SUPPLEMENTARY INFORMATION

Mild cognitive impairment is associated with immunological and inflammatory alterations in non-alcoholic fatty liver disease

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Table S1. Monoclonal antibodies used

Antibodies	Fluorochrome	Clone	Company
CD45	KO (Krome Orange)	J33	Beckman Coulter
CD14	PB (Pacific Blue)	M5E2	Biolegend
CD16	APC (Allophycocyanin) -Alexa Fluor* 750	3G8	Beckman Coulter
CD3	APC (Allophycocyanin)	UCHT1	Biolegend
CD4	ECD (Electron Coupled Dye)	SFCI12T4	Beckman Coulter
CD45RA	PERCP/Cy5.5 (Peridinin Chlorophyll Protein/Cyanin5.5)	D11	Biolegend
CD45RO	APC/Cy7 (Allophycocyanin/Cyanin7)	HI100	Biolegend
CD28	PB (Pacific Blue)	UCHL1	Biolegend
CXCR5	Alexa Fluor® 700	J252D4	Biolegend
CXCR3	Vio® Bright FITC	REA232	Miltenyi Biotec
CCR4	PE-Vio® 770	REA279	Miltenyi Biotec
CCR6	Brilliant Violet 510	G034E3	Biolegend
CCR10	PE	REA326	Miltenyi Biotec
CD25	Brilliant Violet 510	PC61	Biolegend
FoxP3	Alexa Fluor® 488	259D/C7	BD Pharmingen
CD69	Alexa Fluor® 488	FN50	Biolegend

Table S2. Test performance of controls and patients

TEST /parameter	CONTROLS (n=31)	NAFLD patients without MCI (n=51)	NAFLD patients with MCI (n=20)	Global *p Value*
Coordination tests				
Bimanual (min)	1.93 ± 0.05	2.03 ± 0.04	2.42 ± 0.09****$^{/bbbb}$	**<0.0001**
Visuo-motor (min)	2.27 ± 0.05	2.26 ± 0.05	2.72 ± 0.11***$^{/bbbb}$	**<0.0001**
d2 Test				
TR Values	435 ± 10	420 ± 12	272 ± 20****$^{/bbbb}$	**<0.0001**
TA Values	173 ± 4	159 ± 4	101 ± 8****$^{/bbbb}$	**<0.0001**
O Values	9 ± 1	14 ± 1	15 ± 4	0.0942
C Values	1 ± 0.3	1 ± 0.2	5 ± 1.2****$^{/bbb}$	**<0.0001**
O+C Values	10 ± 1	14 ± 1	19 ± 4*	**0.0155**
TOT Values	425 ± 10	4402 ± 10	253 ± 19****$^{/bbbb}$	**<0.0001**
CON Values	169 ± 5	157 ± 5	92 ± 7****$^{/bbbb}$	**<0.0001**
Stroop Test				
Congruent Task (Number of words)	115 ± 3	111 ± 2	90 ± 3****$^{/bbbb}$	**<0.0001**
Neutral Task (Number of colours)	86 ± 3	84 ± 2	69 ± 3***$^{/bbb}$	**0.0002**
Incongruent Task (Number of items)	49 ± 2	46 ± 1	38 ± 2***$^{/bb}$	**0.0004**
Oral SDMT test				
Total items	55 ± 1	53 ± 1	38 ± 2****$^{/bbbb}$	**<0.0001**
Correct pairings	54 ± 1	52 ± 1	36 ± 2****$^{/bbbb}$	**<0.0001**
Errors	0.5 ± 0.1	0.8 ± 0.2	1.7 ± 0.5*	**0.0404**
DIGIT SPAN Test				
Digits forward (right answers)	9 ± 0.4	9 ± 0.3	8 ± 0.5*	**0.0358**
Digits backward (right answers)	7 ± 0.4	6 ± 0.3*	4 ± 0.4***$^{/b}$	**0.0002**
Number-letter sequencing Test (right answers)	10 ± 0.4	9 ± 0.5	7 ± 0.6***$^{/b}$	**0.0004**

Values are expressed as mean ± SEM (standard error of mean). Results were analyzed with one-way ANOVA followed by post-hoc Tukey's multiple comparisons test. MCI, mild cognitive impairment;

NAFL: non-alcoholic fatty liver; NASH; Oral SDMT, Symbol digit modalities test (oral version). D2 test parameters: TR, Total number of characters processed; TOT, Total correctly processed; CON, Concentration performance; RA, Total right answers; O, errors of omission; C, errors of commission; O+C, Total errors. Values significantly different from controls are indicated by asterisks*. Values significantly different in patients with MCI compared to patients without MCI are indicated by b. ($^{*/b}$ $p < 0.05$; ^{bb} $p < 0.01$; $^{***/bbb}$ $p < 0.001$; $^{****/bbbb}$ $p < 0.0001$).

Table S3. Characteristics of patients grouped by liver damage

Parameter	NAFL patients	NASH patients	p value
Number of subjects [n (%)]	39 (55)	32 (45)	
Age[a]	58±1.4	59±1.4	0.628
Sex [n (%)]			0.115
- **Male**	22 (56)	12 (38)	
- **Female**	17 (44)	20 (62)	
Education[a] (years of schooling)	15±0.7	12±0.7	**0.010**
Comorbidity [n (%)]			
- **Diabetes mellitus**	14 (36)	16 (50)	0.235
- **Dyslipidemia**	24 (62)	18 (56)	0.654
- **Arterial Hypertension**	18 (46)	20 (63)	0.172
- **Metabolic Syndrome**	15 (39)	18 (56)	0.138
Laboratory Parameters[a]			
- **AST (U/mL)**	33.3±2.6	39.6±3.1	0.120
- **ALT (U/mL)**	42.1±3.7	49.9±4.5	0.185
- **Albumin (g/dL)**	4.4±0.0	4.3±0.1	**0.045**
- **Bilirubin (mg/dL)**	0.7±0.1	0.7±0.1	0.559
- **Creatinine (mg/dL)**	0.8±0.0	0.8 ±0.0	0.183
- **Platelets (x10^9/L)**	249.1±14.9	240.6±14.8	0.687
- **INR**	1.1±0.1	1.01±0.1	0.335
- **Ammonia (µM)**	16.3±2.2	15.2 ±(1.3	0.677
BMI[a]	30±0.7	34.2±1.0	<0.0001
Diagnosis [n (%)]			
- **Biopsy (NAS)**	19 (49)	29 (91)	
- **Fibroscan (FAST)**	20 (51)	3 (9)	
Fibroscan Parameters[a]			
- **LSM (kPa)**	6.9±0.5	9.9±1.0	**0.006**
- **CAP (dB/M)**	320.8±10.2	326.4±9.3	0.691
Fibrosis grade [n (%)]			**0.023**
- **F0**	6 (30)	3 (11)	
- **F1**	5 (50)	5 (18)	
- **F2**	8 (40)	13 (46)	
- **F3**	1 (5)	7 (25)	
MCI [n (%)]	12 (31)	8 (25)	0.593
MCI Score[a]	-2.2± 0.7	-3.5±0.05	0.041

[a]Data are shown as mean ± SEM (standard error of mean). Between-group comparisons were analyzed by t-student for continuous data and Chi-Square (χ^2) for categorical data. Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; BMI, body mass index; CAP, controlled attenuation parameter; FAST, fibroscan-AST score; INR, international normalized ratio; LSM, liver stiffness measurement; NAFL, non-alcoholic fatty liver; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; MCI, mild cognitive impairment.

Table S4. Monocyte and $CD4^+$ lymphocyte populations of controls and patients grouped by liver damage (NAFL and NASH)

	Control	NAFL patients	NASH patients	Global *p value*
Monocytes				
$CD14^{++}CD16^-$ (Classical)	95.2 ± 0.3	93.4 ± 0.5	93.0 ± 0.7*	0.022
$CD14^+CD16^+$ (Intermediate)	2.4 ± 0.2	6.4 ± 0.5****	7.3 ± 0.6****	< 0.0001
$CD14^+CD16^{++}$ (Non-classical)	0.7 ± 0.1	0.1 ± 0.0****	0.1 ± 0.0****	< 0.0001
T *helper* lymphocytes				
CD4+	61.9 ± 1.9	62.2 ± 2.6	65.5 ± 2.7	0.569
CD4+ naïve	27.5 ± 2.4	36.7 ± 3.3	34.3 ± 3.6	0.063
$CD4^+$ memory	68.1 ± 2.7	69.5 ± 2.3	73.6 ± 3.6	0.415
$CD4^+$ $CD28^-$	11.2 ± 1.5	6.0 ± 1.0**	5.5 ± 1.0**	0.002
$CD4^+$ $CD28^+$	89.0 ± 1.5	95.5 ± 0.9***	94.9 ± 1.3**	0.0004
Activation				
$CD4^+$	0.33 ± 0.03	6.04 ± 0.80****	5.15 ± 0.90****	< 0.0001
$CD4^+$ naïve	0.3 ± 0.1	2.8 ± 0.4****	2.8 ± 0.6****	< 0.0001
$CD4^+$ memory	0.6 ± 0.1	8.8 ± 1.1****	7.3 ± 1.1****	< 0.0001
$CD4^+$ $CD28^-$	0.3 ± 0.1	8.1 ± 1.2****	8.4 ± 1.9****	< 0.0001
$CD4^+$ $CD28^+$	2.5 ± 0.1	6.3 ± 0.8***	5.3 ± 1.0*	0.001

Values are expressed as mean ± SEM (standard error of mean). Results were analyzed with one-way ANOVA followed by post-hoc Tukey's multiple comparisons test. NAFL: non-alcoholic fatty liver; NASH; non-alcoholic steatohepatitis. Values significantly different from controls are indicated by asterisks (*): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). Activation is expressed as the percentage of cells expressing CD69 marker.

Table S5. Plasma cytokine levels in controls and patients

Cytokine concentration (pg/mL)	Control	NMCI patients	MCI patients	Global *p value*
IL-13	3.6 ± 0.5	4.0 ± 0.4	11.1 ± 2.0****$^{/bbbb}$	<0.0001
IL-17A	0.2 ± 0.01	0.2 ± 0.02	0.3 ± 0.003***$^{/bb}$	0.0007
IFN γ	0.22 ± 0.02	0.27 ± 0.03	0.62 ± 0.24*$^{/b}$	0.0114
IL-6	1.6 ± 0.1	2.8 ± 0.2*	4.1 ± 0.8****$^{/b}$	<0.0001
IL-18	144.9 ± 12.1	190.8 ± 11.7	258.7 ± 32.2	0.0002
IL-10	3.3 ± 0.2	5.4 ± 0.3****	5.7 ± 0.7***	<0.0001
IL-22	200.0 ± 18.8	276.9 ± 27.0	418.5 ± 64.5***$^{/b}$	0.0014
TGF β	4759 ± 243.3	8.38 ± 416.0****	8351 ± 832.4****	<0.0001
IL-8	7.3 ± 1.3	18.4 ± 1.6****	15.3 ± 2.1*	<0.0001
IL-15	4.7 ± 1.0	6.1 ± 0.9	5.2 ± 1.1	0.5753
IL-23	11.5 ± 2.9	14.9 ± 2.6	34.2 ± 8.5**$^{/bb}$	0.0026
IL-12 p70	0.17 ± 0.00	0.19 ± 0.02	0.14 ± 0.01	0.2364
TNF α	5.4 ± 0.2	6.5 ± 0.4	6.5 ± 0.9	0.1186
IL-4	65.0 ± 2.3	65.0 ± 2.1	68.4 ± 2.1	0.5108
IL-21	8.2 ± 2.3	31.0 ± 4.3*	62.4 ± 19.6***$^{/b}$	0.0006
IL-1 β	2.2 ± 0.4	3.7 ± 0.5	4.9 ± 1.0**	0,0104
CCL5	7352 ± 830.5	10605 ± 847.6*	11625 ± 1539*	0.0226
CX3CL1	489.2 ± 121.9	799.4 ± 116.8	516.6 ± 141.3	0.0154
CCL20	8.2 ± 1.6	12.0 ± 1.8	17.9 ± 4.5*	0.1686
CCL2	5.1 ± 0.8	11.5 ± 0.8****	12.7 ± 1.5****	0.0369
BDNF	1942 ± 159.4	1994 ± 143.5	2624 ± 165.7	<0.0001

Values are expressed as mean ± SEM (standard error of mean). Results were analyzed with one-way ANOVA followed by post-hoc Tukey's multiple comparisons test. MCI, mild cognitive impairment. Values significantly different from controls are indicated by asterisks*. Values significantly different in patients with MCI compared to patients without MCI (NMCI) are indicated by ^{b}. ($^{*/b}$ $p < 0.05$; $^{**/bb}$ $p < 0.01$; *** $p < 0.001$; $^{****/bbbb}$ $p < 0.0001$).

Table S6. Plasma cytokine levels of NAFL and NASH patients

Cytokine concentration (pg/mL	Control	NAFL patients	NASH patients	Global *p value*
IL-13	3.6 ± 0.5	6.2 ± 1.0	6.1 ± 1.2	0.166
IL17A	0.18 ± 0.02	0.21 ± 0.02	0.20 ± 0.02	0.599
IFN γ	0.18 ± 0.2	0.37 ± 0.13	0.39 ± 0.10	0.216
IL-6	1.6 ± 0.1	2.9 ± 0.4*	3.6 ± 0.4***	0.0005
IL-18	145 ± 12	219 ± 21**	202 ± 14	0.006
IL-10	3.3 ± 0.2	5.1 ± 0.3***	6.0 ± 0.6***	<0.0001
IL-22	200 ± 19	313 ± 41	353 ± 44*	0.031
TGF β	4759 ± 243	8265 ± 464****	7920 ± 648****	<0.0001
IL-8	7.3 ± 1.3	18.03 ± 1.8***	16.7 ± 1.9**	<0.0001
IL-15	4.7 ± 1.0	5.9 ± 1.3	5.9 ± 1.0	0.665
IL-23	11.5 ± 2.9	21.9 ± 4.9	18.2 ± 4.1	0.241
IL-12p70	0.17 ± 0.00	0.17 ± 0.02	0.18 ± 0.02	0.983
TNF alfa	5.4 ± 0.2	5.8 ± 0.3	7.5 ± 0.8**$^{*/b}$	0.003
IL-4	65.0 ± 2.3	67.8 ± 2.1	63.1 ± 2.4	0.389
IL-21	8.2 ± 2.3	36.3 ± 9.5*	36.5 ± 5.6*	0.02
IL-1 beta	2.2 ± 0.4	4.2 ± 0.7 *	3.6 ± 0.5	0.03
CCL5	7352 ± 830.5	11475 ± 1006**	10136 ± 1111	0.012
CX3CL1	489 ± 122	547 ± 83	937 ± 173	0.039
CCL20	8.2 ± 1.6	14.6 ± 3.0	13.0 ± 2.2	0.131
CCL2	5.1 ± 0.8	11.7 ± 1.0****	12.0 ± 0.9****	<0.0001
BDNF	1942 ± 159	2399 ± 135	1783 ± 205^{b}	0.025

Values are expressed as mean ± SEM (standard error of mean). Results were analyzed with one-way ANOVA followed by post-hoc Tukey's multiple comparisons test. NAFL: non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis. Values significantly different from controls are indicated by asterisks*. Values significantly different between NASH and NAFL patients are indicated by ^{b}. ($^{*/b}$ $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

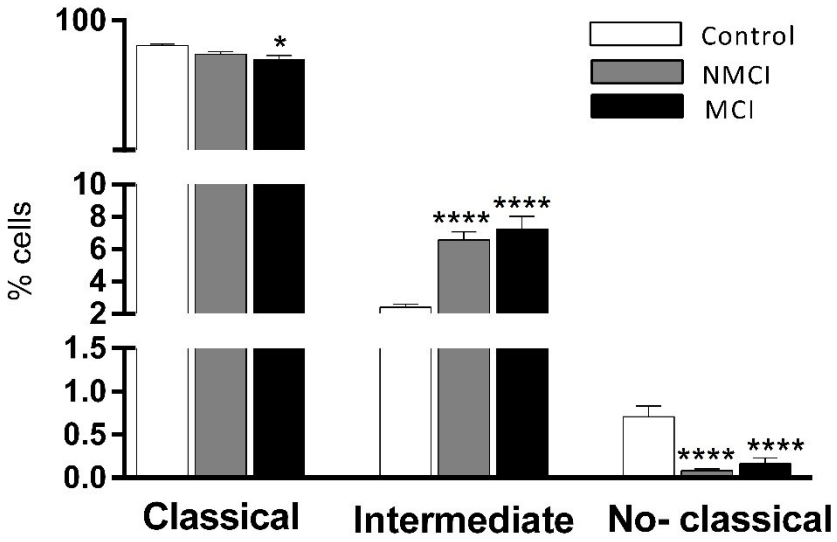

Figure S1. Monocyte populations in peripheral blood from NAFLD patients and controls. Percentage of the three monocyte subsets relative to total monocyte cells: classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$). Values are mean ± SEM of the following groups: control, n = 15; patients without MCI (NMCI), n = 26; patients with MCI (MCI), n = 12. Values significantly different from controls are indicated by asterisks*. ($^{*}p<0.05$; $^{****}p<0.0001$). MCI, mild cognitive impairment.

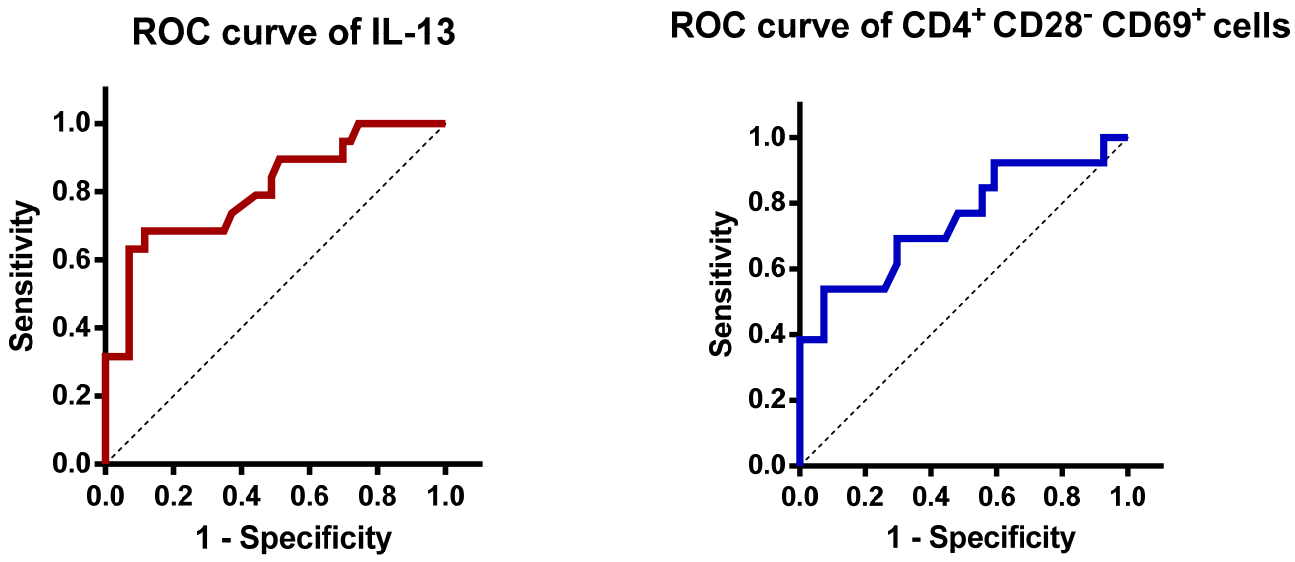

Figure S2. Receiver operating characteristic (ROC) curves for sensitivity and specificity of IL-13 and activated autoreactive cells (%CD69^{+}) in the diagnosis of mild cognitive impairment in NAFLD patients. Area under the curves (AUC) were: IL-13: AUC: 0.804 (95% confidence interval (CI) 0.680-0.928; *P*=0.0001); Activated autoreactive cells: AUC: 0.75 (95% CI 0.575-0.924; *P*=0.01). For IL13, the specificity was 83% and the sensitivity was 68% at a cutoff of 6.98 pg/mL. For activated autoreactive cells, the specificity was 70% and the sensitivity was 69% at a cutoff of 7.86 %.