creatinine ratio. Interestingly, we found a significant higher urinary Lcn2/creatinine ratio in Treg-depleted animals when compared with isotype-treated controls (9.03 ± 3.05 $\mu\text{g}/\text{mg}$ versus 1.99 ± 0.47 $\mu\text{g}/\text{mg}$; $P = 0.029$).

Figure 5 Mortality rate and dystrophic cardiac calcinosis in DBA/2 mice. **A:** Kaplan-Meier survival curve of DBA/2 mice on a high-phosphate diet (HPD) that were depleted of Tregs (dashed line), depleted of T cells (dotted line), or treated with isotype control (solid line) ($n = 5$ per group; P values versus isotype control). An HPD was also associated with dystrophic cardiac calcinosis. Cardiac calcifications were visualized by Alizarin Red staining (**B**) and PAS reaction (**C**). **D:** ETA-F20 transmitters were implanted in anesthetized, female, 8-week-old, DBA/2 mice 5 days before putting them on an HPD. Telemetric electrocardiographic recordings were continuously recorded in freely moving mice and revealed both negative chronotropic and negative dromotropic effects in mice with dystrophic cardiac calcinosis ($n = 5$). Representative electrocardiograms from one animal before and after putting it on an HPD are shown. **E:** Voxel intensities of the native μ -computed tomography images of the explanted hearts were used to calculate the absolute and relative amount of cardiac calcification. Female DBA/2 mice that were depleted of T cells by anti-CD3 ϵ pretreatment (white bars) had a similar amount of calcified cardiac tissue when compared with isotype-treated controls (black bars) ($n = 10$ per group). **F:** Moreover, there was no significant difference in the amount of calcified cardiac tissue between DBA/2 mice that were treated with a Treg-depleting anti-CD25 antibody (gray bars) or an isotype control (black bars) ($n = 15$ per group; $P = 0.28$). HR, heart rate; SCD, standard chow diet. Original magnification: $\times 40$ (**B** and **C**, upper rows); $\times 200$ (**B** and **C**, bottom rows).

Nephrocalcinosis Is Associated with Increased Mortality and Dystrophic Cardiac Calcinosis

Next, we investigated whether the accelerated course of nephrocalcinosis after immunomodulation had any impact on the survival rate in our animal model. Therefore, we again depleted DBA/2 mice of either T cells or Tregs and set them on high-phosphate diet *ad libitum*. Both, T-cell and Treg depletion were not only associated with a deteriorated acute phosphate nephropathy but also with a significantly increased mortality rate when compared with isotype treated controls ($P = 0.004$) (Figure 5A). DBA/2 mice are also prone to develop dystrophic cardiac calcinosis. Therefore, we analyzed cardiovascular tissue for aberrant calcifications and performed telemetric electrocardiographic recordings. In our experimental setting, DBA/2 mice indeed developed dystrophic cardiac calcinosis (Figure 5, B and C) that was associated with both negative chronotropic and negative dromotropic effects as assessed by telemetry (Figure 5D). Continuous ECG telemetric readings displayed normal heart rhythm with progressive reduction of heart rate followed by a gradual prolongation of atrioventricular nodal conduction, ultimately developing into atrioventricular conduction block preceding death in the DBA/2 mice fed with a high-phosphate diet. Microcomputed tomography three-dimensional reconstructions of the explanted hearts were used to quantify the severity of the cardiac calcinosis. However, in contrast to the clear effect on nephrocalcinosis, neither the systemic depletion of T cells by anti-CD3e pretreatment nor the depletion of Tregs by anti-CD25 antibody significantly altered the amount of radio-opaque precipitations in hearts (Figure 5, E and F).

Discussion

Similar to other crystallopathies, acute phosphate nephropathy might be seen as passive deposition of an oversaturated ion product where inflammation is just a circumstantial phenomenon. Our data suggest that the course of acute phosphate nephropathy is actively modulated by these inflammatory repair mechanisms that lead to tissue remodeling, aberrant calcium deposition, and ultimately the loss of functioning renal parenchyma. The present findings are also consistent with those of Mulay et al,⁵ who have recently reported acute calcium oxalate nephropathy to be attenuated by therapeutic IL-1 antagonism. Thus, nephrocalcinosis is not exclusively a passive precipitation of a supersaturated ion product but an actively modulated process of tissue remodeling, where inflammation is not just an innocent bystander. Up-to-date treatment of nephrocalcinosis was directed at correcting the underlying cause. Immunomodulation may nicely complement this therapeutic approach and withhold deleterious renal inflammation, tissue remodeling, loss of renal function, and chronic kidney disease. Naive CD4-positive T cells have been reported to differentiate into one of several distinct phenotypes of Th cells, such as Th1, Th2, or Treg. Each of

these lineages has been found to be regulated by distinct transcription markers, to express distinct cytokines, and to play detrimental and protective roles in various pathologic processes.²⁷ In our studies, T cells, particularly Tregs, play a central role in withholding renal inflammation in acute phosphate nephropathy and thus attenuate nephrocalcinosis. In the Treg-depleted animals, we observed a shift from Th2 toward Th1 response reflected by a significantly decreased renal mRNA expression of the master regulator of Th2 differentiation *Gata3*.²⁴ Furthermore, we found a significant inverse correlation of *Gata3* transcription levels with the degree of renal calcification, which further corroborates our hypothesis that the Th2 lineage may play a role in protecting against aberrant calcification.

Treg depletion led not only to increased renal calcification as shown by the higher calcium score of the kidneys in microcomputed tomography but also to a significant increase in serum Fgf23 levels. In this context these higher Fgf23 levels are most likely a reflection of the increased renal damage in Treg-depleted animals. Most importantly, this accelerated course of acute phosphate nephropathy after Treg depletion was also associated with a significantly increased mortality rate. Thus, the present data reconfirm previous clinical studies showing that elevated circulating Fgf23 levels were associated with increased risk for end-stage renal disease and death in patients with chronic kidney disease.^{28,29} In patients with chronic kidney disease, there was also a cross-sectional association of Fgf23 with left ventricular dysfunction,^{30,31} vascular dysfunction,³² and higher levels of inflammatory markers.³³ In our studies, the continuous telemetric ECG recordings in DBA/2 mice with nephrocalcinosis and dystrophic cardiac calcinosis revealed progressive negative chronotropic and negative dromotropic changes that ultimately led to cardiac arrest and death. However, the present experimental design did not allow the discrimination of whether Fgf23 serum levels mediated these electrophysiologic effects.

Treg depletion had a clear impact on the amount of nephrocalcinosis but did not increase cardiac calcification in our experimental setting. Tregs have been reported to ameliorate angiotensin II-induced cardiac damage^{34,35} but cannot withhold cardiac remodeling in dystrophic cardiac calcinosis. The differential regulation of aberrant calcification in different organs may reflect the influence of strain-specific modifier genes on the complex pathologic process of mineralization.^{11,21,36} Thus, the present data derived from a single mouse strain cannot be easily extrapolated to other mouse strains and warrant further investigations.

Taken together, the present study not only corroborates evidence that tissue remodeling in nephrocalcinosis is accompanied by inflammation^{5,18} but also provides first evidence that the degree of intrarenal aberrant calcium deposition is dependent on this inflammatory response. Moreover, our data suggest a pivotal role of T cells and particularly Tregs in the progression of nephrocalcinosis and emphasize the fact that intrarenal inflammation deteriorates the outcome in acute phosphate nephropathy.

Acknowledgments

We thank Andrijana Kozina for critically reviewing the manuscript.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.04.012>.

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