

Suppression of Tumor Necrosis Factor Production by Alcohol in Lipopolysaccharide-Stimulated Culture

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Many studies have shown that alcohol consumption is associated with alteration in immune responses and increased incidence of infection in the host. Tumor necrosis factor (TNF) is a potent soluble mediator of immunoregulation and inflammation, and plays a very important role in host's defenses against infection and tumor. We propose that one of the mechanisms of alcohol-mediated immunosuppression may be due to a defect in the synthesis and release of the TNF. To determine this, we studied the direct effect of alcohol on lipopolysaccharide (LPS)-induced TNF production by whole blood and total mononuclear cell from normal subjects. Aliquots of blood samples (1 ml) or ficoll-hypaque separated total mononuclear cells (1×10^6 /ml) were cultured with different concentrations of either ethanol or acetaldehyde in the presence or absence of LPS for 4 hr at 37°C. Plasma samples and culture supernatants were assayed for TNF levels in a bioassay using a TNF-sensitive WEHI 164 subclone 13 cell line. LPS at 10 μ g/ml produced a maximal level of TNF compared with lower (1 μ g/ml) or higher concentration (50 μ g/ml) of LPS. Kinetics studies showed that an incubation time of 4 hr with LPS produced a maximum level of TNF production by blood. Alcohol, as low as 0.1% concentration, produced significant suppression of LPS-induced TNF production by whole blood, whereas alcohol at 0.2 and 0.3% concentrations were required to produce a significant suppression of TNF production by separated mononuclear cells. Anti-TNF- α antibodies significantly neutralized the LPS-induced TNF that suggests that blood monocytes may be the primary source of TNF production. Further, significant correlation between TNF production and monocyte numbers was observed. Acetaldehyde one of the primary metabolites of alcohol, did not suppress the LPS-induced TNF production by whole blood. These studies suggest that alcohol-induced inhibition of TNF may be one of the mechanisms for immunosuppression in alcoholic patients.

Key Words: Tumor Necrosis Factor, Immunoregulation, Alcohol.

THE BODY'S ability to mount an antigen-specific response is directly dependent on the production of multiple immunoregulatory proteins, cytokines.^{1,2} Tumor necrosis factor (TNF) is an important cytokine that is known to be involved in defense mechanisms against tumor and infectious and inflammatory diseases.^{1,3} Pre-

vious studies showed that TNF modulates the functions of polymorphonuclear neutrophils,⁴ T-cells,^{5,6} B-cells,^{7,10} monocyte/macrophages,¹¹ natural killer (NK),¹² and lymphokine-activated killer (LAK) cells.¹³ TNF also interacts with other cytokines, particularly interferon- γ , granulocyte-macrophage colony-stimulating factor, and interleukin (IL)-1, IL-2, and IL-4.¹⁴⁻¹⁷ Thus, TNF plays an important role in the host's defense mechanisms. Previous studies have shown that chronic alcohol consumption is associated with dysfunctions of immune system.¹⁸⁻³⁴ We have earlier reported that the lymphocytes from nonalcoholic healthy donors precultured in vitro with different concentrations of alcohol manifested decreased level of NK and antibody-dependent cellular cytotoxic (ADCC), and LAK cell activities.³⁵ We have demonstrated that alcohol has a selective inhibitory effect on NK activity of lymphocytes from acquired immunodeficiency syndrome (AIDS) patients.³⁶ Recent studies showed that acute alcohol intoxication in rats markedly suppressed both serum and lung TNF, elicited in response to lipopolysaccharide (LPS)³⁷ and intratracheal challenge with *Staphylococcus aureus* or *Klebsiella pneumoniae*.³⁸ These studies suggest that altered TNF release may have a role in the reduced immune response to infections in alcoholics. Although a number of in vitro and in vivo studies reported that alcohol modulates immune responses, the mechanisms by which alcohol alters these functions remains unclear. The present study was undertaken to investigate the direct effect of alcohol on TNF production by whole blood cells and separated total mononuclear cells in response to LPS in vitro.

MATERIALS AND METHODS

Blood Donors

Blood donors were apprised of this study, and consents were obtained consistent with the policies of the appropriate institutions and the National Institutes of Health. Peripheral blood from a total of 36 healthy, human immunodeficiency virus (HIV) seronegative, nonalcoholic individuals was drawn into a syringe containing heparin (20 units/ml). Subjects were free of medical or psychiatric illness and were not taking medications known to affect immune functions, including nonsteroidal antiinflammatory agents and substances of abuse. The age of the subjects ranged from 20 to 40 years. For each set of experiments, different blood donors were used, and no blood samples/blood donors were used interchangeably. For each blood sample, a white blood cell count was obtained using a coulter counter (Coulter Electronics, Hialeach, FL). A blood

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smear was also made and stained with Diff-Quik (Scientific Products, McGaw Park, IL) for a differential count.

Experimental Design

Total mononuclear cells were separated by ficoll-hypaque centrifugation, and the cells were washed three times in Ca^{2+} - Mg^{2+} free Hank's balanced salt solution. In experiments using whole blood samples, 1 ml of blood samples was aliquoted rapidly into sterile eppendorf tubes. In experiments using separated mononuclear cells, one million cells were cultured in complete medium containing 5% fetal bovine serum (FBS) (GIBCO), 300 $\mu\text{g}/\text{ml}$ of fresh glutamine, and 80 μg gentamicin/ml (complete medium). Triplicates of blood samples and mononuclear preparations received ethanol (EtOH) at 0.1, 0.2, and 0.3% final concentrations and acetaldehyde at 0.001, 0.002, and 0.003% final concentrations. Triplicates of cultures also received these concentrations of EtOH and acetaldehyde separately plus LPS (*Escherichia coli* no. L-2630 Sigma Chemical Co., St. Louis, MO) at 1, 5, 10, and 50 $\mu\text{g}/\text{ml}$ final concentrations. As controls, triplicate blood samples and mononuclear cells cultures also received LPS or media alone. These control and treated cultures were incubated at 37°C for 2–24 hr in 5% CO_2 and 95% air incubator. At 2-, 4-, 8-, and 24-hr intervals, treated and control samples were centrifuged at $900 \times g$ for 10 min at 4°C, and the plasma and culture supernatants were separated, diluted 1:1 with RPMI 1640 media supplemented with 1% FBS, and stored at -20°C until assayed for TNF. The viability of leukocytes was examined at different periods of culture by trypan blue dye exclusion assay. The viability of alcohol-treated cultures was not affected and was found to be similar to that of control cultures.

TNF Assay

The TNF activities of the treated and control blood plasma fluids/culture supernatants were determined by a cytotoxicity assay using TNF-sensitive WEHI 164 subclone 13 cell line (the generous gift of Dr. Daniel G. Remik, Department of Pathology, University of Michigan, Ann Arbor, MI). The method of measuring TNF was described or detailed earlier³⁹ and was standardized in our laboratory using human recombinant TNF (rTNF). The WEHI cell line is very sensitive to the cytotoxic effect of TNF and can detect as low as 2 pg/ml of TNF. Briefly, plasma/culture supernatants were serially diluted with RPMI 1640 + 1% FBS media and were added at 100 μl quantities directly into the 96 well plates (Costar, Cambridge, MA). WEHI 164 cells resuspended at a concentration of 5×10^5 cells/ml in RPMI 1649 media containing 10% FBS and 300 $\mu\text{g}/\text{ml}$ of fresh glutamine + 0.5 $\mu\text{g}/\text{ml}$ of actinomycin D (Calbiochem, Boehringer Diagnostic, LaJolla CA) was added to each well in 100 μl aliquots. As a standard, human rTNF (Peprotech, Rocky Hill, NJ) at different dilutions was included in the assay. The plates were incubated at 37°C for 20 hr, after which 20 μl of 3-[4,5-dimethylthiazol-2-yl]2,5 diphenyltetrazolium bromide (Sigma, 5 mg/ml) was added to each well and further incubated for 4 hr at 37°C. One hundred and fifty μl of supernatant was removed from each well and 100 μl of isopropanol:0.04 N HCl was added to develop the dark blue crystals. The plates were covered with aluminum foil and allowed to remain at room temperature overnight to dissolve the crystals. Then the plates were read at 550 nm in an ELISA reader. The amount of TNF in the test supernatants was calculated on the basis of the standard curve that was obtained using the rTNF used as a standard in the same assay.

In TNF neutralization assay, antihuman TNF- α and - β antibodies (rabbit polyclonal antisera, Sigma) were used at a concentration of 1:80 dilution to give maximum inhibitory effect as determined by previous experiments. In brief, the test samples containing TNF were incubated for 1 hr at 37°C with anti-TNF sera and were then used in TNF assay using WEHI cell line as described.

To investigate the possibility that metabolites of EtOH may alter the production of TNF, we studied one of the primary metabolites of EtOH, acetaldehyde on LPS-induced TNF production by whole blood. The final concentrations of acetaldehyde used were 0.001, 0.002, and 0.003%.

The choices of these concentrations were based on previously published reports of acetaldehyde concentration as determined in vivo.^{40,41}

Data Analysis

Data were analyzed using repeated measures analyses of variance (ANOVA) (samples \times doses) and Pearson correlation. The dose-response relationship of TNF production to alcohol or acetaldehyde was tested using a single linear contrast effect in the ANOVAs. A Pearson correlation was calculated to show the association of the number of monocytes to TNF production in the untreated condition.

RESULTS

Data presented in Fig. 1 show the kinetics of LPS-induced TNF in the plasma samples harvested at 2, 4, 8, and 24 hr after incubation of whole blood with LPS. Plasma samples from blood incubated with LPS for 2 hr contained 86 ng/ml of TNF. The maximum level of TNF was produced at 4 hr, the values of TNF being 175 ng/ml. The TNF production was found to be lowered at 8 and 24 hr, the values being 140 and 103 ng/ml, respectively. Based on this finding, further experiments were performed using 4-hr incubation periods of blood with LPS.

Data presented in Fig. 2 show a dose-response effect of LPS on TNF production by normal blood. LPS at 1, 10, and 50 $\mu\text{g}/\text{ml}$ produced 86, 189, and 160 ng TNF/ml of plasma, respectively. Because LPS at 10 $\mu\text{g}/\text{ml}$ concentration consistently produced maximal level of TNF in our hand (189 ng/ml), that concentration was used in our subsequent experiments. Data presented in Fig. 3 show a significant correlation between the number of monocytes and TNF production in the blood samples as calculated by the Pearson correlation coefficient. TNF productions was highly correlated with the number of monocytes ($r = 0.86$, $p < 0.0001$). Data presented in Table 1 show the number of monocytes and the levels of TNF produced by whole blood in response to LPS in the presence and absence of EtOH. EtOH produced a dose-dependent

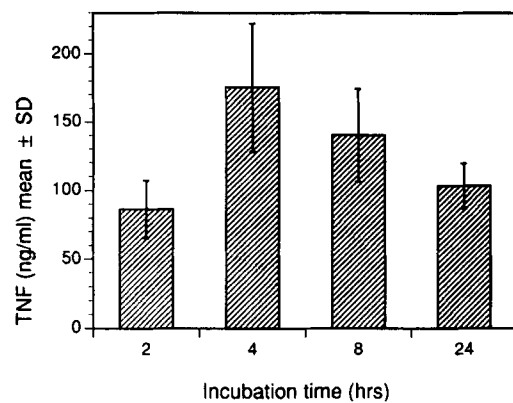


Fig. 1. Kinetics of TNF release in LPS-stimulated human whole blood. Aliquots of whole blood samples (1 ml) were cultured with 10 $\mu\text{g}/\text{ml}$ of LPS for 2, 4, 8, and 24 hr at 37°C. Plasma samples were assayed for TNF levels in a bioassay using a TNF-sensitive WEHI 164 subclone 13 cell line. A total of five separate experiments using five different blood samples were studied. Values are expressed as mean \pm SD. Mean monocyte count in these blood samples was 641 ± 108 (SD)/ml of blood.

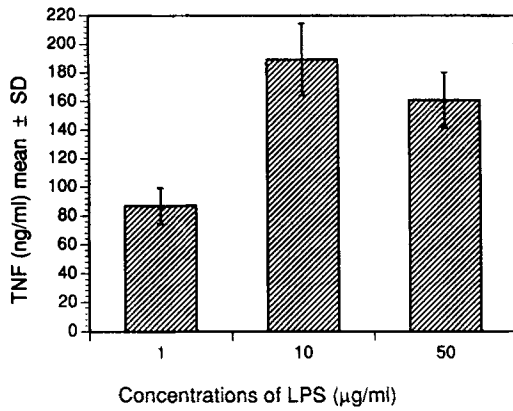


Fig. 2. Dose-response effect of LPS on TNF production by normal whole blood. Aliquots of a 1 ml blood sample were incubated with different concentrations of LPS for 4 hr at 37°C, and the plasma samples were quantitated for TNF using WEHI cell assay. Data represent mean \pm SD of five separate experiments using five different donors performed in triplicate. TNF levels in the plasma of blood cultured without LPS produced a negligible level of TNF. Mean monocyte count in these blood samples was 749 ± 102 (SD)/ml of blood.

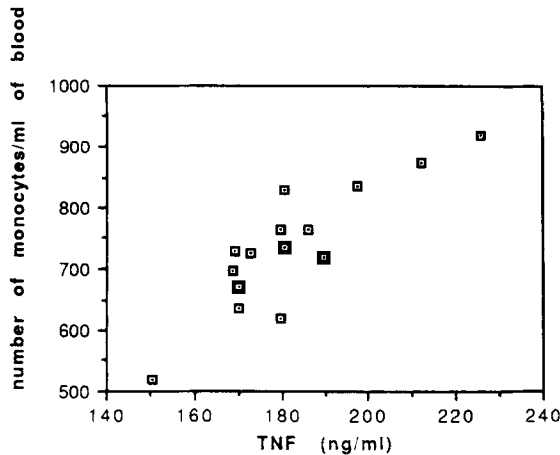


Fig. 3. Correlation between TNF production and number of monocytes. Number of monocytes and LPS-induced TNF production by 15 samples presented in Table 1 have been calculated by the Pearson correlation coefficient evaluation. For descriptions on culture conditions, see table 1 footnotes.

suppression of LPS-induced TNF production. TNF production was affected by alcohol concentration [$F(3,42) = 160.72$, $p < 0.0001$]; the linear component of this dose effect accounted for most of the variance [$F(1,42) = 419.8$, $p < 0.0001$], indicating a strong dose-response relationship. Data presented in Table 2 show the levels of TNF produced by ficoll-hypaque separated total mononuclear cells in response to LPS in the presence and absence of different concentrations of EtOH. Total mononuclear cells cultured with LPS in the presence of 0.1, 0.2, and 0.3% EtOH differed in TNF production [$F(3,9) = 26.11$, $p < 0.0001$], and EtOH produced a dose-dependent suppression of TNF production [linear contrast $F(1,9) = 75.24$, $p < 0.0001$]. These data demonstrate that alcohol at intoxicating levels in vivo can significantly suppress TNF production.

To examine the isotype of TNF produced in response to LPS, neutralization assay using anti-TNF- α and - β antisera was performed. Data presented in Table 3 showed

that anti-TNF- α antibodies significantly ($p < 0.02$) neutralized the TNF activity in the test fluid, whereas anti-TNF- β antibodies only marginally inhibited the TNF activity. A combination of anti-TNF- α and - β almost completely ($p < 0.001$) inhibited the TNF activity. This suggests that LPS-induced TNF in our culture may be primarily produced by monocytes.

To examine the specificity of alcohol-induced suppression of TNF production, experiments were performed to assess the effect of acetaldehyde, a primary metabolite of alcohol on TNF production by normal blood. Data presented in Fig. 4 show that acetaldehyde at 0.001, 0.002, and 0.003% concentrations similar to in vivo concentrations^{40,41} did not inhibit LPS induced TNF production by normal blood. There was no significant concentration effect [$F(3,12) = 0.47$, $p > 0.10$], nor was the linear contrast significant [$F(1,12) = 0.74$, $p > 0.10$].

DISCUSSION

Previous studies have shown that alcohol consumption is associated with abnormalities of humoral¹⁷⁻¹⁹ and cellular immunity,²⁰⁻²⁴ including dysfunctions of suppressor,²⁴⁻²⁷ helper,²⁸ and cytotoxic lymphocyte activities,²⁹⁻³³ and production of various soluble immune mediators.³⁴ Rats fed with diet containing EtOH also indicated a loss in cells from the thymus and lymph nodes, and showed reduced proliferative response to concanavalin A,⁴² whereas their ability to produce IL-2 and the numbers of IL-2 receptors were not affected.²² The effects of alcohol on the production and release of various cytokines by effector cells and on the functions of various effector cells responding to various stimuli have not been clearly elucidated. Previous studies have shown that acute EtOH intoxication in rats markedly suppressed serum and lung TNF elicited in response to LPS, as well as suppressed neutrophil recruitment in the alveoli of rats.³⁷ Recent studies have shown that acute alcohol administration markedly reduced *S. aureus*- or *K. pneumoniae*-induced TNF activity in lung lavage fluids of rats.³⁸ D'Souza et al.⁴³ have shown that acute EtOH administration decreased the circulating serum TNF levels in rats. Administration of rTNF has been shown to enhance the bactericidal capacity of the lung against *S. aureus*,⁴⁴ suggesting that TNF plays an important role against infection. Acute alcohol intoxication of rats also showed an alteration of TNF- α receptors on neutrophils.⁴⁵ In the murine model, dietary alcohol did not significantly affect in vitro TNF production, whereas splenocytes from mice fed either EtOH or control diet produced significantly lower levels of mitogen-induced TNF production when cultured with EtOH.⁴⁶ EtOH also downregulated TNF- α production of the in vivo-preactivated monocytes of trauma patients.⁴⁷ The results presented in the present study show that alcohol at intoxicating levels in vivo added directly to the normal human whole blood and separated total mono-

Table 1. Effect of EtOH on LPS-induced TNF Production by Normal Blood

Sample no.	No. of monocyte/ml of blood	EtOH concentrations (% v/v)			
		0	0.1	0.2	0.3
1	728	169.3	130.2	112.7	100.9
2	829	180.6	116.7	106.7	89.7
3	520	150.7	132.6	118.8	98.0
4	875	212.0	130.1	127.6	110.0
5	634	170.1	135.6	122.3	116.7
6	764	179.7	132.0	110.6	86.3
7	919	225.8	133.2	118.6	104.7
8	725	172.7	111.6	108.2	92.8
9	672	170.3	120.7	108.6	105.6
10	718	189.6	129.9	128.2	101.1
11	618	179.7	132.3	119.0	107.6
12	736	180.5	131.3	114.0	85.6
13	836	197.7	149.7	124.2	111.3
14	697	168.8	132.0	130.0	120.7
15	763	185.9	122.8	120.3	114.6
Mean ± sd	735.6 ± 103.5	182.2 ± 18.6	129.4 ± 8.9 (<i>p</i> < 0.01)	118.0 ± 7.6 (<i>p</i> < 0.001)	103.0 ± 11.0 (<i>p</i> < 0.001)

One ml aliquots of whole blood samples were cultured with 10 µg/ml of LPS with or without different concentrations of EtOH for 4 hr at 37°C. Plasma samples were assayed for TNF levels using a TNF-sensitive WEHI 164 subclone 13 cell line. A total of 15 different blood samples were studied, and the values of TNF were expressed as mean ± sd.

Table 2. Effect of EtOH on TNF Production by Total Mononuclear Cells

Experiment no.	EtOH concentrations (% v/v)			
	0	0.1	0.2	0.3
1	194.4	177.7	173.2	140.2
2	231.0	176.8	163.7	156.2
3	222.5	193.6	164.0	156.1
4	216.6	199.0	152.1	158.2
Mean ± sd	216.6 ± 14.7	186.8 ± 11.2	163.3 ± 8.6	152.7 ± 8.4

Peripheral blood mononuclear cells (1 × 10⁶ cells/ml) were cultured for 4 hr with 10 µg/ml of LPS with or without EtOH. Culture supernatants were examined for TNF using WEHI cell assay. TNF levels in culture supernatants produced without LPS manifested negligible levels of TNF (data not shown).

Table 3. Inhibition of TNF Activity by Anti-TNF Antibodies

Treatment of LPS-induced TNF supernatant	TNF (ng/ml)	% inhibition
None	192.8 ± 14.9	—
Anti-TNF-α (1:80 dilution)	50.3 ± 7.6 (<i>p</i> < 0.02)	73.9
Anti-TNF-β (1:80 dilution)	165.5 ± 15.6 (<i>p</i> < 0.3)	14.1
Anti-TNF-α + -β	15.2 ± 2.9 (<i>p</i> < 0.001)	92.1

Culture supernatants from LPS-stimulated peripheral blood mononuclear cell cultures were incubated with either anti-TNF-α or -β or TNF-α + -β (1:80 dilutions previously determined to yield maximum TNF inhibition) for 1 hr at 37°C and then assayed for TNF using WEHI cell assay. Data represent the mean ± sd of TNF levels from three experiments performed in triplicate determinations. Statistical significances of the differences between untreated and treated samples were evaluated by Student's *t* test.

nuclear cells significantly suppressed the LPS-induced TNF production. Blood cultured with different concentrations of EtOH or acetaldehyde without LPS did not induce the production of TNF (data not shown).

Previous studies have shown that TNF is produced by different subsets of circulating T-cells^{1,5,6} and macrophages.^{10,11} TNF can also influence the functions of different cells, including T, B, polymorphonuclear, NK, LAK, and macrophages.⁴⁻¹³ Alcohol is known to affect a variety of immunological responses of circulating monocytes and T-cells. Because TNF is an important immu-

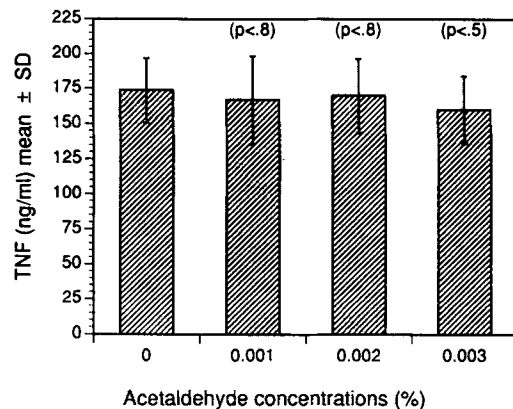


Fig. 4. Effect of acetaldehyde on LPS-induced TNF production by normal blood. Aliquots of whole blood samples were cultured with 10 µg/ml of LPS and different concentrations of acetaldehyde for 4 hr at 37°C. Plasma samples were assayed for TNF levels using a TNF-sensitive WEHI 164 subclone 13 cell line. A total of five separate experiments using five different blood samples were studied, and values were expressed as mean ± sd. Statistical significance of the differences between the control and acetaldehyde-treated samples were evaluated by two-tailed Student's *t* test. Mean monocyte count in these blood samples was 673 ± 93 (sd)/ml of blood.

noregulatory molecule and alcohol is known to affect various functions of circulating effector cells, studies on the effect of alcohol on TNF production by circulating monocytes/macrophages or T-cells may be of significance in understanding the pathogenesis of alcohol-associated immune dysfunctions in clinical situations.

Our experiments using whole blood and separated total mononuclear cells produced similar results, although separated mononuclear cells produced slightly higher levels of TNF (216 vs. 182 in tables 2 and 1, respectively). It is possible that the ex vivo model system using whole blood as originally described³⁹ may, therefore, be a good model system very similar to in vivo. This system potentially eliminates the artifacts due to adherence phenomenon⁴⁸

and also reduces the LPS contamination, because minimal handling of the specimen is required. This system may reduce the confounding variables resulting from isolating cellular subsets for the preparation of TNF. Because cellular interactions are known to influence cytokine productions,^{48,49} the use of whole blood may preserve the cellular integrity. Further, the use of whole blood may also preserve the LPS binding protein facilitating the maximum cellular response to LPS in the assay system. Although the whole blood system may be a limited lymphoid system in comparison with the intact animal and cannot accurately mimic the highly complex in vivo system or intact animals, it is possible that this system has considerable relevance to in vivo situations. Although alcohol levels in tissue and cell membranes may vary, a concentration of 0.3–0.4% in blood results in severe intoxication, whereas 0.5% leads to coma. Chronic alcoholics may manifest greater suppressive effects of alcohol on TNF production, because increased levels of alcohol are maintained over longer periods of chronic drinking. It is interesting to note that alcohol as low as 0.1% could significantly inhibit TNF production by whole blood, whereas alcohol at a similar concentration (0.1%) did not manifest any significant effect on TNF production by the separated total mononuclear cells; rather, high concentrations of alcohol (0.2 and 0.3%) were required to mediate a significant inhibition. Our recent studies showed an increase in the number of cells bearing IL-2 R and I2 markers in alcoholic patients compared with that of matched controls.⁵⁰ The effects of EtOH on the production of TNF by lymphocyte or monocyte subpopulations responding to various specific and nonspecific stimuli in addition to LPS and the number or affinity or avidity of TNF binding receptors present on various subpopulations subjected to isolation procedures in comparison to whole blood needs to be investigated. Our studies using the whole blood samples indicated a significant correlation between TNF production and number of monocytes (Fig. 3). It is important to note that alcohol at 0.1% and 0.2% in culture condition levels or similar to those found in humans under varying degrees of intoxication levels significantly suppressed TNF, a potent mediator of inflammatory cascade and an important immunoregulatory substance against various infections. Furthermore, the fact that acetaldehyde, an active metabolite of alcohol, did not produce similar TNF suppression, points to the specificity of this reaction. In summary, our data show that alcohol at intoxicating levels produces suppression of LPS-induced TNF production by whole blood, as well as separated total mononuclear cells. Results of these studies are consistent with that of other in vivo findings^{37,38,43} and present direct evidence that one of the mechanisms of alcohol-mediated immunosuppression may be associated with decreased production of TNF. This finding may explain the noted increase in susceptibility to infection reported in alcoholic patients.

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