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Coordinated Histone H3 Methylation and Acetylation Regulate Physiologic and Pathologic Fas Ligand Gene Expression in Human CD4+ T Cells

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Activation-induced Fas ligand (FasL) mRNA expression in CD4+ T cells is mainly controlled at transcriptional initiation. To elucidate the epigenetic mechanisms regulating physiologic and pathologic Fasl transcription, TCR stimulation–responsive promoter histone modifications in normal and alcohol-exposed primary human CD4+ T cells were examined. TCR stimulation of normal and alcohol-exposed cells led to discernible changes in promoter histone H3 lysine trimethylation, as documented by an increase in the levels of transcriptionally permissive histone 3 lysine 4 trimethylation and a concomitant decrease in the repressive histone 3 lysine 9 trimethylation. Moreover, acetylation of histone 3 lysine 9 (H3K9), a critical feature of the active promoter state that is opposed by histone 3 lysine 9 trimethylation, was significantly increased and was essentially mediated by the p300-histone acetyltransferase. Notably, the degree of these coordinated histone modifications and subsequent recruitment of transcription factors and RNA polymerase II were significantly enhanced in alcohol-exposed CD4+ T cells and were commensurate with the pathologic increase in the levels of FasL mRNA. The clinical relevance of these findings is further supported by CD4+ T cells obtained from individuals with a history of heavy alcohol consumption, which demonstrate significantly greater p300-dependent H3K9 acetylation and FasL expression. Overall, these data show that, in human CD4+ T cells, TCR stimulation induces a distinct promoter histone profile involving a coordinated cross-talk between histone 3 lysine 4 and H3K9 methylation and acetylation that dictates the transcriptional activation of FasL under physiologic, as well as pathologic, conditions of alcohol exposure. The Journal of Immunology, 2014, 193: 412–421.

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In this regard, heavy alcohol use is known to have deleterious effects, leading to immune system pathology, and is an important predisposing factor to opportunistic infections and certain types of cancer (7–10). Excess alcohol consumption causes a significant reduction in the numbers of CD4+ T lymphocytes, and the recovery of CD4+ T lymphocyte count following alcohol abstinence indicates that alcohol can directly affect CD4+ T lymphocyte survival (11–14). Notably, elevated serum levels of soluble FasL and Fas that correlate with the decline in CD4+ T cells are observed in individuals with a history of alcohol abuse (15). Our earlier work showed that in vitro exposure of human CD4+ T cells to physiologically relevant concentrations of ethanol increases their susceptibility to Fas- and activation-induced apoptotic death (16, 17). Hence, elucidating the mechanisms that govern FasL expression in normal, as well as in alcohol-exposed, CD4+ T cells is highly relevant for understanding the regulation of AICD and overall immune responses under both normal and pathologic conditions.

In resting primary CD4+ T cells, FasL mRNA expression is minimal to nonexistent; it is induced upon activation and is mainly controlled at transcriptional initiation (18–20). Studies examining the FasL promoter region identified several transcription factors that contribute to FasL gene transcription in CD4+ T cells (20–22). However, it is becoming increasingly clear that the access of transcription factors to the promoters of target genes is critically regulated by the state of the chromatin that plays a primary role in activation of transcription (23). Genomic DNA is packaged into chromatin, which is usually repressive for transcription and requires epigenetic modification to allow binding of specific transcription factors and regulators. Histones are the major structural proteins of chromatin; they undergo different types of
covalent modifications, primarily at their N-terminal tails, which can modify chromatin structure and influence transcriptional regulation (24–26). Among these modifications, a coordinated cross-talk between histone methylation and acetylation appears to be particularly important in regulating the interconversion between transcriptionally permissive and repressive chromatin states (27).

Earlier work, based on DNase hypersensitivity of the FasL 5′-regulatory region, indicated that chromatin remodeling is a primary event in the transcriptional activation of FasL gene expression (28). To further elucidate the epigenetic mechanisms underlying FasL promoter chromatin remodeling and transcriptional activation, we examined the TCR stimulation–responsive histone modifications in primary human CD4+ T lymphocytes under normal conditions, as well as under the pathologic conditions of alcohol exposure. Particularly, pathogenic epigenetic mechanisms mediated by alcohol exposure, leading to augmented FasL expression and AICD, were investigated in primary CD4+ T cells exposed to alcohol in vitro as well as in vivo (i.e., obtained from individuals with a history of heavy alcohol use). The findings from this study identify the coordinated cross-talk between FasL promoter histone H3 lysine 4 (H3K4) and histone H3 lysine 9 (H3K9) methylation and acetylation occurring in response to TCR stimulation. Further, the data also demonstrate the critical regulatory role of p300 histone acetyltransferase (HAT) in H3K9 acetylation (H3K9Ac) and recruitment of transcription factors and RNA polymerase II (RNA Pol II), which are required for FasL mRNA expression and AICD under both normal and pathologic conditions.

Materials and Methods

Human CD4+ T lymphocyte culture

CD4+ T lymphocytes from healthy volunteers were isolated, cultured, and treated as described previously (29). The purity of sorted populations was determined by flow cytometry and was always >90% (Supplemental Fig. 1).

Reagents and Abs

Cell culture reagents were obtained from Invitrogen (Carlsbad, CA). Ethyl alcohol, protease inhibitor mixture, and garcinol were purchased from Sigma Aldrich (St. Louis, MO). FasL, β-actin, NF-κB (p65), NFAT, Sp-1, and histone H3 Abs were obtained from Cell Signaling Technology (Beverly, MA). Anti-CD3, anti-CD28, and PE-labeled anti-CD4 Abs were obtained from BD Biosciences (San Jose, CA).

Alcoholic patient study

Five heavy drinkers of alcohol and five controls were recruited at the University of Louisville hospital. Heavy alcohol use was defined as at least one of the following criteria: 1) chart documentation of at-risk drinking, as per National Institutes of Health–National Institute of Alcohol Abuse and Alcoholism guidelines; ≥14 drinks/wk or ≥4 drinks/d for men and ≥7 drinks/wk or ≥3 drinks/d for women or 2) alcohol consumption ≥50 g alcohol/d for men and ≥30 g alcohol/d for women (30–32). Five healthy individuals who did not abuse alcohol were included as age/gender/ smoking status–matched controls. Blood samples were collected, for CD4+ T lymphocyte isolation, under a University of Louisville Institutional Review Board–approved protocol (IRB #188.04).

RNA isolation and real-time PCR analysis

Total RNA was isolated using TRIzol reagent (Invitrogen), and cDNA was made using Quanta qScript (Quanta BioSciences, Gaithersburg, MD). The real-time PCR was performed with Quanta Perfecta SYBR Green Fast Mix and an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA). The following primers were used: FasL: FP 5′-TTCACTGCCAAAAGTGTAGGG-3′ and RP 5′-TGGTGAGCAGCTAGGAGG-3′, and 18s RNA: FP 5′-CTCAACACCGGAAACTCCTAC-3′ and RP 5′-CGCTCCACACTAAGAAGC-3′. The gene expression was analyzed by relative quantification using the 2–ΔΔCT method by normalizing with 18s rRNA.

Chromatin immunoprecipitation assay

Histone modifications at the human FasL promoter region were detected using a chromatin immunoprecipitation (ChIP) assay kit (Millipore, Billerica, MA), as per the manufacturer’s protocol. ChIP Abs to detect anti-H3K9Ac, anti-trimethylated H3K3 (H3K9Me3), anti-trimethylated H3K4 (H3K4Me3), p300, NF-κB (p65), Sp-1, and RNA Pol II (Upstate Biotechnology, Lake Placid, NY) and NFAT (Santa Cruz Biotechnology, Dallas, TX) were used for immunoprecipitation along with a nonspecific control rabbit and mouse IgG Ab (Cell Signaling Technology). ChIP quantitative real-time PCR (qPCR) was performed as described earlier (33).

The following ChIP primers were used for analysis: region I of FasL promoter: FP 5′-TTCAGCTGCAAAAAAGTTGAGTGGG-3′ and RP 5′-GCTGACTGTCGAGA-3′; region II of FasL promoter: FP 5′-ACCTGTTGTTGAGTCACACGAC-3′ and RP 5′-TTGCACGTGAAGTCGAGAAG-3′; region III of FasL promoter: FP 5′-CTCCCCCTAGACCATTTCT-3′ and RP 5′-TAAAAATCCCCAAAATAAACCTCAAAC-3′; and region IV of FasL 3′ untranslated region (UTR): FP 5′-GGGGGACGTGTGCAATTTTA-3′ and RP 5′-TGGGAAGAAGGCTCAAAGG-3′.

Semiquantitative ChIP PCR was performed and analyzed by ethidium bromide–stained agarose gel electrophoresis using FasL promoter–specific primers (Supplemental Fig. 2).

p300 small interfering RNA transfection

Scrambled control and p300–specific small interfering RNA (siRNA) were purchased from Dharmaco (Lafayette, CO). Molt-4 cells (ATCC CRL-1582) were transfected by electroporation with 150 nM control siRNA or siRNA specific for p300 mRNA using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer’s protocol. The transfected T cells were subsequently cultured in RPMI 1640 supplemented with 10% FBS, but no antibiotics, for 48 h before use.

Western blot analysis

Total cellular and nuclear extracts were prepared, and proteins were analyzed by SDS-PAGE, as described previously (29), with β-actin as a loading control. Quantification was performed with Scion Image analysis software (Scion, Frederick, MD).

DNA fragmentation ELISA

DNA fragmentation was measured using a commercial ELISA kit (Cell Death Detection ELISA; Roche Applied Sciences, Indianapolis, IN), in accordance with the manufacturer’s instructions.

Statistical analyses

A repeated-measures mixed effects (RMME) model (34) was used to analyze the CD4+ T lymphocytes data collected from each experiment to evaluate the association between the experimental conditions (untreated, CD3/CD28 Ab treated, ethanol treated, and ethanol + CD3/CD28 Ab treated) and the FasL promoter–associated responses under investigation (H3K9Ac, NFAT recruitment, NF-κB recruitment, Sp-1 recruitment, RNA Pol II occupancy, p300 HAT, and FasL mRNA expression). The model had the following form:

\[ y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_{ij} + u_k + e_{ijk} \]

where \( I = 1,2 \) indicates the presence/absence of ethanol treatment, \( k = 1,2,\ldots,N \) indicates the subject (\( n = 3 \) or 4 in all cases). The responses \( y_{ijk} \) are normalized ΔCT values. The parameters \( \alpha_i, \beta_j, \gamma_{ij} \) represent main effects for ethanol, CD3/CD28 Abs, and interaction between the two factors, respectively, with \( \alpha_i = \beta_j = 1, \gamma_{ij} = 1, \gamma_{ij} = 2, \gamma_{ij} = 0 \) for identifiability purposes. The \( u_k \) are random effects representing the subject-specific variability, whereas the \( e_{ijk} \) are random residual errors. In addition to testing for main effects and interaction between the two experimental factors, we tested whether the CD3/CD28 Ab– and ethanol-treated cells differed from the untreated cells, as well as whether the combined ethanol + CD3/CD28 Ab–treated cells differed from the cells treated solely with CD3/CD28 Ab or ethanol. For the garcinol-inhibitor experiments, the model was simplified to:

\[ y_{ijk} = \mu + \alpha_i + u_k + e_{ijk} \]

where the \( \alpha_i \) represents different treatment groups with \( \alpha_i = 0 \) and \( u_k \) and \( e_{ijk} \) as defined above. Interest was in comparing the anti-CD3/CD28 Ab–stimulated cells (both untreated and ethanol treated) under the same conditions with conditions that were subsequently inhibited by garcinol. Lastly, to evaluate the association between histone H3 acetylation and p300 binding, we calculated an adjusted Pearson correlation coefficient between the two sets of measurements and a partial correlation coefficient, which accounts for the experimental design (treating the subject as a fixed
effect in this case). The latter measures the residual correlation between the two measurements, after taking into account any correlation due to the shared design structure. To account for multiple comparisons between experimental groups, p values were adjusted using the Bonferroni correction. All statistical analyses were conducted using R version 2.15.0 or greater (http://www.r-project.org).

Results
This study examines promoter-associated epigenetic histone modifications involved in the transcriptional activation of FasL gene upon TCR stimulation under normal, as well as pathologic conditions of, ethanol exposure. TCR stimulation–induced promoter histone modifications under normal conditions were examined in CD4⁺ T cells obtained from healthy individuals with no history of alcohol abuse. In comparison, CD4⁺ T cells subjected to ex vivo alcohol exposure, as well as CD4⁺ T cells obtained from individuals with a history of heavy alcohol consumption, were examined for pathologic histone modifications. The purity of isolated CD4⁺ T cells used in the study was always ≥90% (Supplemental Fig. 1). Promoter histone modifications were examined by ChIP qPCR analysis (33), using PCR primer sets representing three specific regions in the proximal FasL promoter that were demonstrated to bind relevant transcription factors and regulate FasL gene expression in T lymphocytes (Fig. 1) (20–22). In region I we interrogated histone modifications in the area that overlaps the transcription start site (TSS); in regions II and III we examined the histone H3 status located at −200 and −400 bp upstream of the TSS.

FIGURE 1. FasL promoter schematic. Locations of key transcription factor binding sites and ChIP PCR primer pairs for analysis of epigenetic modifications are denoted as regions I–IV. The coordinate locations shown are with respect to the TSS site in REFSEQ NM_000639.1

FIGURE 2. TCR stimulation–induced coordinate histone H3 modifications at the FasL promoter in control and ethanol-treated CD4⁺ T lymphocytes. Freshly isolated CD4⁺ T cells from healthy individuals (n = 3–5) were examined by ChIP qPCR. The cells were left untreated (UT) or exposed to 25 mM ethanol (E) for 24 h and subsequently stimulated with anti-CD3/CD28 Ab (1 μg/ml) for 6 h (S and E+S). Histone modifications were assessed by analyzing chromatin that was immunoprecipitated with anti-H3K4Me3 (A), anti-H3K9Me3 (B), and anti-H3K9Ac (C) Abs. Levels of histone modification were measured using primer for regions I–IV shown in Fig. 1. Differences are expressed as fold over UT after normalizing for input DNA. Results are presented as mean ± SE. a* (p < 0.05) and a** (p < 0.01), versus UT, b* (p < 0.05) and b** (p < 0.01) versus S, c* (p < 0.05) and c** (p < 0.01), versus E, RMME models with Bonferroni correction for multiple comparisons.
Additionally, region IV, located ~6.8 kb downstream from the promoter region/TSS spanning the 3’ exon and the stop codon in the 3’ UTR, was examined as a comparative negative control. The specificity of each ChIP was established by using corresponding isotype-specific control Abs (IgG). Real-time ChIP qPCR results were validated further by visualization of single PCR products and semiquantitative agarose gel analysis (Supplemental Fig. 2).

**TCR stimulation increases FasL promoter H3K4Me3**

To examine the promoter histone modifications that regulate the induction of FasL gene in response to TCR stimulation, we initially examined histone H3K4Me3 levels, because the methylation status of this H3-lysine is critically linked to increased transcriptional activity (35, 36). Chromatin was prepared from normal or ethanol-treated (25 mM; 24 h) CD4+ T lymphocytes that were stimulated with anti-CD3/CD28 for 6 h, and ChIP analysis was performed using an Ab that selectively recognizes H3K4Me3. In normal CD4+ T lymphocytes, TCR stimulation led to an increase in the levels of H3K4Me3 at all the three regions examined in the FasL promoter (Fig. 2A). In comparison, TCR-stimulated H3K4Me3 levels were further enhanced in CD4+ T cells treated with ethanol. Interestingly, ethanol treatment alone, in the absence of TCR stimulation, led to an increase in H3K4Me3 in all three regions. In contrast to the three promoter regions, region IV, located at the 3’ end of the FasL gene, did not show any changes in H3K4Me3 in response to TCR stimulation or ethanol treatment. These data show that TCR stimulation and ethanol treatment induce H3K4Me3 primarily in the promoter region.

**FIGURE 3.** TCR activation–induced recruitment of transcription factors and RNA Pol II at the FasL promoter and gene expression in control and ethanol-treated CD4+ T cells. Freshly isolated CD4+ T cells from healthy individuals (n = 3) were left untreated (UT) or were treated with ethanol (E) for 24 h, followed by anti-CD3/CD28 stimulation (E+S and S) for 12 h. ChIP qPCR quantification was performed on anti-NFAT (A), anti–NF-κB(p65) (B), anti–Sp-1 (C), and anti–RNA Pol II (D) immunoprecipitated chromatin. Differences are expressed as fold over UT after normalizing for input DNA. Results are presented as mean ± SE. a* (p < 0.05) and a** (p < 0.01), versus UT, b** (p < 0.01), versus S, and c** (p < 0.01), versus E, RMME models with Bonferroni correction for multiple comparisons. (E) Total RNA was isolated, and mRNA levels were quantified by real-time PCR after 24 h of stimulation. Data are expressed as fold induction over untreated. Results are presented as mean ± SE (n = 5). *p < 0.01, versus UT, **p < 0.01, versus control stimulated cells, one-way ANOVA with Bonferroni correction for multiple comparisons.
TCR stimulation decreases FasL promoter H3K9Me3

During transcriptional activation, an increase in promoter H3K4Me3 is functionally linked with a decrease in H3K9Me3 (37, 38). Because a significant increase in H3K4Me3 was observed in TCR-stimulated and ethanol-exposed CD4+ T cells, we next examined the H3K9 methylation status.

In contrast to H3K4Me3, TCR stimulation of normal CD4+ T lymphocytes significantly decreased the levels of H3K9Me3 at all three regions in the FasL promoter (Fig. 2B); TCR stimulation of ethanol-exposed CD4+ T cells further decreased H3K9Me3 levels. In relation to H3K4Me3, ethanol treatment alone, in the absence of TCR stimulation, led to a correspondent decrease in H3K9Me3 at regions I and III, whereas region II showed a trend toward a decrease in H3K9Me3. Thus, TCR stimulation alters histone H3 methylation to create a transcriptionally permissive configuration of the FasL promoter in normal cells, which is enhanced in alcohol-exposed cells.

TCR stimulation increases FasL promoter H3K9Ac

H3K9Ac, an essential mark for transcriptional activation, is mechanistically linked to H3K4Me3 and is mutually exclusive with H3K9Me3 (39–41). Hence, alterations in H3K9Ac were analyzed at the FasL promoter. TCR stimulation of CD4+ T cells significantly increased FasL promoter H3K9Ac in all three proximal promoter regions (Fig. 2C). In comparison, TCR stimulation of ethanol-treated CD4+ T cells led to a further enhancement of FasL promoter H3K9Ac (Fig. 2C). Ethanol exposure alone, in the absence of TCR stimulation, induced promoter H3K9Ac similar to alterations in methylation. Analysis of 3′UTR (region IV) showed no change in H3K9Ac levels under any treatment conditions. These data demonstrate that TCR stimulation ultimately leads to transcriptionally activating FasL promoter H3K9Ac, which is augmented under the pathologic condition of ethanol exposure.

TCR stimulation–induced histone modifications lead to the recruitment of relevant transcription factors and RNA Pol II to the FasL promoter

Because transcriptionally permissive H3K9Ac in response to TCR stimulation was increased in the regions known to bind the essential transcription factors NFAT, NF-κB, and Sp-1, their recruitment to the FasL promoter was also examined by ChIP analysis. Specifically, NFAT binding in regions I and II (Fig. 3A), NF-κB (p65) binding in regions I and III (Fig. 3B), and Sp-1 DNA binding in regions I, II, and III (Fig. 3C) were evaluated. The results obtained from TCR-stimulated control and ethanol-treated CD4+ T cells revealed that all three transcription factors showed increased FasL promoter binding at their respective sites, commensurate with the increase in H3K9Ac. To determine whether the increase in transcription factor recruitment on the FasL promoter was due to an increase in their nuclear translocation, we examined the nuclear levels of each transcription factor. As anticipated, TCR stimulation increased nuclear translocation of all three transcription factors; however, no additional change was observed with ethanol treatment (Supplemental Fig. 3). These data indicate that the extent of recruitment of transcription factors to the FasL promoter is not dictated by their nuclear levels, but rather by promoter-specific epigenetic modifications.

Further, RNA Pol II binding at the FasL gene promoter (region I) in response to stimulation also was increased (Fig. 3D). Overall, these data showed that formation of the transcription initiation complex (TIC) occurred at the FasL promoter in response to TCR stimulation. Importantly, the TCR-dependent increase in TIC formation and FasL mRNA expression in normal cells was enhanced in ethanol-treated cells (Fig. 3E). Interestingly, ethanol treatment alone also led to the formation of TIC, as demonstrated by transcription factor and RNA Pol II binding, but it did not induce FasL mRNA expression. These data indicate that, in ethanol-treated cells in the absence of TCR signaling, there may be a deficiency in other essential factors that are required for FasL transcriptional initiation and elongation. FasL protein expression (Fig. 4A) and AICD (Fig. 4B) also were correspondingly increased in normal and ethanol-treated CD4+ T cells. Moreover, inhibition of ethanol metabolism by 4-methyl pyrazole (4MP; inhibitor of alcohol dehydrogenase) showed that ethanol metabolism is essential for its effect on FasL promoter histone acetylation (Fig. 5A) and mRNA expression (Fig. 5B).

p300 HAT is a critical regulator of TCR stimulation–mediated FasL promoter H3K9Ac and mRNA expression

p300 was demonstrated to be the major HAT that effects H3K9Ac and subsequent transcriptional activation of several gene promoters (39, 42, 43). ChIP analysis showed that TCR stimulation leads to the recruitment of p300 to the FasL promoter, with a further increase in ethanol-treated cells commensurate with H3K9Ac (Fig. 6A). Similar to the acetylation data, the 3′UTR region IV did not show a significant difference in p300 recruitment under different treatment conditions (Fig. 6A).

Based on these results, the contribution of p300 to FasL promoter histone acetylation was examined by evaluating the linear correlation between p300 binding and H3K9Ac using Pearson correlation coefficient analysis. A strong positive correlation was obtained, with region II showing the highest correlation (coefficient $p = 0.92$, $p < 0.001$), followed by region 3 (coefficient $p = 0.87$, $p < 0.001$), versus ethanol + stimulation, † $p < 0.001$, versus anti-FasL+S, †† $p < 0.001$, versus ethanol + stimulation, † $p < 0.001$, versus anti-FasL+S, †† $p < 0.001$, versus ethanol+anti-FasL+S.
Effect of inhibition of ethanol metabolism was examined by treating the cells with an alcohol dehydrogenase inhibitor, 4MP (1 mM, 1 h), followed by ethanol for 24 h (4MP+E) and stimulation with CD3/CD28 Ab (4MP+E+S). (**p < 0.01, versus E+S, ANOVA.)

To further confirm the involvement of p300, we inhibited p300 activity using a competitive p300 inhibitor (garcinol) (44, 45) and by p300 gene knockdown with siRNA. The effect of garcinol on...

**FIGURE 6.** Increased p300 binding at FasL promoter and its correlation with H3K9Ac in control and ethanol-treated CD4+ T lymphocytes. Freshly isolated CD4+ T cells from healthy individuals (n = 3) were left untreated (UT) or exposed to 25 mM ethanol (E) for 24 h and subsequently TCR activated with anti-CD3/CD28 Ab (1 μg/ml) (S and E+S). (A) p300 binding at FasL promoter was analyzed after 6 h by ChIP qPCR assay using anti-p300 Ab. All regions (I–IV) were examined, and differences are expressed as fold over UT after normalizing for input DNA. Results are presented as mean ± SE. Statistical analysis was performed as described in Materials and Methods. a* (p < 0.05) compared to UT, b* (p < 0.05) and b** (p < 0.01) compared to S, and c* (p < 0.05) compared to E. (B) Correlation between p300 and histone H3K9Ac was assessed using the Pearson coefficient analysis. Normalized ΔCt values for p300 binding versus histone H3K9Ac levels of the FasL promoter region were plotted. Original values for region 2 (p = 0.92, p < 0.001), followed by region 3 (p = 0.87, p < 0.004), and region 1 (p = 0.65, p < 0.001) (upper panel). Residual values after accounting for the experimental design factors (lower panel). Pearson partial correlation coefficient ρ = 0.33 (p < 0.05).
p300 recruitment, histone H3K9Ac, and FasL mRNA expression was evaluated. ChIP analysis showed that garcinol significantly and specifically inhibited TCR-inducible p300 binding (Fig. 7A), as well as H3K9Ac (Fig. 7B), in all three regions in the proximal FasL promoter, with an ~2–3-fold reduction in p300 binding and an ~5-fold reduction in H3K9Ac. Additionally, garcinol-mediated inhibition of p300 binding and H3K9Ac abrogated TCR-inducible FasL mRNA expression (Fig. 7C).

Because primary CD4+ T cells are highly refractory to transfection, p300 siRNA gene-knockdown experiments were carried out in a human CD4+ T cell line (Molt-4). SiRNA-mediated knockdown of p300 prevented TCR activation–induced FasL mRNA expression, whereas control nonspecific scramble siRNA did not cause any change (Fig. 7D). Taken together, these data demonstrate that p300 is the critical HAT that is recruited upon TCR stimulation and regulates promoter H3K9Ac and transcriptional activation of FasL in CD4+ T cells.

**FasL mRNA expression in CD4+ T lymphocytes from alcoholic patients is regulated by p300-mediated promoter histone acetylation**

To verify the pathologic role of promoter histone modifications in the regulation of FasL gene expression in vivo, CD4+ T cells obtained from alcoholic subjects were examined. Specifically, recruitment of p300 HAT and resultant promoter H3K9Ac were examined by ChIP analysis. Similar to the results with CD4+ T cells treated with ethanol in vitro, a significantly increased p300 binding (Fig. 8A), as well as histone H3K9Ac (Fig. 8B), was observed at the proximal FasL promoter regions I, II, and III in CD4+ T lymphocytes obtained from alcoholic patients in comparison with nonalcoholic control subjects. Further, commensurate with the increased p300 binding and H3K9Ac, CD4+ T cells from alcoholic patients had significantly enhanced FasL mRNA expression, supporting the relevance of promoter histone acetylation in the regulation of FasL mRNA under pathologic conditions (Fig. 8C).

**Discussion**

Transcriptional regulation of the FasL gene, particularly in relation to transcriptional signaling components and transcription factors, has been studied extensively (20, 21). However, FasL gene–specific epigenetic mechanisms that regulate the access of transcription factors and transcriptional activation under normal and pathologic conditions are only beginning to be understood. Hence, the mechanistic role of promoter histone H3 modifications in regulating transcriptional activation of FasL in normal and alcohol-exposed primary human CD4+ T cells was determined.
Both transcriptional activation and suppression can be affected by specific histone methylation changes. TCR stimulation of normal, as well as alcohol-exposed, cells led to discernible changes in FasL promoter histone H3 methylation, as documented by an increase in the levels of transcriptionally permissive H3K4Me3 and a concomitant decrease in the repressive H3K9Me3 (36, 46, 47). These modifications in histone methylation are particularly significant because they are known to be mutually exclusive with a functional relationship that is highly conserved (48, 49). Importantly, these data show that coordinated H3K4/H3K9 methylation/demethylation plays a significant role in the regulation of activation-induced FasL gene expression under normal, as well as pathologic, conditions. Similar coordinated modulation of H3K4 and H3K9 methylation was observed to be a regulatory feature of other actively transcribed genes (22, 23, 28, 50–52).

In relation to effecting transcription, an increase in promoter H3K4Me3 is also functionally correlated with an increase in H3K9Ac, a major modification of initiated and transcribed genes (39, 40, 53). Moreover, H3K9Ac is mutually exclusive with the transcriptionally repressive H3K9 methylation (41). In view of this, an increase in promoter H3K9Ac levels (Fig. 2C), correspondent to the decrease in H3K9Me3 levels (Fig. 2B), shows that the cross-regulation between H3K9 methylation and acetylation plays a significant role in establishing a transcriptionally active state of the FasL promoter in response to TCR stimulation. The essential role of FasL promoter H3K9Ac in transcriptional activation is supported by earlier work in which treatment of CD4+ T cells with a histone deacetylase inhibitor, trichostatin A (known to increase histone acetylation), led to enhanced activation-induced FasL expression, and apoptosis (54). Overall, the type of transcriptionally permissive modifications entailing histone methylation and acetylation induced by TCR stimulation were similar in normal and alcohol-exposed CD4+ T cells. However, commensurate with the pathological elevation in FasL gene expression, these histone modifications occurred to a significantly higher degree in CD4+ T cells exposed to alcohol.

H3K9Ac actively neutralizes the basic charge of the affected lysine and ultimately leads to chromatin decompaction, making DNA more accessible to transcription factors and RNA polymerases (55–59). Indeed, corresponding with an increase in TCR-inducible H3K9Ac, alcohol-exposed CD4+ T cells showed significantly enhanced binding of all of the key transcription factors and RNA Pol II at the FasL promoter, along with increased mRNA expression, compared with normal cells. Our data also indicate that the extent of recruitment of transcription factors and RNA Pol II is regulated, not by their nuclear levels, but by coordinated promoter histone modifications dictating FasL mRNA expression under normal and pathologic conditions.

Interestingly, alcohol-exposed unstimulated CD4+ T cells showed a similar trend of promoter histone changes and transcription factor and RNA Pol II recruitment to normal TCR-stimulated cells, but without induction of FasL mRNA expression. Of note, epigenetic mechanisms, including histone modification, are essential, but not sufficient, with regard to gene transcriptional activation. The data show that alcohol, in the absence of T cell stimulation, is able to institute transcriptionally essential promoter histone modifications but is unable to provide all of the requisite molecular components that are required for transcriptional activation of FasL. In the context of RNA Pol II–dependent gene transcription, it is known that initiation and elongation are preceded by the formation of a preinitiation complex consisting of transcription factors and RNA Pol II on the promoter region closer to the TSS (60). Several lines of evidence show that recruitment of RNA Pol II is not the rate-limiting step for transcription. Indeed, preassembled preinitiation complexes containing RNA Pol II are present on the promoters of several hundred genes in the absence of transcription (61, 62). Also, examination of promoters at a large number of silent genes revealed epigenetic marks, including H3K9Ac, which usually are associated with transcription initiation (63, 64). Taken together, these data support the notion that alcohol alone can effect FasL promoter histone modifications and formation of the preinitiation complex; however, the initiation and/or elongation factors required for mRNA expression in alcohol-exposed CD4+ T cells are lacking in the absence of TCR stimulation.

Genome-wide analysis of chromatin signatures demonstrated that p300 binding and enrichment of histone acetylation (H3K9Ac) are important features of H3K4Me3-containing transcriptionally
active promoters and enhancers (39, 56–59). Accordingly, the mechanistic role of p300 HAT in regulating H3K9Ac, and, consequently, TCR-inducible FasL transcriptional activation, was investigated. Chromatin analysis showed that p300 is targeted to the FasL promoter in TCR-stimulated normal and alcohol-exposed CD4+ T cells. Moreover, alcohol exposure, which enhances promoter histone acetylation and FasL mRNA expression, correspondingly increases p300 binding (Fig. 6). Importantly, inhibition of p300 HAT activity by a competitive inhibitor, as well as its expression by gene knockdown, further confirmed its critical role in H3K9Ac at the FasL promoter and regulation of gene expression (Fig. 7). In addition, linear modeling analysis of p300 binding and H3K9Ac levels showed the correlation of histone H3 acetylation with p300 HAT (Fig. 6B). Taken together, these data clearly indicate a direct effect of p300 on transcriptional activation of FasL; however, it is also possible that p300 could indirectly affect FasL expression as a result of its ability to transcriptionally activate other related genes.

A critical factor in immunosuppression caused by alcohol abuse is the loss of Th CD4+ lymphocytes, leading to the impairment of multiple immune functions. Although data obtained from several experimental and clinical studies showed that ethanol decreases CD4+ T lymphocytes (11–14), the mechanism(s) leading to this depletion have not been completely elucidated. Notably, recruitment of p300 to the FasL promoter and H3K9Ac were significantly increased in CD4+ T cells from alcoholic patients in comparison with matched nonalcoholic healthy controls. Consistent with the promoter histone modifications, FasL mRNA expression also was significantly greater in CD4+ T cells from alcoholic patients. These findings are in agreement with the earlier studies that documented increased levels of soluble FasL in the sera of alcoholic patients (15). The consistency between the epigenetic mechanisms and FasL gene expression seen in CD4+ T cells exposed to alcohol in vitro and those obtained from alcoholic patients further reveals the clinical relevance of the direct effects of ethanol in the development of immune suppression in alcoholic patients (Fig. 8). This inference is also supported by several experimental and clinical studies showing that ethanol abuse is the loss of Th CD4+ lymphocytes, leading to the impairment of peripheral T-cell apoptosis. Cell Death Differ. 10: 36–44.


