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Efficacy of the Combination of Metformin and CTLA4Ig in the (NZB × NZW)F1 Mouse Model of Lupus Nephritis

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ABSTRACT

CTLA4Ig, a reagent that inhibits CD28 signaling, has shown therapeutic efficacy in mouse models of lupus nephritis (LN) when combined with several other biologics or standard of care drugs. Unfortunately, clinical trials treating LN patients with CTLA4Ig (abatacept) have not met endpoints. Metformin, a drug used to control hyperglycemia that inhibits mitochondrial metabolism, lowered the effective dose of glucocorticoids and prevented major flares when added on to the standard of care treatment of lupus patients with low disease activity. Metformin combined with inhibition of glycolysis by 2-deoxyglucose showed therapeutic efficacy in multiple mouse models of LN. Because CD28 signaling triggers glucose metabolism in T cells, we hypothesized that combining CTLA4Ig treatment with metformin would have the same effect. In this study, we showed that the combination of metformin and CTLA4Ig decreased the development of LN in (NZB × NZW)F1 mice treated at the early stage of disease. This preventive effect was associated with a decreased expansion of CD4+ T cell effector subsets. However, contrary to the combination with 2-deoxyglucose, metformin combined with CTLA4Ig did not alter autoantibody production, suggesting different mechanisms of symptom mitigation. Overall, this study shows therapeutic efficacy of the combination of metformin and CTLA4Ig, two drugs with established safety records, in a preclinical mouse model of LN. ImmunoHorizons, 2020, 4: 319–331.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease whose complexity and heterogeneity have impeded the development of alternative therapies to glucocorticoids and immunosuppressive drugs despite intense preclinical research and numerous clinical trials (1). CTLA4 is a negative regulator of CD4+ T cell activation that competes with coreceptor CD28 for binding to CD80 and CD86. Based on the obligate involvement of CD4+ T cells in SLE (2), preclinical studies have tested CTLA4Ig, a soluble fusion protein that blocks CD28 interactions with CD80/86, in mouse models of lupus. Although CTLA4Ig alone did not show any clinical efficacy in established nephritis, lasting disease remission was achieved in combination with cyclophosphamide (3, 4), anti-CD40L (5), TACI-Ig (6), or BAFF-R (7). Interestingly, the inhibition of T cell costimulation appeared to have a long-lasting effect on end-organ injury rather than immune activation and autoantibody production (4). These studies have...
provided a rationale for a randomized clinical trial in which active class III or IV lupus nephritis (LN) patients treated with CTLA4Ig (abatacept) were compared with standard of care alone (8). The primary endpoint, which was defined as a sustained improvement of LN clinical parameters, was not met. However, a modest improvement was observed in the patients with the most severe disease, as well as a reduction in serum anti-DNA Abs and an increase in C3 and C4 levels, indicating that the treatment had a biological effect. This modest clinical effect was independently confirmed in a small cohort (9). A post hoc analysis of the PBMCs showed a heterogeneous response to the treatment based on the activation of specific immune cell types in which the most responsive patients shared a distinctive transcriptional signature of plasma cells, activated dendritic cells, NK cells, and neutrophils (10). In addition, structural variants in CTLA4g and/or its ligands may also affect the response to treatment (11). Moreover, despite the efficacy of CTLA4Ig in combination therapies in lupus mice (12), the outcome of abatacept treatment in LN patients was not improved by its combination with low-dose cyclophosphamide and azathioprine in the ACCESS trial (13).

Cellular metabolic programs (i.e., the type and amount of metabolic substrates used by a cell, as well as the manner by which they are used), control the effector phase of the immune system. Targeting T cell metabolism has been proposed as a novel approach to manipulate the immune response toward therapeutic outcomes in immune mediated diseases (14). CD4$^+$ T cells from mouse models and patients with SLE present numerous metabolic abnormalities, including an increased glycolysis and oxidative phosphorylation (15). We have shown that 2-deoxyglucose (2DG) and metformin, which are inhibitors of glycolysis and the mitochondrial electron transport chain, respectively, normalized the functions of human and murine lupus CD4$^+$ T cells. Furthermore, treatment with the combination of these two drugs reversed autoimmune pathology in multiple mouse models of the disease (16, 17). Moreover, these inhibitors have little impact on the non-autoreactive functions of the immune system, including its response to immunization and infection (18). These findings suggest that metabolic inhibitors can improve clinical outcomes in lupus perhaps with less systemic side effects than currently used therapies.

In SLE patients, metformin can enhance the efficacy of existing therapies as a glucocorticoid-sparing add-on treatment (19) and by reducing the rate of major flares in SLE patients with low disease activity (20). In mice, metformin treatment expands the regulatory T (Treg) cell subset while inhibiting the expansion of Th1 and Th17 effector cells, reducing the severity of multiple autoimmune diseases, such as experimental autoimmune encephalomyelitis (21), insulinitis (22), and scleroderma (23). In lupus-prone mice, metformin decreases the production of IFN-$\gamma$ and the expansion of memory CD4$^+$ T cells while increasing the production of IL-2 (16, 17). Metformin has, however, little effect on follicular helper T (Tfh) cells or autoantibody production, which are targeted by glycolysis inhibition with 2DG (18). Therefore, the combination of metformin and 2DG has the potential to provide an optimal effect in reversing early disease in lupus mice by targeting different arms of autoimmune activation. Because of the limitation and potential side effects of using 2DG clinically, we investigated whether metformin could enhance the efficacy of another treatment in a preclinical model of lupus.

The concept to simultaneously target a select immune pathway and cellular metabolism has been validated by combining a PI3K$\alpha$ inhibitor with checkpoint blockade in multiple preclinical tumor models (24). Furthermore, there is evidence that blocking specific immune pathways affects the metabolism of the targeted immune cells. We focused on CTLA4Ig because it is a safe biologic with documented modest effects in LN patients. Furthermore, checkpoint blockade with Abs against CTLA4 restored T cell glycolysis and effector functions in a mouse tumor xenograft model (25). Because CD28 signaling activates glycolysis (26), CTLA4Ig should inhibit glycolysis in T cells.

The goal of this study was to test the hypothesis that metformin would improve the efficacy of CTLA4Ig in (NZB × NZW)F1 (BWFI) mice treated at a preclinical stage of the disease, a model of lupus in which a short course of CTLA4Ig has shown efficacy in combination with other drugs (12). We showed that although neither metformin nor CTLA4Ig alone were effective, the combination of metformin with CTLA4Ig reduced renal pathology to a similar extent as the combination of metformin with 2DG. The mechanisms of these two combination therapies are, however, different because the CTLA4Ig combination minimally affects the production of autoantibodies, which is eliminated by the combination with 2DG. Given the safety record of both CTLA4Ig and metformin in SLE patients, these results suggest that their combination may offer an effective venue to treat LN.

**MATERIALS AND METHODS**

**Mice**

BWFI females were purchased from The Jackson Laboratory at 6 wk of age. Treatments started when the mice were 27–32 wk of age. All age-matched cohorts included single treatments and a no-treatment control. CTLA4Ig was administered as previously described (5). Briefly, 100 $\mu$g of CTLA4Ig was given via i.p. injection six times over a 2-wk period. 2DG (6 mg/ml; Sigma-Aldrich) or metformin (3 mg/ml; Sigma-Aldrich) was administered as previously described in drinking water (16), either separately or in combination with another treatment for 4 or 8 wk. Mice were sacrificed at week 4 or 8. Serum for Ab measurements was collected on the day the treatment started, then at 4 and 8 wk. Peripheral blood was collected at wk 2 for flow cytometry. Proteinuria was measured semiquantitatively with Albustix strips. C57BL/6J (B6) mice as well as the lupus-prone triple congenic B6.Sle1.Sle2.Sle3 (TC) (27) mice were bred and maintained at the University of Florida. All experiments were conducted according to protocols approved by the University of Florida Institutional Animal Care and Use Committee.

**Autoantibody measurement**

For the detection of anti-nuclear Abs (ANAs), indirect immunostaining of Hep-2 slides (Bio-Rad Laboratories) with Alexa Fluor 488–conjugated goat anti-mouse IgG (BD Biosciences) was performed with sera diluted 1:40 as previously described (16).
Fluorescence intensity was analyzed using the ImageJ program. Detection of anti-dsDNA IgG was performed with sera diluted 1:100 as previously described (16). Results were normalized to a serial dilution of a pool of titer TC sera in which the 1:100 dilution was set to 100 U.

**Flow cytometry**

Peripheral blood was collected in heparinized tubes. Single-cell suspensions were prepared using standard procedures from spleens. Cells were stained in FACS staining buffer (2.5% FBS and 0.05% sodium azide in PBS). Fluorochrome-conjugated Abs against B220 (RA3-6B2), CD25 (PC61.5), CD38 (90/CD38), CD4 (RM4-5), CD44 (IM7), CD62L (MEL-14), CD95 (15A7), CD138 (28L2), CXCR5 (2G8), Foxp3 (FJK-16S), GL-7, S473P-AKT (SDRNR), PD-1 (RPMI-30), and P-S6 (D57.2.2E) were purchased from BD Biosciences, eBioscience, BioLegend, and Cell Signaling Technology. Follicular T cells were stained in a three-step process using purified CXCR5, followed with biotinylated anti-rat IgG (Jackson ImmunoResearch Laboratories) and PerCP-Cy5 streptavidin in FACS on ice. GLUT1 expression was measured with a rabbit anti-mouse Ab (EPR3915; Abcam), followed by an Alexa Fluor 640–conjugated goat anti-rabbit IgG–(H + L chain), F(ab’)2 fragment (Cell Signaling Technology). Intracellular staining was performed with a Fixation/Permeabilization kit (eBioscience). Dead cells were excluded with fixable viability dye (eFluor780; Thermo Fisher Scientific). All samples were acquired on an LSRFortessa flow cytometer (BD Biosciences) and analyzed with the FlowJo software (Tree Star) as previously described (28).

**Histology**

Renal pathology was scored on periodic acid Schiff–stained sections as previously described on a 0–4 scale (4). The score for each mouse was calculated as the average of 20 glomeruli in each of two sections (40 glomeruli per mouse). The surface area of every glomerulus from one coronal section per mouse was measured on scanned slides with the Aperio ImageScope software (Leica Biosystems), then averaged to give one value per mouse. The presence of IgG2a/C3 immune complex in glomeruli was detected in frozen sections with FITC-tagged anti-IgG2a and anti-C3 Abs, as previously reported (16), and quantitated with ImageJ.

**Metabolic analysis**

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), a measure of glycolysis, were assessed on splenic B cells and CD4+ T cells purified by negative selection (Miltenyi Biotec) from treated mice during a mitochondrial stress test using a Seahorse XF96 instrument (Agilent Technologies) as previously reported (16). One age-matched B6 mouse was included in each assay for normalization. We also performed mitochondrial stress tests on B cells and CD4+ T cells purified from 2-mo-old B6, TC, and BWF1 mice. To measure glucose uptake, splenocytes were incubated with FITC-labeled 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2NBDG) (Sigma-Aldrich) at 37°C for 30 min. FITC mean fluorescence intensity was evaluated by flow cytometry in the B220+ B cell and CD4+ T cell gates.

**Statistics**

Statistical analyses were performed with the GraphPad Prism 8.0 software. Differences between the control and treatment groups were evaluated by one-way Brown–Forsythe and Welch ANOVA with Dunnnett T3 multiple comparisons tests with individual variances computed for each comparison. Additional tests for specific datasets were used as described in the figure legends. To reduce instrument-induced variations between cohorts, mean fluorescence intensity results were normalized for each cohort to the mean value of the control group set as 1. The result is expressed as means ± SEM. The levels of statistical significance were set at *p < 0.05, **p < 0.01, and ***p < 0.001.

**RESULTS**

**Effect of the single treatments on circulating lymphocytes**

We evaluated the effect of a 2-wk treatment with CTLA4Ig compared with the metabolic inhibitors metformin and 2DG on PBLS. We observed no change in the frequency of total or memory B cells with any of the treatments (Fig. 1A, 1B), but 2DG decreased the number of plasma cells (Fig. 1C). Both metformin and 2DG decreased the frequency of circulating CD4+ T cells (Fig. 1D). 2DG decreased the frequency of memory CD4+CD44+ T cells (Fig. 1E), whereas metformin increased the frequency of Treg cells (Fig. 1F), as reported by others (21). Overall, short-term treatments with metformin and 2DG resulted in specific alterations in circulating lymphocytes, whereas treatment with CTLA4Ig had no effect on these phenotypes.

**The combination of CTLA4Ig and metformin reduced autoimmune pathology after a 4-wk treatment**

The efficacy of a treatment with metformin and CTLA4Ig was first evaluated compared with CTLA4Ig, 2DG, or metformin alone in mice sacrificed 4 wk after the treatment was started. Only the 2DG treatment reduced both anti-dsDNA IgG and ANA production (Fig. 2A, 2B). There was a modest, but variable, decrease in ANA levels in mice treated with metformin and CTLA4Ig (Fig. 2B). The efficacy of 2DG indicated that a 4-wk treatment is long enough to eliminate anti-DNA–producing cells in this lupus model, which has a large contribution of short-lived autoreactive plasmablasts (29). The amount of IgG2a/C3 immune complexes deposited in glomeruli of the metformin and CTLA4Ig–treated mice was similar to that of control mice (Fig. 2C–E), confirming a modest effect, at best, of the combination treatment on autoantibodies. The combined treatment showed, however, a reduction of renal pathology. The size of glomeruli, which is directly correlated with the severity of glomerulonephritis (GN) (30), was decreased by the metformin and CTLA4Ig treatment (Fig. 2F). The GN severity scores were also decreased in mice that received the combination treatment (Fig. 2G). Finally, only 1/8 mice treated with metformin and CTLA4Ig presented with 2000 mg/dl terminal proteinuria, as compared with 5/8 controls (Fig. 2H). Metformin alone showed only a trend in reducing renal pathology, consistent across our three measurements of glomerular size, GN score, and proteinuria.
which confirmed previous findings with another lupus-prone model (17). CTLA4Ig alone had no effect on renal pathology, assessed either with immune complex deposition, GN scores, or proteinuria (data not shown). Overall, these results showed that a short treatment combining metformin and CTLA4Ig reduced renal pathology as compared with untreated mice, whereas the single treatments showed either only a trend or no effect as compared with untreated mice. This effect on renal pathology occurred without affecting the production of autoantibodies in BWF1 mice.

**The combination of metformin and CTLA4Ig reduced CD4+ T cell activation after a 4-wk treatment**

The total number of splenocytes was not affected by any of the treatments (data not shown). We evaluated the distribution of B and CD4+ T cell effector subsets in the spleen. Globally, the B cell populations were not affected, as shown by the frequency of total B cells, memory B cells, germinal center (GC) B cells, and plasma cells (Fig. 3A–D). However, both metformin and metformin and CTLA4Ig increased the frequency of total CD4+ T cells (Fig. 3E). Metformin and CTLA4Ig also increased the frequency of naive CD4+ T cells (Fig. 3F), and it decreased the ratio of effector memory T (Tem) cells over naive T (Tn) cells (Fig. 3H) as well as the frequency of Tfh cells (Fig. 3I). Metformin alone did not change the frequency of effector T cells, and CTLA4Ig alone increased the frequency of Tem cells (Fig. 3G). Finally, there was no effect on the Treg population (Fig. 3J).

We next assessed whether changes in the metabolism of B and CD4+ T cells of treated mice corresponded to their immune activation. B cells of mice treated with metformin, either alone or in combination with CTLA4Ig, increased their glucose uptake measured with the glucose analogue 2NBDG (Fig. 4A). This is an expected response to the inhibition of respiration induced by metformin, but it was not associated with an increased expression of GLUT1, the main glucose transporter in lymphocytes (Fig. 4B) (31). Metformin alone or in combination with CTLA4Ig also decreased S473 pAKT expression in total B cells and memory B cells (Fig. 4C, 4D), but not in GC B cells (Fig. 4E). AKT is phosphorylated at S473 by PI3K in response to BCR and TCR/coreceptor stimulation, which then initiates mTORC1 activation (32). mTOR activation, however, was unchanged when measured by pS6 levels (data not shown). As for B cells, CD4+ T cells from mice treated with metformin with or without CTLA4Ig increased their glucose uptake (Fig. 4F), although a small decrease in GLUT1 expression was observed on CD4+ T cells from mice treated with metformin alone (Fig. 4G). Unlike B cells, however, S473 pAKT expression on CD4+ T cells was not changed by any of the treatments (Fig. 4H–J).

We have previously shown that CD4+ T cells from TC mice present higher respiration and glycolysis than B6 controls even before autoimmune manifestations (16), but it is unknown whether it is also the case in BWF1 mice. In this study, we compared the
FIGURE 2. The combination of CTLA4Ig and metformin reduces renal pathology after a 4-wk treatment.
Mice were treated with 2DG, CTLA4Ig, metformin, or the combination of metformin and CTLA4Ig for 1 mo. The metformin alone and metformin and CTLA4Ig treatments represent three cohorts along with their controls. Serum autoantibodies were compared before and after treatment for anti-dsDNA IgG (A) and ANA (original magnification ×20) (B) as fold change. Fold change values after 4 wk of treatment with 2DG for mice that were sacrificed after 8 wk of treatment are shown for reference. Representative ANA images (original magnification ×20) are shown for one control and two mice treated with metformin and CTLA4Ig. (C–E) Immune complex deposition in the glomeruli of control mice and mice

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metabolism of total B cells and CD44+ memory CD4+ T cells in 2-mo-old B6, TC, and BWF1 mice before they produced autoantibodies. B cells showed higher respiration (Supplemental Fig. 1A, 1B) and glycolysis (Supplemental Fig. 1C, 1D) in BWF1 mice, with TC B cells showing intermediate values. Unexpectedly, the metabolism of activated CD4+ T cells was similar between B6 and BWF1 mice, whereas we confirmed a higher metabolism in CD44+ T cells from TC mice (Supplemental Fig. 1E—H). Similar results were obtained with naive CD44+CD4+ T cells of old TC and BWF1 mice as well as with untreated control older mice (data not shown). The results suggest that global metabolic alterations affect B cells to a greater extent than CD4+ T cells in the BWF1 model. This finding may, at least in part, explain why the treatments reduced pAKT levels in B cells, but not in CD4+ T cells. With these results as a baseline, we next evaluated the effect of the 4-wk treatments on respiration and glycolysis. Metformin reduced respiration and glycolysis in both B and CD4+ T cells (Fig. 4K–R). CTLA4Ig treatment reduced respiration and glycolysis in CD4+ T cells (Fig. 4O–R), consistent with CD28 signaling being the gatekeeper of glucose metabolism in T cells (26) and glucose being the main substrate for respiration in CD4+ T cells of lupus-prone mice (17). Interestingly, the combination of

**FIGURE 3.** The combination of CTLA4Ig and metformin alters CD4+ T cells after a 4-wk treatment.

Frequency of B220+ B cells (A), CD38+ memory B cells (B), GL7+CD95+ GC B cells (C), CD138+ B220low plasma cells (D), CD4+ T cells (E), CD62L+ CD44+ Tn cells (F), CD62L−CD44+ Tem cells (G), Tem/Tn ratio (H), frequency of Tfh (I), and Treg (J) cells. Mean ± SEM compared by one-way Brown–Forsythe and Welch ANOVA with Dunnett T3 multiple comparisons tests. Each symbol represents a mouse (n = 13 for controls in three cohorts, n = 9 for metformin in two cohorts, n = 4 for CTLA4Ig in one cohort, and n = 9 for metformin and CTLA4Ig in two cohorts). *p < 0.05, **p < 0.01, ***p < 0.001.
FIGURE 4. Metabolic effects of the 4-wk treatments.

B cell glucose uptake (A) and GLUT1 expression (B). S473 pAKT expression in total B cells (C), CD38+ memory B cells (D), and GC B cells (E). CD4+ T cell glucose uptake (F) and GLUT1 expression (G). S473 pAKT expression in total CD4+ T (H), Tem (I), and Treg (J) cells. (K–N) B cell mitochondrial stress test. OCR time course (K) and basal OCR (L); ECAR time course (M) and basal ECAR (N). (O–R) CD4+ T cell mitochondrial stress test. OCR time course (O) and basal OCR (P); ECAR time course (Q) and basal ECAR (R). In the time-course plots, the arrows on the x-axes correspond to the injection of oligomycin, trifluoromethoxy carbonyl cyanide phenylhydrazone (FCCP), and antimycin A and rotenone, in that order. Basal OCR and ECAR correspond to the average values before the addition of oligomycin. For clarity, the metformin and CTLA4Ig plots are not shown. Mean ± SEM compared by one-way Brown–Forsythe and Welch ANOVA with Dunnett T3 multiple comparisons tests. Each symbol represents a mouse (n = 13 for controls in three cohorts, n = 5 for metformin, n = 4 for CTLA4Ig, and n = 5 for metformin and CTLA4Ig, each in one cohort). *p < 0.05, **p < 0.01, ***p < 0.001.
FIGURE 5. The combination of CTLA4Ig and metformin reduces renal pathology after an 8-wk treatment.

(A) Terminal serum anti-dsDNA IgG. Fold changes between the terminal and initial values for serum anti-dsDNA IgG (B) and ANA (C) with representative images of ANA (original magnification ×20) for each treatment (D). (E) Representative images (original magnification ×20) of C3 (top) and IgG2a (bottom) glomerular deposits with corresponding quantification, with each symbol representing the average of four glomeruli from four to nine mice (F and G). (H) Representative periodic acid Schiff–stained kidney sections from a control and metformin and CTLA4Ig–treated mouse. Scale bars, 200 μm.

(I) Mean glomerulus size in one coronal section per mouse. (J) GN pathology scores. (K) Terminal proteinuria. Mean ± SEM compared by one-way Brown–Forsythe and Welch ANOVA with Dunnett T3 multiple comparisons tests. Each symbol represents a mouse (n = 14 for controls in three cohorts; n = 5 for 2DG, metformin, and CTLA4Ig, each in one cohort; and n = 10 for metformin and CTLA4Ig in two cohorts). *p < 0.05, **p < 0.01, ***p < 0.001.

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metformin and CTLA4Ig had no metabolic effect in pAKT levels, respiration, or glycolysis in either B or T cells. Although our sample size for these assays was small, it was sufficient to yield significant results with the single treatments in this study and with other metabolic inhibitors in previous studies (16, 17). These results suggest that the efficacy of this treatment on renal pathology is not associated with a global metabolic reprogramming of total B and CD4+ T cells.

**The combination of CTLA4Ig and metformin reduced autoimmune pathology**

The 4-wk treatment with the combination of metformin and CTLA4Ig resulted in a significant, but modest, therapeutic effect on renal pathology. We have shown that the potent combination of metformin and 2DG required an 8-wk treatment for full effect (16). To better assess the efficacy of a treatment combining CTLA4Ig and metformin, we prolonged the treatment with metformin for a total of 8 wk and compared the results to treatments with metformin alone, as well as with 2DG and 2DG and metformin, which are two effective treatments in the BWF1 model (16, 18). As expected, 2DG either alone or in combination with metformin eliminated the production of anti-dsDNA IgG (Fig. 5A, 5B). The combination of metformin and CTLA4Ig had variable effects on these Abs with a reduction in a small number of mice, but overall no significant effect (Fig. 5A–D). It is possible that a larger sample size would clarify this issue; however, the 8-wk treatment confirmed the results obtained with the shorter treatment (Fig. 2). Accordingly, treatments with either 2DG or 2DG and metformin reduced the amount of C3 and IgG2a immune complexes in the kidneys (Fig. 5E–G). The treatment with metformin and CTLA4Ig reduced C3 deposits, and there was a trend for IgG2a (Fig. 5E–G). These results suggest that the autoantibodies produced by metformin and CTLA4Ig–treated mice are not reduced in quantity but may have a lower pathogenic effect. This was confirmed by reduced GN severity (Fig. 5H), glomerular size (Fig. 5I), GN score (Fig. 5J), and proteinuria to a similar extent as the treatment with metformin and 2DG (Fig. 5K). It should be noted that every 8-wk treatment, except CTLA4Ig alone, reduced the size of glomeruli (Fig. 5J). These results validated the findings obtained with a 4-wk treatment that metformin and CTLA4Ig reduced renal pathology without a major effect on autoantibody production, differing from the metformin and 2DG treatment, which reduced both.

**The combination of CTLA4Ig and metformin reduced CD4+ T cell activation after an 8-wk treatment**

Contrary to the 4-wk treatment, mice treated with CTLA4Ig alone or in combination with metformin presented with a lower count of total splenocytes 8 wk after the treatment began, suggesting that the 4-wk treatment was too short to be effective. The same results were observed in mice treated with either 2DG or 2DG and metformin (Fig. 6A). The treatments with 2DG or 2DG and metformin also reduced the frequency of B cells (Fig. 6B), GC B cells (Fig. 6C), and plasma cells (Fig. 6D). However, similar to the

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**FIGURE 6.** The combination of CTLA4Ig and metformin reduces plasma cell and effector CD4+ T cell differentiation after an 8-wk treatment.

(A) Splenocyte numbers. Frequency of total B cells (B), GC B cells (C), and plasma cells (D). Frequency of total CD4+ T cells (E), Tem cells (F), Tfh cells (G), and Treg cells (H) with CD25 expression on Treg cells (I). Mean ± SEM compared by one-way Brown–Forsythe and Welch ANOVA with Dunnett T3 multiple comparisons tests. Each symbol represents a mouse (n = 14 for controls in three cohorts; n = 5 for 2DG, metformin, metformin and 2DG, and CTLA4Ig, each in one cohort; and n = 10 for metformin and CTLA4Ig in two cohorts). *p < 0.05, **p < 0.01, ***p < 0.001.

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results observed after the 4-wk treatment (Fig. 3), metformin and CTLA4Ig only reduced the frequency of plasma cells (Fig. 6D). The combination treatments of metformin with either 2DG or CTLA4Ig had no effect on the frequency of total CD4+ T cells (Fig. 6E), but they reduced the frequency of Tem and Tfh cells (Fig. 6F, 6G). Each of the single treatments was effective at reducing the frequency of Tem cells, and both 2DG and metformin reduced the frequency of Tfh cells (Fig. 6G). Finally, the frequency of Treg cells was also decreased by the metabolic treatments (Fig. 6H), as we have previously reported in TC mice (16), with a modest increase of CD25 expression on these Treg cells in mice treated with metformin and 2DG (Fig. 6I). This suggests that the clinical improvement associated with any of the treatments was not linked to improved Treg suppression.

Last, we compared the metabolic effects of metformin combined with either 2DG or CTLA4Ig. The metformin and 2DG treatment decreased glucose uptake and GLUT1 expression in B cells, where it had no effect on S473 pAKT expression but...
Reduced mTOR activation (Fig. 7A–D). In contrast, the metformin and CTLA4Ig treatment did not change any of these parameters in B cells. Similar results were obtained for CD4+ T cells, with the exceptions that metformin and 2DG decreased S473 pAKT expression (Fig. 7G) and that metformin and CTLA4Ig decreased mTOR activation in CD4+ T cells (Fig. 7H). Neither treatment changed the metabolism of B cells (Fig. 7I, 7J), but metformin and CTLA4Ig increased both respiration and glycolysis in CD4+ T cells (Fig. 7K, 7L). The results suggest that the initial metabolic effect of CTLA4Ig was lost 6 wk after that short-course treatment was stopped. It also confirms in this preclinical model that clinical improvement by metformin and CTLA4Ig is uncoupled from splenic B and CD4+ T cell metabolism. Although the sample size in these metabolic studies was small, it was sufficient to observe an effect with the single treatments, suggesting that their combination may cancel each other’s effect.

**DISCUSSION**

This study used BFW1 mice, a classical model of spontaneous lupus, to test the hypothesis that the combination of metformin and CTLA4Ig would have a beneficial clinical outcome when treatment is initiated at the early clinical stage of disease before the onset of proteinuria. These two drugs have an established safety profile in SLE patients. There is considerable evidence for the efficacy of CTLA4Ig in preclinical models of lupus, with durable remission achieved in early stages of LN when administered in combination with either a brief induction with cyclophosphamide or with other biologics (12). Unfortunately, these promising results have not been translated into clinical success, with the failure of abatacept clinical trials to meet endpoints, although some modest biological activity was observed in the patients with active disease (8, 9). Metformin prevents or delays the development of disease in lupus-prone mice when treatment started before they develop autoantibodies (17) but showed little therapeutic efficacy (16). Metformin used as add-on treatment to standard of care showed steroid-sparing activity (19), and the ability to reduce the appearance of major flares in patients with low disease activity (20).

Beyond their safety and biological activity records in preclinical models and SLE patients, the rationale for combining metformin and CTLA4Ig is that a treatment combining metformin with 2DG, a glycolysis inhibitor, is very effective in multiple mouse models of lupus (16). Because CD28 signaling induces glycolysis in T cells (26), we predicted that CTLA4Ig could at least partially substitute for 2DG. We combined a short 2-wk CTLA4Ig treatment with either a 4- or 8-wk metformin treatment, and we found significantly reduced renal pathology at both time points with little, if any, effect on autoantibody production. This result is consistent with a previous study showing that the therapeutic effect of CTLA4Ig was largely downstream of immune complex deposition in the kidneys, possibly by preventing dendritic cells’ ability to recruit inflammatory cells (4). Therefore, although we confirmed our hypothesis that CTLA4Ig inhibits glucose metabolism in CD4+ T cells, its effect in combination with metformin is different from that of 2DG, which diminishes autoantibody production (Refs. 16 and 18 and this study). Our study confirmed that monotherapy with CTLA4Ig is not therapeutic (33). Although our sample size was small for this group, we observed in these mice physiological effects that were consistent with the function of CTLA4Ig. This indicates that the combination of metformin and CTLA4Ig results in a unique therapeutic effect that is not achieved by either monotherapies.

We have reported that T-dependent B cell responses are very sensitive to CTLA4Ig, which limits both class-switching and somatic hypermutations in young (<20 wk old) BWF1 mice that are treated before autoantibodies arise (33). In this study of older mice with early clinical disease, however, the only B cell subset that was affected by the metformin and CTLA4Ig treatment was plasma cells. Because this was not associated with a reduction of autoantibody levels, the significance of this finding is unclear. In the short term, this treatment decreased pAKT phosphorylation, but no major changes in B cell metabolism were observed.

Consistent with its inability to induce remission when administered alone to nephritic BWF1 mice, it has been reported that CTLA4Ig does not affect the expansion of memory CD4+ T cells (33). In this study, we even found an expanded Tem cell population in mice treated with CTLA4Ig alone. The combination of metformin and CTLA4Ig, however, effectively reduced the expansion of Tem cells, which are tightly associated with lupus pathogenesis. This reduction in inflammatory effector phenotypes was, however, not associated with a reduction in CD4+ T cell metabolism. It is possible that metabolic changes occurred earlier in the treatment and were missed at the terminal endpoints or that they occurred in specific subsets rather total CD4+ T cells.

CTLA4Ig inhibits the development and homeostasis of Treg cells, which depend on CD28 signaling (34). This potentially proinflammatory side effect, however, has not been reported in multiple models of lupus (12). We observed a reduced frequency of Treg cells in all treated BWF1 mice, with only a modest increased CD25 expression in the metformin and 2DG treatment, supporting our previous findings that the clinical efficacy of metabolic inhibitors in lupus-prone mice does not involve Treg cells (16).

Overall, this preclinical study supports the hypothesis that metformin is an old drug that can be repurposed to treat autoimmune diseases such as lupus (35), and it can be an effective and simple approach to boost the efficacy of CTLA4Ig, which despite great promise, did not meet endpoints in clinical trials.

**DISCLOSURES**

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