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Diabetes is linked to increased inflammation and susceptibility to certain infectious diseases including tuberculosis (TB). We previously reported that aerosol TB in mice with chronic (≥12 wk) hyperglycemia features increased bacterial load, overproduction of several cytokines, and increased immune pathology compared with normoglycemic controls. A similar phenotype exists in human patients with diabetes and TB. The mechanisms of increased T cell activation in diabetes are unknown. In the current study, we tested the hypothesis that hyperglycemia modifies the intrinsic responsiveness of naive T cells to TCR stimulation. Purified T cells from chronically hyperglycemic (HG) mice produced higher levels of Th1, Th2, and Th17 cytokines and proliferated more than T cells from normoglycemic controls after anti-CD3e or Ag stimulation. In this way, naive T cells from HG mice resembled Ag-experienced cells, although CD44 expression was not increased. Chromatin decondensation, another characteristic of Ag-experienced T cells, was increased in naive T cells from HG mice. That phenotype depended on expression of the receptor for advanced glycation end products (RAGE), presumably in response to endogenous ligands upregulated in diabetic hosts including high-mobility group box 1 (HMGB1) and S100 proteins (9, 10) in addition to glycated proteins. This T cell phenotype, which is maintained after adoptive transfer into euglycemic hosts, may be a contributing factor in the pathological inflammation characteristic of TB and a wide range of other infectious and noninfectious complications of diabetes.

We previously reported that a low aerosol dose of M. tuberculosis in chronically hyperglycemic (HG) mice was associated with higher bacterial burden, increased lung immune pathology, and higher levels of proinflammatory cytokines compared with euglycemic mice with tuberculosis (TB) (4). An association between type 2 diabetes and high levels of Th1 and Th2 cytokines also has been described in patients with TB (5–7). Diabetes is associated with a delayed innate response to inhaled bacilli in mice and leads to delayed priming and expression of adaptive immunity and consequently higher lung bacterial burden (8). Although higher Ag load may drive cytokine overexpression in diabetic hosts, we speculated that this hyperinflammation also could result from impaired immune regulation as a complication of hyperglycemia.

In the current study, we investigated the effects of hyperglycemia on T cell responses to TCR stimulation in the absence of infection. Our data show that T cells from HG mice have enhanced proliferation and cytokine production in response to stimulation with anti-CD3e mAb or Ag. Naive T cells from HG mice behave functionally like Ag-experienced T cells, despite having similar CD44 expression as euglycemic controls. We found that naive T cells from chronically HG mice have a significantly higher frequency of decondensed nuclei, as occurs normally after primary encounter with Ag. This decondensation of DNA, increasing cytokine production and proliferation following TCR stimulation. This mechanism may contribute to pathological inflammation associated with diabetes and might offer a novel therapeutic target.


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Materials and Methods

Mice

Age-matched (6–8 wk old) male C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME), male C57BL/6 OT-II mice were a gift from K. Rock (University of Massachusetts Medical School [UMMS]), and RAGE− mice were donated by MedImmune (Gaithersburg, MD). Mice were housed in the Animal Medicine facility at UMMMS where experiments were performed under protocols approved by the Institutional Animal Care and Use Committee and Institutional Biosafety Committee.

Mice were treated with 150 mg/kg body weight of streptozotocin (STZ; Sigma-Aldrich, St Louis, MO) by i.p. injection dissolved in phosphate citrate buffer (pH 4.5). All mice were at least 8 wk old with minimum weight of 25 g when treated with STZ. Blood glucose measurements were performed with a BD Logic glucometer (BD Biosciences, Franklin Lakes, NJ). Ten d after STZ treatment and prior to experiment, Mice were considered diabetic when blood glucose was >300 mg/dl, and euglycemic or control when blood glucose was <200 mg/dl. Urine ketones were tested by dip stick (LW Scientific, Lawrenceville, GA); mice with diabetic ketoacidosis were excluded from the study. All mice were HG for 12 wk before starting the experiment.

Cell preparation

Splenocyte and lymphocyte isolation. Splenocytes were isolated by mechanical disruption of the spleen and passed through a 40-μm strainer. RBCs were lysed, and splenocytes were washed and resuspended in RPMI 1640 medium. RBCs were lysed and incubated with 150 U/ml collagenase type IV and 60 U/ml DNAse in PBS with 5% FBS at 37°C under mild shaking for 40 min. After digestion, cells were passed through a 40-μm strainer, washed with medium, and pooled together with the splenocytes.

T cells separation. Total T cells or CD4+ or CD8+ T cells were isolated using Dynabeads Untouched Mouse T cells (Life Technologies, Grand Island, NY), according to the manufacturer’s guidelines. Briefly, pooled splenocytes and lymphocytes were resuspended in PBS with 0.1% BSA and 2 mM EDTA and incubated with FBS and the Ab mix at 4°C for 20 min. After the incubation, cells were washed and resuspended in the buffer with the magnetic beads. Cells were incubated at room temperature under mild shaking for 15 min and were separated with a magnet. T cell purity was determined by flow cytometry (LSRII; BD Biosciences, San Jose, CA) with anti-CD3 (145-2C11), anti-CD4 (GK1.5), and anti-CD8 (53-6.7) Abs (eBioscience, San Diego, CA).

Non-T cell separation. Non-T cells were isolated using Dynabeads Mouse pan T (Thy1.2) (Life Technologies) by positive selection of T cells by magnetic beads following the manufacturer’s instructions. Pooled splenocytes and lymphocytes were resuspended in PBS with 0.1% BSA, 2 mM EDTA and incubated with the magnetic beads with gentle tilting and rotation at room temperature for 20 min. Cells were separated via magnet isolation, and purity was checked by flow cytometry with anti-CD3, anti-CD4, and anti-CD8 Abs.

Thymocytes isolation. Thymocytes were isolated by mechanical disruption of the thymus. Double-negative (CD4−CD8−), double-positive (CD4+CD8+) and CD4+ and CD8+ populations were sorted after staining with the specific mAb and using BD FACSAria cell sorter (BD Biosciences).

Cytokine detection

Production of cytokines (IFN-γ, TNF-α, IL-12p40, IL-2, IL-10, IL-13, IL-17, and GM-CSF) was determined by multiplex ELISA in accordance with the manufacturer’s guidelines (R&D Systems, Minneapolis, MN) in medium from pooled or individual samples of splenocytes from control or HG mice. Medium was taken after 24, 48, and 72 h of in vitro stimulation with plate-bound anti-CD3e (2.5 μg/ml). Absorbance was analyzed with a Multiskan Ascent microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Intracellular staining for IFN-γ was performed in 2 × 10^6 splenocytes 48 h after in vitro stimulation with plate-bound anti-CD3e (2.5 μg/ml). IL-2 (20 ng/ml) was included during the stimulation time, and brefeldin A (1:1000 dilution; BioLegend, San Diego, CA) and anti-CD28 (2 μg/ml) were added 4 h before starting the staining. Cells were incubated with 1:50 dilution of Fc block (BD Biosciences) for 10 min and then with the surface mAbs, anti-CD4 and anti-CD8 for 20 min. After the cells were washed twice with PBS with 10% FBS and 0.05% sodium azide, they were fixed and permeabilized with BD Cytofix/Cytoperm solution (BD Biosciences) for 30 min at 4°C. After the cells were washed twice with perm/wash buffer (BD Biosciences), they were incubated with anti–IFN-γ (XMG1.2; BioLegend) for 30 min at 4°C, and data were acquired on an LSRII (BD Biosciences). Results were analyzed using FlowJo version 7 (Tree Star, Ashland, OR). Cells were gated based on forward light scatter (FSC)/side scatter (of light) for lymphocytes and FSC-A/FSC-H for singlets.

CFSE labeling

Cells were labeled with 1 μM CFSE (Vybrant CFDA SE Cell Trace kit; Life Technologies) for in vitro experiments or 2 μM for in vivo experiments in PBS at 37°C for 10 min. RPMI 1640 medium with 10% FBS was added after the labeling, and the cells were washed twice. Labeling efficiency was determined by both fluorescent microscopy and flow cytometry.

In vitro culture stimulation by anti-CD3e or OVA

Ninety-six-well round bottom plates were coated with 2.5 μg/ml anti-CD3e mAb overnight at 4°C. Wells were washed twice with PBS, and 0.15 × 10^6 cells/well were plated for the procedures. Supernatants and cells were taken after 24, 48, and 72 h of stimulation. CD4+ and CD8+ T cells proliferation were analyzed by CFSE dilution by flow cytometry after 48 and 72 h. OVA stimulation was performed by adding 100 μg/ml soluble OVA (Sigma-Aldrich) to 0.15 × 10^6 OT-II T cells. Cells were taken after 62 and 84 h of stimulation, and proliferation of CD4+ T cells was assessed by CFSE dilution by flow cytometry.

In vivo adoptive transfer

Pooled splenocytes and lymphocytes from control or HG OT-II mice were CFSE labeled, and 20 × 10^6 cells were transferred to control or HG C57BL/6 mice via tail vein injection. After 24 h, 10 μg OVA in yeast β-glucan particles (provided by Dr. G. Ostrow, UMMS) were delivered to the lungs by intratracheal instillation. Thoracic lymph nodes were harvested after 62 h, and proliferation of CD4+ T cells was assessed by flow cytometry and CFSE dilution.

Flow cytometry

Approximately, 1 × 10^6 cells were used for flow cytometry staining for surface markers as described above. The following mAb were used: anti–CD3, anti-CD4, anti-CD8, anti-CD44 (IM7), anti-CD28 (PC61), anti-CD25 (N418), and anti-CD11c (M1/70). Regulatory T cells were stained using mouse Foxp3 buffer set (BD Biosciences) following manufacturer’s guidelines. Briefly, 2 × 10^6 cells were stained for surface markers CD4 and CD25 (PC61) as described previously. Cells were washed and fixed for 30 min at 4°C. Following several washes with the permeabilization buffer, cells were incubated in this same buffer for 30 min at 37°C. The Ab anti-Foxp3 (FKJ-16s) was used to stain the cells for 20 min at room temperature. Data were acquired on an LSR II and analyzed with FlowJo version 7. Cells were gated based on FSC/side scatter (of light) for lymphocytes and FSC-A/FSC-H for singlets.

Immunoblotting

Total protein was obtained from isolated T cells from control and HG mice and quantified by a bicinchoinic acid assay kit (Pierce Biotechnology, Rockford, IL). Approximately 20 μg total protein was SDS-PAGE separated and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). After blocking the membrane with 5% BSA in TBST for 1 h, it was incubated with Abs rabbit anti-mouse for P38 (Thr180/Tyr182), total p38, P-ERK1/2 (Thr 202/Tyr204), ERK1/2, P-Akt (Ser 473), Akt, P-FoxO3a (Thr32), and FoxO3a (Cell Signaling Technology, Danvers, MA) overnight under mild shaking at 4°C. The membrane was washed with TBST and incubated with the secondary Ab goat anti-rabbit IgG HRP-conjugated (Santa Cruz Biotechnology, Dallas, TX) for 1 h. After several washes with TBST, the membrane was incubated with SuperSignal West Pico chemiluminescence substrate (Pierce Biotechnology) for 5 min, and film was exposed and developed. Bands were quantified by ImageJ 1.4r (National Institutes of Health, Bethesda, MD).

T cell and thymocyte chromatin decondensation analysis

Control and HG mice T cells were isolated from pooled splenocytes and lymphocytes and then CD25+ T cells were depleted by magnetic bead
separation (Miltenyi Biotec, Auburn, CA). The remaining cells were fixed with 4% paraformaldehyde for 10 min at room temperature or overnight at 4°C. Four populations of thymocytes from control and HG mice (CD4+, CD8+, double-positive, and double-negative) were fixed and individually sorted using a FACSAria II cell sorter (BD Biosciences). T cells and thymocytes were adhered to slides by cytocentrifugation, washed with PBS, and mounted with ProLong Gold antifade reagent with DAPI (Life Technologies). Images were taken on a fluorescence microscope Nikon Eclipse E400. Chromatin decondensation was assessed by measuring the diameter of the nucleus using ImageJ. Percentages of T cells with nuclear diameter > 6 μm and of thymocytes with diameter > 6.5 μm were calculated.

Bone marrow chimeras

Bone marrow cells were isolated from control CD45.1 or HG CD45.2 mice. T cells were depleted or not from the bone marrow by CD90.2 (Thy1.2) microbeads (Miltenyi Biotec). Chimeras of bone marrow from control and HG mice were transferred via tail vein into lethally irradiated CD90.1 C57BL/6 mice. After 7 wk, pooled splenocytes and lymphocytes from control or HG donor mice were stained with anti-CD90.1, anti-CD25, anti-CD3, anti-CD25, anti-CD19, anti-CD45.1, and anti-CD45.2 and sorted gating on CD90.2+ and then CD3+CD19−CD25− for the donor cells and then CD45.1+ for control and CD45.2+ for HG cells. T cells were fixed, adhered to slides by cytocentrifugation, and mounted with ProLong Gold antifade reagent with DAPI. Images were taken on a fluorescent microscope, and the percentage of cells with the nucleus diameter > 6 μm was analyzed for each population.

Statistics

Statistical analysis was performed using Sigma Plot 11.0 and GraphPad Prism 6.0, and variance test was performed before the statistical analysis. When normality was confirmed, data were analyzed by Student t test or one-way ANOVA. Nonparametric data were analyzed by the Mann–Whitney U test. The coefficient of correlation for DAPI fluorescent intensity and nucleus diameter in T cells was calculated by Pearson Product Moment Correlation. A p value < 0.05 was considered statistically significant.

Results

Hyperglycemia is associated with increased cytokine production by T cells

HG mice with TB overexpress a broad range of Th1, Th2, and Th17 cytokines (4) and a similarly augmented cytokine response by diabetic humans with TB has been reported previously (5–7). To investigate whether the cytokine overexpression phenotype is restricted to Ag-specific responses in HG mice or whether it is a generalized phenomenon, splenocytes from uninfected euglycemic control mice or HG mice were stimulated ex vivo with plate-bound anti-CD3e. Multiplex analysis of pooled samples demonstrated greater production of Th1, Th2, and Th17 cytokines from the HG group (Fig. 1A–C). Parallel samples from individual mice were tested by ELISA and confirmed the relative overexpression of IFN-γ, IL-10, and IL-17 by anti-CD3e–stimulated splenocytes from HG mice (Fig. 1D). Basal cytokine expression in unstimulated splenocytes was below the limit of detection in samples from control and HG mice. Further confirmation of cytokine production by CD4+ or CD8+ T cells was done for IFN-γ by intracellular staining (Fig. 1E). Despite a trend for an increase in the percentage of CD4+IFN-γ+ T cells from HG mice, only the mean fluorescent intensity (MFI) was significantly higher in HG mice. There was no change in the percentage of IFN-γ− T cells among CD8+ T cells, but the MFI of IFN-γ staining was higher in the HG mice (Supplemental Fig. 1A).

Hyperglycemia is associated with increase in CD4+ T cell proliferation

To determine whether the effects of hyperglycemia on T cell activation were limited to cytokine expression or extended to cell cycle regulation, splenocytes or lymphocytes from control or HG mice were isolated and labeled with CSFE as described in Materials and Methods. Cells from control and HG OT-II mice were stimulated in vitro with OVA for 62 and 84 h (Fig. 2A). The proportion of CD4+ T cells with CSFE dilution was significantly higher in the HG group. Similar results were obtained using cells from wild-type C57BL/6 control or HG mice stimulated with plate-bound anti-CD3e for 48 and 72 h (Supplemental Fig. 1B).

Furthermore, the accelerated proliferative response of HG CD4+ T cells to anti-CD3e stimulation was not shared by CD8+ T cells (Supplemental Fig. 1C). The hyperproliferative phenotype was observed only with T cells from mice that were hyperglycemic for ≥12 wk; it was not detected using cells from mice that were HG for ≤4 wk (Supplemental Fig. 1D). The requirement for prolonged hyperglycemia is consistent with mechanism of other diabetic complications and mirrors the acquisition of TB susceptibility in diabetic mice (4).

Although we focused on effects of hyperglycemia on intrinsic T cell functions, a concurrent alteration of APC function also might occur in the context of diabetes. To address that question, we measured the proliferation of OVA-stimulated OT-II T cells coincubated with autologous APC from control or HG mice. Increased proliferation of HG compared with control CD4+ T cells was observed in cultures with HG or control APC, indicated that the phenotype of accelerated proliferation could not be attributed to an effect of diabetes on APC function (Fig. 2B). Indeed, the proliferation of control T cells in the presence of HG APC was slightly lower than that seen in the presence of control APC. To further investigate the impact of diabetes on APC function and to validate the physiological relevance of results from in vitro culture conditions, we performed a reciprocal adoptive transfer experiment between control and HG OT-II mice. Following transfer of CFSE-labeled donor T cells, recipient mice were challenged by tracheal instillation of OVA packaged in β-glucan particles. Lung-draining lymph nodes were harvested 62 h later, and proliferation of transferred CD4+ T cells was assessed by flow cytometry (Fig. 2C). Using this in vivo system, we observed that the hyperproliferative phenotype of HG T cells was maintained following transfer to a euglycemic host, supporting our interpretation to our in vitro data. Furthermore, the accelerated proliferation of HG T cells was reduced upon transfer to diabetic hosts. Thus, chronic hyperglycemia appeared to exert opposing effects on T cells and at least certain APC populations in vivo. Alternatively, the in vivo results might be explained by increased migration of activated proliferating T cells from the spleen and lymph node. In either case, the in vivo data were consistent with the increased proliferative response of HG T cells observed in vitro.

T cell and APC populations in control and HG mice

Naive T cells (CD44−CD62Lhi) become primed during Ag presentation by DC and they proliferate to become effector cells, whereas ~10% survive to become memory T cells (CD44hi) with self-renewal capacity (11). Memory T cells have a reduced threshold for proliferation and cytokine production within several hours of TCR restimulation. The hyperproliferative phenotype we observed in vitro and in vivo would be predicted to lead to an increased proportion of Ag-experienced T cells in diabetic hosts. In fact, we found a similar basal percentage of naive and memory T cells in control and HG mice (Fig. 3A). We also compared APC from these two groups, finding no impact of chronic hyperglycemia on the relative populations of CD19+, CD11c+, and CD11b+ cells (Fig. 3B). Therefore, it appeared unlikely that the observed functional differences in T cell activation were the result of a basal increase in the population of Ag-experienced T cells or a shift in steady state of APC populations in chronically HG mice. This increased proliferation of HG T cells also could be because of a defective population of regulatory T cells. We analyzed the
proportion of regulatory T cells (CD4+Foxp3+CD25+) in control and HG mice (Fig. 3C), finding an increased percentage of these cells in HG mice.

Increased MAPK pathway activation in T cells from HG mice

The breadth of the activation responses observed in T cells from HG mice suggested the involvement of one or more downstream regulators of TCR signaling. Candidates for such regulators include the MAPK family pathways (12, 13). We therefore compared basal, PMA-, and anti-CD3e/CD28–stimulated p38 and ERK phosphorylation in purified T cells from control or HG mice (Fig. 4). Under basal conditions, p38 phosphorylation was significantly greater in T cells from HG mice, whereas no difference in basal ERK phosphorylation was identified in T cells from control and HG mice. Following stimulation with PMA, both p38 and ERK phosphorylation were significantly elevated in the HG group. When cells were stimulated with anti-CD3e/CD28, only p38 presented significantly higher activation in T cells from HG mice. This upregulation of the MAPK pathway in T cells from HG mice could explain the augmented cytokine production and proliferation phenotype, confirmed by the reduction of cytokine levels with p38 inhibitor (Supplemental Fig. 2A) in splenocytes stimulated with plate-bound anti-CD3e for 48 h. Because differential activation was identified using PMA stimulation, which bypasses the TCR, it suggests that hyperactivation phenotype in diabetes is not due to any perturbation of the cell surface receptor components.

Stimulation of the TCR recruits and activates PI3K that modulates the activation of Akt and leads to the downstream inactivation of forkhead box transcription factor family O (FOXO) transcription factors or mammalian target of rapamycin complex 1 (mTORC1) activation regulating T cell proliferation, growth and glycolytic metabolism (14). The increased proliferation observed in T cells from HG mice could reflect differential regulation in the Akt/mTOR pathway as well. Increased Akt activation in renal tissue from acute HG mice has been described (15), but this has not been previously examined in T cells. In contrast to the effects

FIGURE 1. Cytokine production by plate-bound anti-CD3e stimulated splenocytes from control and HG mice. Medium from cultures of splenocytes isolated from control or HG mice was collected after 24, 48, and 72 h of plate-bound anti-CD3e stimulation. (A) Pooled samples from three individual cultures were analyzed for Th1 cytokines IFN-γ, TNF-α, IL-12p40, and IL-2 by multiplex ELISA. (B) Pooled samples from three individual cultures were analyzed for Th2 cytokines IL-10 and IL-13 by multiplex ELISA. (C) Pooled samples from three individual cultures were analyzed for Th17 cytokines IL-17 and GMCSF by multiplex ELISA. (D) IFN-γ, IL-10, and IL-17 production were analyzed in medium from individual cell cultures by ELISA. The line represents the mean of the values (n = 5). (E) The representative histogram of IFN-γ staining (left), percentage of IFN-γ+ cells (center) and MFI (right) in CD4+ T cells stimulated with plate-bound anti-CD3e (2.5 μg/ml) for 48 h. Statistical differences were analyzed by Student t test for each time point. *p < 0.05, **p < 0.01.
FIGURE 2. In vitro and in vivo T cell proliferation in OT-II control and HG mice recognizing OVA. (A) Representative histograms (left) of CD4+ splenocytes and lymphocytes proliferation from control or HG mice stimulated with 100 μg/ml OVA in vitro for 84 h. The percentage of CD4+ T cells proliferation from splenocytes (n = 4–5) or lymphocytes (n = 2–4) in control or HG mice stimulated with 100 μg/ml OVA in vitro for 62 and 84 h (right graphs). The proliferation of CD4+ T cells was detected by flow cytometry and CFSE dilution. (B) Splenocytes depleted of T cells by negative selection with magnetic beads (non-T cells) from control or HG mice were used as APCs. T cells from euglycemic OT-II mice or HG OT-II mice were isolated by negative selection by magnetic beads and labeled with CFSE. Purity of T cells was 75% and of non-T cells 97% after isolation. In vitro cultures of 30% T cells and 70% non-T cells were stimulated with 100 μg/ml OVA in vitro for 87 h (n = 3). Proliferation of CD4+ T cells was detected by flow cytometry and CFSE dilution. Two-way ANOVA showed no statistically significant interaction between the source of T cells and of APC. (C) Pooled splenocytes and lymphocytes from euglycemic OT-II mice (n = 4) or HG OT-II mice (n = 7–8) were labeled with CFSE and adoptively (Figure legend continues)
of diabetes on the kidney, we found no difference in basal Akt or FOXO3 phosphorylation between control and HG mice (Supplemental Fig. 2B). These results rule out Akt signaling as a mechanism contributing to the hyperproliferative phenotype of T cells from HG mice.

Hyperglycemia induces chromatin decondensation in T cells

Naive T cells are characterized by a quiescent state, G0 phase, in which nuclear chromatin is condensed, leaving many gene promoters inaccessible by the transcription factors. When T cells are stimulated via TCR, chromatin is decondensed and the cells
The effect of hyperglycemia on chromatin decondensation in the thymus

Chromatin condensation in T cells occurs during thymic development in the transition from double-negative to double-positive thymocytes (16). We considered whether chromatin decondensation of mature T cells in HG mice reflected changes exerted during thymocyte development. Double-negative, double-positive, and single-positive thymocytes from control and HG mice were purified and stained with DAPI to assess nuclear diameter. There was no difference in the proportion of double-negative and double-positive thymocytes with nuclear diameter > 6 μm between control and HG mice (Supplemental Fig. 3A). Consistent with normal trends in thymocyte development, the proportion of cells with decondensed nuclei was lower in double-positive compared with double-negative cells. In marked contrast to results obtained with mature splenic and lymph node T cells, the nuclear diameter of single-positive thymocytes was significantly lower in HG mice compared with controls (Supplemental Fig. 3A). The total number of the different cell populations in the thymus was not altered in HG mice (Supplemental Fig. 3B), and the total cell number in thymus was similar in the two groups (Supplemental Fig. 3C). These data suggest that the diabetic environment does influence the state of chromatin condensation in single-positive thymocytes but in a way opposite to that seen in mature peripheral T cells. It remains to be determined whether these two phenomena are linked or discrete effects.

become larger and develop proliferative capacity (16). Although the proportion of Ag-experienced CD44+ T cells was not increased in HG mice, their enhanced activation response to TCR stimulation was reminiscent of previously activated T cells. We therefore compared the status of chromatin condensation/decondensation in T cells of HG and control mice. Cytosin preps of CD25+ T cells were stained with DAPI and examined by fluorescent videomicroscopy (Fig. 5A) to measure the distribution of nuclear diameters grouped in 12 bins (Fig. 5B). Using a cutoff point of 6 μm, which reflects the upper limit of nuclear diameters of mature T cells with visibly condensed chromatin, we determined that a higher proportion of T cells from HG mice had decondensed nuclei (30.3 ± 6.9%) as compared with controls (10.8 ± 1.7%; Fig. 5C). As expected, stimulation with PMA for 5 h or plate-bound anti-CD3e for 24 h increased the percentage of T cells with nuclear diameter > 6 μm and abrogated the differences between control and HG mice observed in unstimulated T cells (Fig. 5D). The enlarged nuclei also had lower fluorescence intensity, and there was an inverse correlation between the nucleus diameter and fluorescence signal (coefficient of correlation R = −0.81, p < 0.0001) (Fig. 5E), confirming that the chromatin is decondensed as described by Rawlings et al. (16). By isolating CD4+ and CD8+ T cells, we determined that only CD4+ T cells from HG mice presented an increased proportion of cells with nuclear diameter > 6 μm (Supplemental Fig. 2C).

Activation of protein kinase C (PKC) modulates histone acetylation and therefore chromatin decondensation (17). Because the biochemical mechanisms of diabetic complications include the activation of certain PKC isoforms (2), we tested the effect of PKC inhibitors on nuclear condensation/decondensation in unstimulated T cells from HG and control mice. Cells were treated with two doses of the PKC inhibitor Gö6983: 7 nM for inhibition of PKC-α and -β and 60 nM for inhibition of PKC-ζ. Neither of these treatments changed the percentage of T cells with decondensed chromatin from control or HG mice (Supplemental Fig. 2D). The Ca2+-calcineurin pathway is involved in T cell proliferation and effector functions (18) and also in the maintenance of anergy (19). Treatment with cyclosporine A to inhibit calcineurin did not change the difference in basal nuclear diameter of untreated T cells from HG mice (Supplemental Fig. 2E) indicating that the effect of the diabetic environment on chromatin decondensation is not calcineurin dependent.

Because we observed that hyperglycemia was associated with p38 MAPK activation under basal conditions and activation of both p38 and ERK1/2 following stimulation with PMA, we tested the possible involvement of these MAPKs in T cell chromatin decondensation. Treating unstimulated T cells with the p38 inhibitor SB203580 reduced the proportion of cells with nuclear diameter > 6 μm in the HG mice, eliminating the difference between the control and HG groups (Fig. 5F). In contrast, the ERK inhibitor U0126 did not reduce the proportion of T cells with decondensed nuclei in the HG group. Chromatin decondensation following TCR activation with anti-CD3e was slightly inhibited by both inhibitors in control T cells (Fig. 5G). These results indicated that increased basal activation of p38 is required to maintain the elevated proportion of T cells with decondensed nuclei from HG mice and the TCR signaling in both control and HG mice. Increase CD44 expression follows TCR stimulation and is characteristic of memory T cells. We stimulated HG and control T cells with plate-bound anti-CD3e and observed similar increase in the proportion of CD44+ cells (Supplemental Fig. 2F). Surface expression of CD44 on activated T cells was not inhibited by treatment with SB203580 or U0126. These results indicate that p38 and ERK are not required for induction of CD44 following TCR stimulation. Because chromatin decondensation was dependent on p38 activation, we conclude that discrete signal pathways regulate chromatin decondensation and CD44 expression in T cells.

FIGURE 4. MAPK activation in control and HG mice. MAPK phosphorylation in T cells isolated by negative T cell selection by magnetic beads from control or HG mice, unstimulated or stimulated with 40 nM PMA or anti-CD3e/CD28 (2.5 μg/m) for 15 min. p38 phosphorylation (A) and ERK1/2 phosphorylation (B) mean ± SD (n = 6–8). All experiments were repeated at least twice. Statistical differences were analyzed by Student t test. *p < 0.05, **p < 0.01, ***p < 0.001.
The effects of hyperglycemia on T cell function do not involve bone marrow progenitors

Epigenetic mechanisms may drive some complications of diabetes, and lymphocytes have been identified as targets for such chromatin modification (20, 21). Epigenetic effects of chronic hyperglycemia are thought to be responsible for the phenomenon of metabolic memory in diabetes in which complications progress after normalization of plasma glucose (22). We therefore questioned whether chromatin modification of lymphoid precursors in bone marrow was responsible for the phenotypic effects we observed in mature peripheral T cells from HG mice. To test that hypothesis, we created bone marrow chimeras that were lethally irradiated, control mice were reconstituted with bone marrow progenitors transferred from HG and control donor mice. After 7 wk, CD25^+ T cells were isolated from chimeric mice and stained with DAPI to assess nuclear diameter. In this experiment, a significantly higher proportion of T cells from mice reconstituted by HG donors exhibited chromatin decondensation (Fig. 6A, 6B). This indicated that bone marrow progenitors from HG donors retained the chromatin decondensation phenotype despite differentiation and maturation in a euglycemic environment. Adult bone marrow contains hematopoietic stem cells that can give rise to all blood cells types, Thy1^+ prothymocytes that are periodically mobilized from the bone marrow and imported to

![Figure 5](http://www.jimmunol.org/Downloadedfrom)

**Figure 5.** Chromatin decondensation in T cells from control and HG mice. T cells from control or HG mice were isolated by negative selection with magnetic beads. CD25^+ cells were depleted by magnetic beads and the remaining cells were DAPI stained. T cell purity was analyzed by flow cytometry staining, and >90% of the cells were CD4^+ or CD8^+ and CD25^- The diameter of the nucleus was measured in individual cells by fluorescence microscopy. (A) Representative images of T cells stained with DAPI from control and HG mice; scale bar, 10 µm. (B) Nucleus diameter measurements grouped by size in the indicated bins, expressed as the percentage of all T cells; mean ± SD (n = 5–6). (C) The percentage of T cells with nuclei > 6 µm in diameter (n = 5–6). (D) The percentage of T cells stimulated or not with 40 nM PMA or plate-bound anti-CD3e for 5 and 24 h, respectively, with nuclei > 6 µm in diameter (n = 4–6). (E) The correlation of Pearson between DAPI fluorescence intensity and nucleus diameter in T cells from a representative control mouse stimulated with anti-CD3e for 24 h. (F) The percentage of T cells with nuclei > 6 µm in diameter following treatment with 1 µM SB203580 (p38 inhibitor) or 1 µM U0126 (ERK inhibitor) for 5 h (n = 3–5). (G) The percentage of T cells with nuclei > 6 µm in diameter following stimulation with 40 nM PMA and treatment with 1 µM SB203580 or 1 µM U0126 for 5 h (n = 3–5). All experiments were repeated at least three times. Statistical differences were analyzed by Student t test. *p < 0.05, **p < 0.01, ***p < 0.001.
Chimeric mice reconstituted with whole bone marrow from control or HG donor mice (isolated from chimeric mice reconstituted with bone marrow depleted of Thy1.2+ cells prior to transfer. Depletion of Thy1.2+ donor cells abrogated the difference in mature T cell nuclear diameter between groups (Fig. 6C). If epigenetic reprogramming is responsible for the observed mature T cell phenotype, this must occur at a developmental stage later than common lymphoid progenitor cells. Because bone marrow containing Thy1.2+ cells reconstituted the mature T cell chromatin decondensation phenotype of donor HG mice, this could have reflected modification of prothymocytes or resident mature T cells. Regardless of their identity, these transferred cells retained an increased proportion with chromatin decondensation even in a euglycemic environment, along with the hyperproliferative phenotype demonstrated in the experiments shown in Fig. 2C.

**Chromatin decondensation in T cells from HG mice depends on RAGE expression**

Endogenous ligands for RAGE, including S100 proteins, HMGB1 and glycated proteins are elevated in hyperglycemic hosts and pathological RAGE signaling has been implicated in diabetic complications (26). To investigate the possible involvement of RAGE signaling in the T cell chromatin decondensation and activation phenotypes linked to chronic hyperglycemia, we prepared T cells from control RAGE−/− mice and HG RAGE−/− mice 12 wk after the induction of diabetes with STZ. The absence of RAGE expression abrogated in the effects of chronic hyperglycemia on chromatin decondensation in resting T cells (Fig. 7A) and reduced the ~2-fold increase in basal p38 MAPK phosphorylation that we previously identified in wild-type HG mice (Fig. 4A) to ~0.5-fold (Fig. 7B). RAGE deficiency did not inhibit the increased phosphorylation of p38 or ERK stimulated by PMA treatment of RAGE−/− control or HG T cells (Fig. 7B) nor did it influence Akt phosphorylation (Supplemental Fig. 4A). To further confirm that chromatin decondensation is dependent on RAGE, we incubated CD4+ T cells with high-glucose medium (30 mM D-glucose) or medium containing 100 ng/ml S100A1 for 5 h and analyzed the nucleus diameter (Supplemental Fig. 4B). Only cells treated with S100A1 had a significant higher proportion of cells with nuclear diameter > 6 μm. Cytokine production was analyzed in plate-bound anti-CD3ε stimulated splenocytes from RAGE control and HG mice by ELISA as an evaluation of the final result of the signaling (Fig. 7C). As expected, no differences between control and HG RAGE−/− mice were observed. These data implicate the RAGE pathway in the basal increased p38 MAPK signaling, chromatin decondensation, and cytokine production that occurs in chronically hyperglycemic hosts.

**Discussion**

We previously reported that mice with chronic but not acute STZ-induced diabetes have increased susceptibility to *M. tuberculosis* characterized by higher plateau lung bacterial load and increased immune pathology (4). This phenotype mimics at least some manifestations of TB disease in diabetic people (27). Susceptibility in mice is at least partly attributable to delayed recruitment of naive myeloid cells into alveoli harboring resident macrophages initially infected by inhaled *M. tuberculosis* (8). This defect of innate immunity results in delayed initiation of the adaptive immune response that is required to restrict bacterial replication. The subsequently increased inflammatory response in the lung might simply result from a higher load of pathogen-associated molecular patterns and Ag, but a diabetes-associated impairment of immune regulation also could be a contributing factor. Diabetes is associated with chronic inflammation and its delayed resolution in noninfectious inflammatory conditions (28, 29). Patients with metabolic syndrome, with a major risk factor for type 2 diabetes, have increased serum concentrations of both Th1 and Th2 cytokines that correlate positively with fasting glucose levels (30).

We studied naive T cells isolated from control and HG mice to investigate whether diabetes causes an intrinsic perturbation of T cell activation in the absence of infection. Our data show that T cells from mice with chronic (≥12 wk) but not acute (≤4 wk) hyperglycemia express a broad range of cytokines at higher levels and proliferate more than T cells from control mice after TCR stimulation in vitro and in vivo. In this respect, naive T cells from HG mice behave like Ag-experienced T cells despite no increase of CD44 expression. Developing T cells normally undergo nuclear condensation in the transition from double to single positive in the thymus. Compact nuclear chromatin acts as a barrier to inadvertent T cell activation by limiting access of transcription factors to binding sites on DNA. Priming of naive T cells by dendritic cells that present high-affinity Ag to the TCR in the context of

**FIGURE 6.** Chromatin decondensation in T cells from lethally irradiated control mice reconstituted with bone marrow from control or HG mice. Seven weeks after bone marrow transfer, T cells were isolated from recipients by negative selection using magnetic beads. CD25+ cells were depleted by positive selection, and the remaining T cells were DAPI stained for measurement of nuclear diameter. (A) Nucleus diameter measurements grouped by size in the indicated bins, expressed as the percentage of all T cells; mean ± SD (n = 4). (B) Percentage of T cells with nuclei > 6 μm in diameter, isolated from chimeric mice reconstituted with whole bone marrow from control or HG donor mice (n = 4). (C) Percentage of T cells with nuclei > 6 μm in diameter, isolated from chimeric mice reconstituted with bone marrow depleted of Thy1.2+ cells prior to transfer (n = 4). All experiments were repeated at least twice. Statistical differences were analyzed by Student t test. *p < 0.05, **p < 0.01, ***p < 0.001.
costimulatory molecules overcomes this barrier by triggering chromatin decondensation. This allows transcription factors such as Stat5 to bind promoters involved in T cell proliferation and cytokine expression (16). Our data show that naive T cells in diabetic hosts are preactivated via RAGE signaling and have an exaggerated response to initial TCR stimulation. This dysregulated response might contribute to excessive immune pathology in the context of chronic infections like TB.

Several biochemical mechanisms for diabetic complications have been identified including increased polyol pathway flux, increased hexosamine pathway flux, increased protein glycation that alters function and produces ligands for RAGE, and PKC activation resulting from increased diacylglycerol synthesis and increased RAGE signaling (2). All these pathways lie downstream of a common initiating factor: hyperglycemia-driven mitochondrial overproduction of superoxide. We found that RAGE deficiency reversed the preactivated phenotype of T cells in HG mice, linking that phenotype to an established diabetic complication pathway. Hyperglycemia promotes increased expression of RAGE and increased levels of its endogenous ligands S100 calgranulins and HMGB1 that bind with higher affinity than advanced glycation end products and are likely the most physiologically relevant ligands even in diabetic hosts (9, 10). We confirmed in vitro that S100A1, and not high glucose medium, was enough to increase the percentage of T cells with the nucleus decondensed.

Ligand binding to RAGE activates several signaling pathways including NF-κB, focal adhesion kinase, PI3/Akt, Rho GTPase, Jak/STAT, Src kinases, and MAPK (31–35). Histone actetyltransferases such as p300 are activated through some of these pathways, including p38 MAPK, and in turn are linked to chromatin decondensation (36–40). We found that treating unstimulated T cells from HG mice with SB203580 (p38 inhibitor) reduced the proportion of cells with decondensed chromatin to approximately the level present in control mice, indicating a role for p38 MAPK in this process. Nuclear size in T cells from control mice was not further reduced by p38 inhibition. It appears that p38 was involved in the basal chromatin decondensation of HG T cells, but our results from adoptive transfer and radiation chi-
mice and euglycemic hosts suggest that the increased basal p38 activation in HG T cells does not depend on continuous RAGE stimulation but instead reflects a persistent alteration of cell function suggestive of epigenetic remodeling. Increased nuclear decondensation was only observed in CD4+ T cells from HG mice, and this correlated with increased proliferation. This specific pre-activation of CD4+ T cells by hyperglycemia might be explained by a different role of p38 in T cell subsets; proliferation in CD4+ T cells versus apoptosis in CD8+ T cells (41).

A growing body of evidence indicates that epigenetics is an important mechanism of diabetic complications and metabolic memory. This latter concept refers to the progression of diabetic complications at the tissue level despite improved glycemic control. It is a well recognized phenomenon in human diabetes and in animal models (22). The persistence of chromatin decondensation in unstimulated T cells derived from HG donor mice for up to 7 wk after transfer into euglycemic hosts is consistent with an epigenetic effect. Our data suggest that this occurs at a stage of T cell development later than the common lymphoid progenitor.

Our data indicate that prolonged hyperglycemia expanded a population of T cells from control or HG mice (Supplemental Fig. 2F). Because the regulatory T cell population was not defective in HG mice we questioned whether the augmented response of T cells from HG mice reflected an increase of memory T cells (CD44+ CD25−) in the diabetic host environment but found no evidence for this. The increased proportion of T cells with decondensed chromatin in HG mice was not matched by any change in the proportion of memory T cells compared with controls. This suggests that distinct pathways downstream of TCR regulate CD44 expression and chromatin decondensation. That conclusion is further supported by our finding that the marked increase of CD44 expression in cells stimulated by plate-bound anti-CD3e was not modulated by the p38 inhibitor SB203580 which was found to reverse chromatin decondensation in T cells from HG mice. We also found no evidence that diabetes repressed CD44 expression that was equally induced by anti-CD3e stimulation of T cells from control or HG mice (Supplemental Fig. 2F).

Data suggest that prolonged hyperglycemia expands a population of preactivated T cells independent of TCR signaling or CD44, which function like Ag-experienced cells. In summary, we found that naive T cells from chronically HG mice mount a stronger response to TCR stimulation than T cells from control mice. In this way, these naive cells act like Ag-experienced T cells yet they lack the increased expression of CD44 that characterizes memory T cell generated after normal immune priming. The breadth of cytokines expressed, spanning Th1, Th2, and Th17, suggests that hyperglycemia exerts a global effect on T cells rather than one limited to an isolated signaling pathway. We propose that chromatin decondensation, which is increased in T cells from HG mice, enables this widespread activation response by facilitating transcription factors access to their cognate binding sites in promoters of genes linked to cytokine expression and proliferation. Increased chromatin decondensation in HG mice depends on RAGE expression and is maintained by increased basal activation of p38 MAPK. The persistence of this phenotype for weeks after T cell transfer from a diabetic to a euglycemic host suggests an effect of epigenetic remodeling linked to RAGE signaling. This model is consistent with evidence that RAGE expression is increased in T cells from people with diabetes and that diabetes promotes epigenetic remodeling in lymphocytes (20, 22, 26, 43). An expanded population of preactivated T cells in diabetic hosts may be a contributing factor to the increased immune pathology characteristic of TB and other infectious and noninfectious inflammatory conditions linked to diabetes. Our data support a model for complications driven by hyperglycemia, which is common to type 1 and type 2 diabetes (2). Our study did not address glucose-independent complication mechanisms that might be unique to type 2 diabetes and insulin resistance (44). However, hyperglycemia and hyperlipidemia are both associated with oxidative stress which may be a proximal common factor driving complications (44). A more complete understanding of T cell preactivation as a novel complication of hyperglycemia may reveal targets for host-directed therapies to limit morbidity and mortality from infectious and noninfectious inflammatory disorders in people with diabetes.

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References


