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MURINE MODELS OF LUPUS INDUCED BY HYPOMETHYLATED T CELLS

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Abstract

CD4+ T cell DNA hypomethylation may contribute to the development of drug induced and idiopathic human lupus. Inhibiting DNA methylation in mature CD4+ T cells causes MHC-specific autoreactivity in vitro. The lupus-inducing drugs hydralazine and procainamide also inhibit T cell DNA methylation and induce autoreactivity, and T cells from patients with active lupus have hypomethylated DNA and a similarly autoreactive T cell subset. Further, T cells treated with DNA methylation inhibitors demethylate the same sequences that demethylate in T cells from patients with active lupus. The pathologic significance of the autoreactivity induced by inhibiting T cell DNA methylation has been tested by treating murine T cells in vitro with drugs which modify DNA methylation, then injecting the cells into syngeneic female mice. Mice receiving CD4+ T cells demethylated by a variety of agents including procainamide and hydralazine develop a lupus-like disease. Further, transgenic mice with an inducible T cell DNA methylation defect also develop lupus-like autoimmunity. This chapter describes the protocols for inducing autoreactivity in murine T cells in vitro, and inducing autoimmunity in vivo using an adoptive transfer approach or transgenic animal models.

Keywords

Lupus; drug-induced lupus; DNA methylation; animal models; autoimmunity

1. Introduction

1.1. Background

DNA methylation is an essential determinant of chromatin structure and gene expression. DNA methylation refers to the post-synthetic methylation of deoxycytosine (dC) bases at the

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5 position to form deoxymethylcytosine (d^mC). Nearly all d^mC is found in CpG dinucleotides, although only 70–80% of the CpG pairs are methylated. In general, the methylation of CG pairs in regulatory sequences results in transcriptional suppression, while demethylation permits active transcription. Methylation patterns are established during differentiation, and serve to suppress expression of genes not necessary for the function of fully differentiated cells. The enzymes responsible for establishing methylation patterns include Dnmt3a and Dnmt3b, referred to as de novo methyltransferases. The methylation patterns are then maintained during mitosis by maintenance methyltransferases including Dnmt1 (1).

The importance of DNA methylation is evidenced by its role in differentiation, genomic imprinting, and X chromosome inactivation (1). Disrupting both *DNMT1* alleles in embryonal stem cells results in embryonic death, indicating that DNA methylation is important in ontogeny (2). Dnmt3a and 3b are also essential for mammalian development: homozygous Dnmt3a deficiency causes runting and death at 4 weeks of age, while Dnmt3b deficiency is embryonic lethal (3). Inhibiting DNA methylation in differentiated cells can have profound effects on cells. For example, treating the mouse fibroblast cell line 10T1/2 with the irreversible DNA methyltransferase inhibitor 5-azacytidine (5-azaC) causes the cells to differentiate into myocytes, adipocytes and chondrocytes (4).

1.2. DNA methylation and T cell function

DNA methylation is also important in regulating T lymphocyte gene expression. Methylation patterns change during thymic maturation (5), similar to the changes that occur during differentiation of other cell types. DNA methylation is implicated in the differentiation of Th0 cells into Th1 and Th2 phenotypes as well: the interferon-γ (IFN-γ) gene is methylated in non-expressing Th2 cells, but demethylated in Th1 cells, while the IL-4 gene methylated in Th1 but not Th2 cells (6, 7). Demethylation of the *Foxp3* locus is also important in the differentiation and function of regulatory T cells (8). 5-azaC can also modify T cell gene expression. Examples include effects on IFN-γ and perforin expression in CD4+ T cells (9, 10).

Demethylating T cell DNA with 5-azaC can change T cell reactivity and function. Treating CD4+ T cell clones, as well as polyclonal CD4+ T cells, with DNA methylation inhibitors causes autoreactivity. The treated cells lose restriction for nominal antigen, and respond to self class II MHC molecules without added antigen (11, 12). The autoreactivity is due at least in part to overexpression of the adhesion molecule LFA-1 (CD11a/CD18), and causing LFA-1 overexpression by transfection induces a similar MHC-restricted autoreactivity (13–15). The autoreactivity may reflect overstabilization of the normally low affinity interaction between the TCR and class II MHC molecules presenting inappropriate antigen (16). 5-azaC increases steady state levels of CD11a but not CD18 mRNA, and the increase in CD11a mRNA appears to be due to demethylation of repetitive elements 5′ to the CD11a promoter (14, 17). In contrast, CD8+ T cells do not become autoreactive following 5-azaC treatment (12), and the reason is unexplored.

1.3. T cell DNA hypomethylation and autoimmunity

The pathologic significance of 5-azaC induced autoreactivity has been tested in animal models. The approach is to treat stimulated CD4+ T cells with 5-azaC in vitro, culture for at least 1–2 cell cycles, then inject the treated cells into syngeneic recipients. 5-azaC and other DNA methylation inhibitors prevent the methylation of newly synthesized DNA during S phase, referred to as passive demethylation. Thus, these agents are only effective when added to dividing cells. Further, 1–2 rounds of cell division are often required before changes in gene expression are observed (18). Adoptive transfer of murine CD4+ T cells, made autoreactive either by treatment with 5-azaC or by transfection with CD18, causes a lupus-like disease in syngeneic recipients (15). The disease induced closely resembles chronic graft-vs-host disease in mice, in which features of lupus-like autoimmunity are also induced by CD4+ T cells responding to host class II MHC molecules (19).

The DNA hypomethylation model has been used successfully with polyclonal CD4+ T cells in DBA/2 mice (20), cloned Th2 cells in AKR mice (21), and cloned Th1 cells in B10.A mice (22). We have also used a panel of DNA methylation inhibitors, including 5-azaC, procainamide, hydralazine, and the ERK pathway inhibitor U0126 to induce autoimmunity (23, 24). 5-azaC and procainamide are DNA methyltransferase inhibitors (18, 25), while hydralazine and the ERK pathway inhibitors prevent the upregulation of Dnmt1 and Dnmt3a during T cell stimulation (24). All the DNA hypomethylation models develop anti-DNA antibodies but vary to some degree with respect to the histologic changes induced, due either to the different repertoire of effector functions displayed by the treated cells, or to host specific genetic influences. The mechanism common to all models is promiscuous killing of host macrophages (Mø). This may contribute to the development of anti-DNA antibodies by increasing the total amount of potentially antigenic apoptotic material (26), and/or by removing the cells responsible for removing apoptotic debris, analogous to “knockout” mice with defective clearance of apoptotic material, which develop anti-DNA antibodies (27).

Next, we designed experiments to provide further evidence that the T cell ERK signaling defect and DNA hypomethylation described in lupus patients plays a pathogenic role rather than merely being a result of the disease process. We generated transgenic mice that express a dominant negative MEK (dnMEK) under the control of a promoter sequence that contains multiple tetracycline-responsive elements. By breeding these mice onto another transgenic mouse strain that expresses a reverse tetracycline transactivator (rtTA) under the control of a CD2 promoter, we can induce T cell-specific expression of the dominant negative MEK by adding doxycycline to the drinking water of the double transgenic mice (B6.dnMEK/CD2-rtTA). This allowed us to study the effects of an induced T cell ERK signaling defect, caused by over-expressing the dominant negative MEK, in these mice (28).

T cell specific expression of the dominant negative MEK and the resulting reduction of phosphorylated ERK only in the presence of doxycycline were confirmed. As expected, inducing a T cell ERK signaling defect resulted in reduced expression of Dnmt1, the main maintenance DNA methylation enzyme that is known to be regulated by ERK signaling. The expression of the methylation sensitive genes *Itgal* (CD11a) and *Tnfsf7* (CD70) were increased in the spleen, similar to that observed in T cells from lupus patients. Further,

B6.dnMEK/CD2-rtTA mice given doxycycline developed anti-dsDNA antibodies, and gene expression profiling in the spleen revealed an interferon-signature consistent with that described in PBMCs from lupus patients (28). No clinical phenotype was observed in the B6 autoimmune-resistant genetic background. This emphasizes the importance of genetic susceptibility in the pathogenesis of lupus and indicates that DNA demethylation is not sufficient to induce clinical autoimmunity in the absence of a genetic susceptibility background. Indeed, this is similar to what we observe in patients who develop drug-induced lupus as a result of treatment with procainamide or hydralazine (both are DNA methylation inhibitors). The majority of patients treated with these medications develop autoantibodies, but only a small fraction develops clinical drug-induced lupus. Those who go on to develop clinical disease are arguably those who also carry appropriate genetic susceptibility loci.

We therefore studied the effect of the dnMEK/CD2-rtTA on mice with a more autoimmune susceptible genetic background by crossing B6.dnMEK/CD2-rtTA with SJL mice and examining the development of autoimmunity in B6.SJLdnMEK/CD2rtTA F1 mice (29). Female but not male mice developed glomerular IgG deposition suggestive of immune-complex glomerulonephritis. Platelet thrombi were also observed in the small vessels of the lungs of female but not male mice. To determine if the development of autoimmunity in female B6.SJLdnMEK/CD2rtTA F1 mice is due to a gene dosage effect from the X chromosome (presumably due to demethylation and reactivation of the inactive X chromosome) or due to hormonal factors, both male and female B6.SJLdnMEK/CD2rtTA F1 mice were neutered and implanted with time-release pellets delivering placebo or a supra-estrus dose of estrogen. Doxycycline induced anti-dsDNA antibodies in intact and neutered, placebo-treated female transgenic mice. No anti-DNA antibodies were fournd in doxycycline-treated intact or neutered, placebo-treated male transgenic mice. Doxycycline induced even greater amounts of anti-dsDNA antibodies in neutered females given high dose estrogen but none were detected in neutered males given high dose estrogen. No anti-DNA antibodies developed in the absence of doxycycline treatment or without the transgenes. These experiments confirm the X-chromosome gene-dose effect in lupus as an important factor for the female predominance of the disease. Indeed, doxycycline induced overexpression of the X-linked methylation-sensitive gene *Cd40lg* on T cells in female but not male mice, consistent with demethylation and reactivation of the second X chromosome in the females.

1.4. Relevance to human lupus

At least 6 lines of evidence support the contention that the DNA hypomethylation model has relevance to human lupus. First, the two drugs that most clearly cause a lupus-like disease in people, procainamide and hydralazine, are T cell DNA methylation inhibitors (30). Cloned murine Th2 cells treated with these drugs induce a lupus-like disease identical to that caused by 5-azaC (23), suggesting a mechanism by which they might cause lupus in humans. Second, T cells from patients with active lupus have decreased levels of total genomic d^mC, similar to 5-azaC treated cells (31). Third, T cells from patients with active lupus overexpress LFA-1 on an autoreactive T cell subset (14), and the overexpression is associated with hypomethylation of the same sequences flanking the CD11a promoter that demethylate following 5-azaC treatment (17) Fourth, T cells from patients with active lupus

have a selective defect in ERK pathway signaling, the pathway inhibited by hydralazine (32), and inhibiting this pathway with hydralazine or U0126 causes a lupus-like disease in the adoptive transfer model and in the dnMEK/CD2-rtTA mouse model (24–28). Fifth, LFA-1 overexpressing T cells isolated from patients with active lupus spontaneously kill autologous monocytes with a specificity identical to experimentally hypomethylated T cells (14), and by the same mechanisms (FasL, TRAIL and TWEAK) as experimentally hypomethylated T cells (33, 34). Finally, the X-linked methylation sensitive gene *CD40LG* is overexpressed in CD4+ T cells from female but not male lupus patients (35). The overexpression of CD40LG in female patients with active lupus is associated with hypomethylation of the *CD40LG* promoter sequence in both alleles, allowing for transcriptional activity of the *CD40LG* gene from the normally heavily methylated and inactive X chromosome (35). These findings are consistent with a gene-dose effect on the X-chromosome as demonstrated by the dnMEK/CD2-rtTA mice, experiments and suggest that chromosomal sex is perhaps more important than hormonal sex in explaining the female predominance of lupus. Together, these studies strongly suggest that similar mechanisms contribute to the development of autoimmunity in the DNA hypomethylation models and in drug induced and idiopathic human lupus.

2. Materials

2.1. Mice

Young (6–8 week) female AKR and B10.A mice are obtained from Jackson Labs, and DBA/2 mice from Charles River. The dnMEK transgenic mice were generated by subcloning a dominant negative MEK1 into the pTRE2 construct (Clontech), containing multiple tetracycline response elements and a minimal CMV promoter. These mice were then bred with CD2-rtTA mice, obtained from Dr. Rose Zamoyska, to produce dnMEK/CD2-rtTA double transgenic mice. Doxycycline, added to the drinking water at a concentration of 2 mg/ml, was used to induce expression of the dominant negative MEK in vivo.

2.2. Cell lines

D10.G4.1 (D10) cells are obtained from the American Type Culture Collection (ATCC). AE7 cells were obtained from Dr. Ronald Schwartz.

2.3. 5-azacytidine

5-azacytidine (Aldrich) is dissolved in tissue culture media, typically at 0.25–8.0 μM, and is made fresh just before use (see notes). The solution is filter sterilized before adding to culture. 5-aza-2′-deoxycytidine may also be used, and is more potent (see notes). 5-azacytidine is potentially mutagenic, and should be handled with appropriate precautions.

2.4. IL-2

The IL-2 secreting T cell line MLA-144, obtained from the ATCC, was cultured in RPMI 1640 supplemented with 3% fetal calf serum (FCS). Three times a week the cells were sedimented by centrifugation, the conditioned media filtered to remove any remaining cells, then stored frozen at −20° C.

2.5. Media

2.5.1. Polyclonal T cells—RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 40% IL-2 containing conditioned media, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-5} M 2-mercaptoethanol.

2.5.2. D10 cells—Click's medium supplemented with 10% FCS, 40% IL-2 containing conditioned media, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-5} M 2-mercaptoethanol.

2.5.3. AE7 cells—50% Click's medium/50% RPMI 1640 supplemented with 10% FCS, 40% IL-2 containing conditioned media, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-5} M 2-mercaptoethanol.

3. Methods

3.1. Cells and 5-azacytidine treatment

3.1.1 Polyclonal CD4+ T cell lines—Spleens are removed from young (6–8 week old) female DBA/2 (H-2^d) mice and dissociated with forceps followed by forcing through a sterile disposable plastic screen with a syringe piston. Splenocytes are then isolated by density gradient centrifugation through Lympholyte-M (Cedarlane). CD8+ cells are depleted with magnetic beads (Miltenyi) according to the manufacturer's instructions, then 10^6 CD4+ cells are cultured in 2 ml of IL-2 containing media (see Materials) and stimulated with either 10^6 irradiated (3000R) allogeneic splenocytes (e.g. C57BL/6, H-2^b) or 5 μg/ml Concanavalin A (Pharmacia) using flat bottom 24 well plates. The cultures are maintained at 37°C in 5% CO2 and humidified atmosphere, and rocked on a rocker platform (Bellco) at 5–6 cycles/minute (see notes). The lines are maintained by the addition of fresh IL-2 containing media every 2–3 days, and restimulating every 7–10 days, using ~10^6 cells/well and equal numbers of irradiated allogeneic splenocytes or $0.5–1.0 \times 10^6$ irradiated syngeneic splenocytes + 1 μg/ml Concanavalin A, as appropriate. One day after restimulation the cells are treated with 5-azaC and 6 days later the cells tested for autoreactivity and/or used for adoptive transfer. The cells should also be tested for CD4 and CD8 expression by flow cytometry at this point to exclude overgrowth by CD8+ cells.

3.1.2. D10 cells—D10.G4.1 (D10) cells (American Type Culture Collection) are maintained in IL-2 containing media (see Materials) using flat bottomed 24 well plates and a rocker platform as for polyclonal cells. The line is maintained by challenging $0.1–1.0 \times 10^6$ D10 cells with 5×10^5 irradiated (3000R) AKR splenocytes and 100 μg/ml conalbumin (Sigma) every 7–10 days. The D10 line contains an autoreactive subset (see notes), and must be subcloned by limiting dilution and a non-autoreactive subclone selected prior to use. Cells are treated with 5-azaC and used for functional characterization or given in adoptive transfer at least 6 days after treatment.

3.1.3. AE7 cells—AE7 cells are maintained in IL-2 containing media (see Materials) and stimulated weekly with irradiated syngeneic (B10.A) splenocytes and antigen (100 μg/ml pigeon cytochrome C) on a rocker platform as described for polyclonal CD4+ cells and D10

cells. To induce autoreactivity the cells are treated with 5-aza-2′-deoxycytidine (see notes) and used 6 days later.

3.1.4. Variations—See notes.

3.2. Autoreactivity assays

3.2.1. Proliferation assays—For D10 cells, 2×10^4 treated or untreated cells are cultured with graded numbers ($2–10 \times 10^4$) of irradiated syngeneic (AKR) splenocytes in 200 μl of the same media but without IL-2, with or without 100 μg/ml conalbumin, using round bottom 96 well plates. Proliferation is tested 4–5 days later by adding 1 μCi tritiated thymidine/well and 6 hours later determining ^3H incorporation into DNA. Polyclonal CD4+ T cells are similarly tested, using 5×10^4 T cells and ~10^5 irradiated syngeneic splenocytes/well (range 5×10^4–5×10^5), using allogeneic splenocytes or Concanavalin A as appropriate for the positive control. In all cases determinations are performed in triplicate or quadruplicate.

3.2.2. Cytotoxicity assays—For D10 cells, thioglycollate elicited syngeneic (AKR) Mø are labeled with 100 μCi ^{51}Cr in 1 ml of RPMI/10% FCS for 1 hour 37°C in a round bottom culture tube. The cells are washed, then 5000 labeled Mø cultured with 125,000 D10 cells with or without 100 μg/ml conalbumin in a total volume of 200 μl of sterile media lacking IL-2, using round bottom microtiter plates. 18 hours later chromium release is measured using a scintillation spectrometer (33). AE7 killing assays are performed similarly, except that an effector:target ratio of 10:1 is used, and the antigen (positive control) is 100 μg/ml pigeon cytochrome C. Splenocyte killing assays are similarly performed, using an effector:target ratio of 25:1. Percent cytotoxicity is calculated as [(experimental − background release)/(total incorporation − background release)] × 100 (20).

3.3. Adoptive transfer of autoreactive cells

All adoptive transfer models are performed similarly. The treated cells are washed, dead cells removed by centrifugation through Lympholyte M, then 5×10^6 viable cells are suspended in 0.2 ml sterile PBS and injected into the tail vein of young (< 12 weeks) syngeneic female mice, using a 26 gauge needle. A total of 6 injections are performed, spaced 2 weeks apart. The rationale for repeated adoptive transfers derives from the observation that 5-azaC induced autoreactivity is self-limited (12). 4 weeks after the last injection the mice are euthanized and studied for the development of serologic and histologic evidence of autoimmunity. The development of proteinuria and hematuria may be monitored by holding Chemstrips (Boehringer Mannheim) under the mouse while picking it up (see notes).

3.4. IgG, IgM, and anti-DNA antibody assays

Total serum IgG and IgM concentrations are measured using Immulon 4 plates (Dynatech Laboratories) coated with 2.5 μg anti-mouse IgG or IgM (Sigma) in 100 μl 0.01M PBS, pH 7.4 for 18 hours at 4°C. The plates are washed x 3 with PBS containing 0.05% Tween 20, then 200 μl of PBS supplemented with 3% BSA, 0.1% gelatin and 0.05% Tween 20 are added and incubated 2 hours 23°C. Serum samples or purified standards (murine IgG or

IgM, from Sigma) are diluted to the desired concentrations in PBS containing 3% BSA and 0.1% gelatin, added to the wells and incubated 18 hours at 4°C, then washed x 3. 100 µl horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (heavy and light chains) or IgM (µ chain specific) are added at a final dilution of 1:2500 in PBS containing 0.05% Tween 20 and incubated for 2 hours at room temperature. The plates are developed using Sigma Fast tablets (Sigma) according to the manufacturer's instructions.

Anti-ssDNA and anti-dsDNA titers are determined by coating Immulon 4 plates with 2.5 µg purified ssDNA (Sigma) or dsDNA (cesium chloride-purified KS^{+}-SV2CAT plasmid) in 100 µl 0.01M PBS, pH 7.4 for 18 hours at 4°C. The protocols used are the same as for the immunoglobulin ELISAs described above, except for coating the wells. HRP-conjugated goat anti-mouse polyvalent (IgG, IgM, IgA) antibody (Sigma) is again used as the secondary antibody before developing with Sigma Fast tablets. Positive controls should include pooled serum from ≥ 6 month old female NZB/W mice or ≥ 5 month old MRL/lpr mice.

For the experiments performed in the dnMEK/CD2-rtTA mice, anti-dsDNA antibody was detected using an ELISA kit (Alpha Diagnostic Intl. Inc.).

4. Notes

4.1. Cells and 5-azacytidine treatment

4.1.1. 5-azacytidine—5-azacytidine and 5-aza-2′-deoxycytidine are unstable in aqueous media (18), and must be prepared just before use. The purchased chemicals have some variability in potency, and each lot should be tested. Typical concentrations for 5-azacytidine are 0.25–8.0 µM, with 1 µM most often being effective. 5-aza-2′-deoxycytidine is more specific for DNA methylation inhibition and is also more potent (18), so lower concentrations may be used. Both compounds inhibit both DNA methylation and DNA synthesis, and concentrations inhibiting DNA methylation are only slightly lower than concentrations inhibiting DNA synthesis (18). Further, treated cells must undergo 1–2 cycles of cell division for the changes in gene expression to occur (18), highlighting the importance of establishing optimal concentrations. Significant cell death also occurs during treatment. Changes in T cell gene expression are typically seen 3–6 days after treatment, and kinetic analysis should be performed in order to determine the optimal time.

4.1.2. Polyclonal cell lines—Cell-cell contact is maximized in flat bottom culture plates by using a rocker platform, and the cultures should be rocked for at least 24 hours following stimulation.

4.1.3. D10 cells—With prolonged culture, D10 cells tend to lose the restriction for antigen and proliferate to syngeneic APC without added antigen. The mechanism is unknown, but adoptive transfer of these cells does not induce autoimmunity (unpublished observations). The cells also tend to lose the requirement for IL-2 for sustained growth over time, causing high backgrounds in proliferation assays. Consequently, it is necessary to repeatedly subclone this line, and select antigen-specific cells. Alternatively, multiple aliquots of quality tested subcloned cells may be stored in liquid nitrogen, and thawed as needed.

4.1.4. AE7 cells—AE7 cells are more refractory to the induction of autoreactivity than normal T cells or D10 cells. Treatment with 5-aza-2′-deoxycytidine is required, and concentrations up to 8 μM are sometimes needed (22). Also, in our hands, proliferation assays are less reliable than cytotoxicity assays for both antigen reactivity and autoreactivity.

4.1.5. Variations—Activated T cells can be modified with other DNA methylation inhibitors, or by transfection as needed. Our group has found D10 cells to be the best suited for these studies, and have successfully compared procainamide with N-acetylprocainamide, and hydralazine with phthalazine in this model (23). Similarly, we have used the ERK pathway inhibitor U0126 (24), and used D10 cells transfected with CD18 (15). Other modifications of the cells may be similarly tested.

4.2 Autoreactivity assays

If desired, MHC specificity of the autoreactivity assays may be tested using monoclonal antibodies to the relevant class II MHC molecules, or congenic mouse strains.

4.3. Adoptive transfers for autoimmunity

Following injection of 5-azaC treated polyclonal CD4+ T cells from DBA/2 mice, hematuria is first seen between weeks 1–3, and usually lasts 7–14 days then resolves. Proteinuria, defined as > 30 mg/dl, was more persistent. Immunofluorescent evidence of renal Ig deposition correlates with active hematuria, and resolves at later time points.

4.4. Autoantibody assays

Sometimes the control sera give a relatively high background in the anti-DNA ELISAs. The specificity of the ELISAs may be tested by adding 5 μg/ml of soluble ss-DNA or ds-DNA as appropriate to replicate wells. Lack of inhibition is indicative of nonspecificity, while inhibition is indicative of autoantibodies.

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