Online Supplement data

Monocytes inhibit NK activity via TGFβ in patients with obstructive sleep apnea

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Supplementary Methods

Study participants

The diagnosis of OSA was established by respiratory polygraphy (Embletta GOLD, ResMed), which included continuous recordings of oronasal flow and pressure, heart rate, thoracic and abdominal respiratory movements and oxygen saturation (SpO_2). Those tests in which the patients claimed to sleep less than 4 hours or in which there were less than 5 hours of nocturnal recording were repeated. Exclusion criteria were the following: previous or current treatment with oxygen or mechanical ventilation; underweight (body mass index [BMI] <18.5 Kg/m^2) or morbid obesity (BMI >40 Kg/m^2); history of respiratory or cardiovascular disease, including chronic obstructive pulmonary disease, asthma, respiratory failure, hypertension, heart failure and coronary artery disease; any infectious disease in the previous 3 months; diagnosis of malignancies or chronic inflammatory diseases; and the use of inhaled or systemic corticosteroids or other anti-inflammatory drugs.

Blood samples and peripheral blood mononuclear cells isolation

Blood samples were obtained from the participants between 8:00 and 9:00 AM, following an overnight fast. PBMCs were isolated by centrifugation in Ficoll-Histopaque Plus (Amersham Biosciences) for flow cytometry and real-time quantitative PCR (RT-qPCR) analysis. For mechanistic and mRNA expression studies involving $CD14^+$ and NK cells, a specific isolation kit from Miltenyi Biotec was used. Cell purity was checked by flow cytometry showing >95% positive cells.

Reagents and cell lines

DMEN (Invitrogen) and RPMI 14640 (Invitrogen) were used for the cell cultures. SB431542 (Biogen), and TGFβ1 antibodies (R&D Systems) were used for the inhibition assays. All reagents used for the cell cultures were tested for endotoxins using the Limulus amoebocyte lysate test (Lonza). The cell line K562 is authenticated and free from contamination by bacteria such as mycoplasma.

Cytometric bead array

TGFβ1 levels in the sera and in the culture supernatants were determined using the TGFβ Flex Set (BD Biosciences), following the manufacturer's protocol, and they were analyzed by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences). To activate the latent TGFβ1 (TGFβ-LAP) sera samples have to be acidified to PH3.0 or lower for 10 minutes and neutralized back to PH 7.2 before testing. The active TGFβ1 on supernatants were analyzed without acidification treatment.

Cytotoxicity assay

NK cytotoxicity was monitored using a conventional 2-h europium lysis-TDA release assay (Perkin-Elmer). The primary NK cells were used as effector cells. The TDA-labeled K562 cell line was used for target cells at effector-to target cell (E:T) ratios of 4:1, 2:1 and 1:1.

Supplementary tables and figures

Table E1. The detail Antibodies for flow cytometry.

Antibodies	Clone	Dye	Isotype
Anti-human CD3	SP34-2	PerCP	IgG1
Anti- human CD14	M5E2	APC or FITC	IgG2a
Anti-human CD56	B159	FITC	IgG1
Anti-human CD56	NCAM16.2	APC	IgG2b
Anti-human CD16	3G8	PE or FITC	IgG1
Anti-human CD4	L200	PerCP	IgG1
Anti-human CD8	SK1	PerCP	IgG1
Anti-human CD25	2A3	APC	IgG1
Anti-human FOXP3	236A/E7	PE	IgG1
Anti-human NKp30	P30-15	PE	IgG1
Anti-human NKp44	P44-8	PE	IgG1
Anti-human NKp46	9E2/NKp46	PE	IgG1
Anti-human Smad2/Smad3	O72-670	PE	IgG1
Anti-human CD117	YB5.B8	PE	IgG1
Anti-human T-bet	O4-46	PE	IgG1
Anti-human RORC	Q21-559	PE	IgG2b
Anti-human Perforin	δG9	Alexa fluor 488	IgG2b
Anti-human EOMES*	WD1928	FITC	IgG1
Anti-human CD28	L293	PE	IgG1
Anti-human GARP	7B11	APC	IgG2b
Anti-human TGF	TW4-E2	PE	IgG1
Anti human TCR γδ	B1	FITC	IgG1
Anti-human IgG1	G18-45	FITC, APC, PE	IgG1
Anti-human IgG1	MOPC-21	PerCP	IgG1
Anti-human IgG2a	G155-178	FITC or APC	IgG2a
Anti-human IgG2b	27-35	FITC, APC, PE Alexa 488.	IgG2b

*Anti-human EOMES was purhased from TermoFisher

Table E2. Comparison of the three study groups at the inclusion moment*

	OSA group (n=60)	**CPAP group** (after treatment)† (n=32)	**Healthy volunteers** (n=29)	**p-value‡**
Body mass index, Kg/m^2	28.5 ± 4.6	27.1 ± 4.6	27.4 ± 5.0	0.514
CPAP pressure, cmH_2O	-	9 ± 1	-	-
Epworth Sleepiness Scale (ESS)	10.8 ± 4.5	6.7 ± 2.5 ¶	4.1 ± 3.6 ¶	<0.001
Daytime sleepiness (ESS ≥10)	33 (55.0)	3 (9.4)	1 (3.4)	<0.001
AHI, events/h	55.1 ± 18.2	6.7 ± 4.8 ¶	2.7 ± 1.2 ¶	<0.001
Oxygen desaturation index, events/h	49.6 ± 22.3	6.8 ± 4.3 ¶	1.9 ± 1.1 ¶‖	<0.001
Sleep time with SpO_2 <90%, %	33.1 ± 29.0	5.6 ± 3.4 ¶	2.3 ± 2.8 ¶	<0.001
Sleep time with SpO_2 <80%, %	5.6 ± 9.6	0.6 ± 1.1 §	0	0.003
Low nocturnal SpO_2, %	77.6 ± 6.9	82.7 ± 5.6 ¶	87.6 ± 2.3 ¶‖	<0.001
Mean nocturnal SpO_2, %	89.9 ± 4.0	91.4 ± 1.9 §	92.3 ± 1.5 §	0.034

* Values are mean ± SD or percentage.

†Values of the treated OSA patients refer to measurements performed 6 months after treatment with CPAP.

‡Comparisons between groups were performed by ANOVA or chi-squared test: § $p<0.05$ vs. OSA group; ¶ $p<0.001$ vs. OSA group; ‖ $p<0.05$ vs. CPAP group.

Abbreviatures: AHI = apnea-hypopnea index; SpO_2 = oxyhemoglobin saturation.

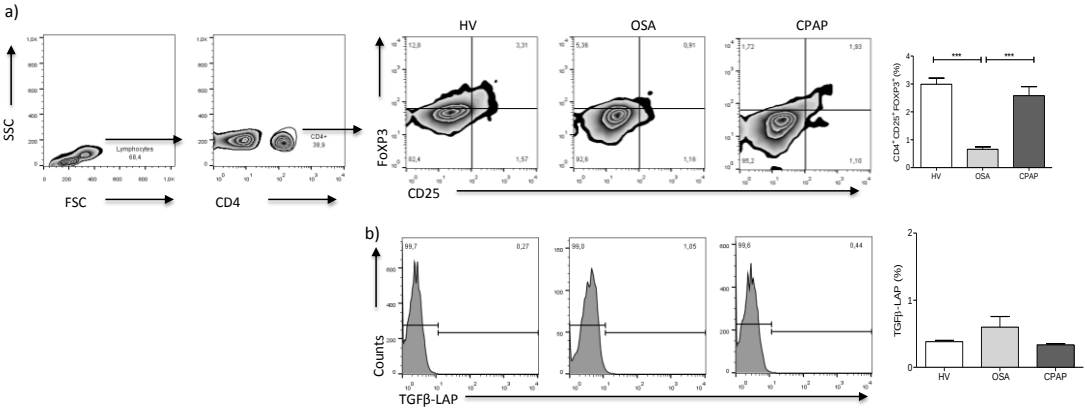

Figure E1. Treg distribution and phenotype.

PBMCs from HV (open bars, n=29), OSA (light grey bars, n=20 randomly selected), CPAP (dark grey boxes, n=20 randomly selected) were analyzed by flow cytometry. a) Gating strategy for the detection of Tregs. Numbers adjacent to outlined areas indicate the percentage of lymphocytes and $CD4^+$ cells. Representative dot blots of CD25 and FoXP3 expression on $CD4^+$ cells are shown. b) The percentage distribution of TGFβ-LAP on gated Tregs is shown. Standard histograms are shown. ***p<0.001 using Kruskal-Wallis test with a Dunn's multiple comparison.

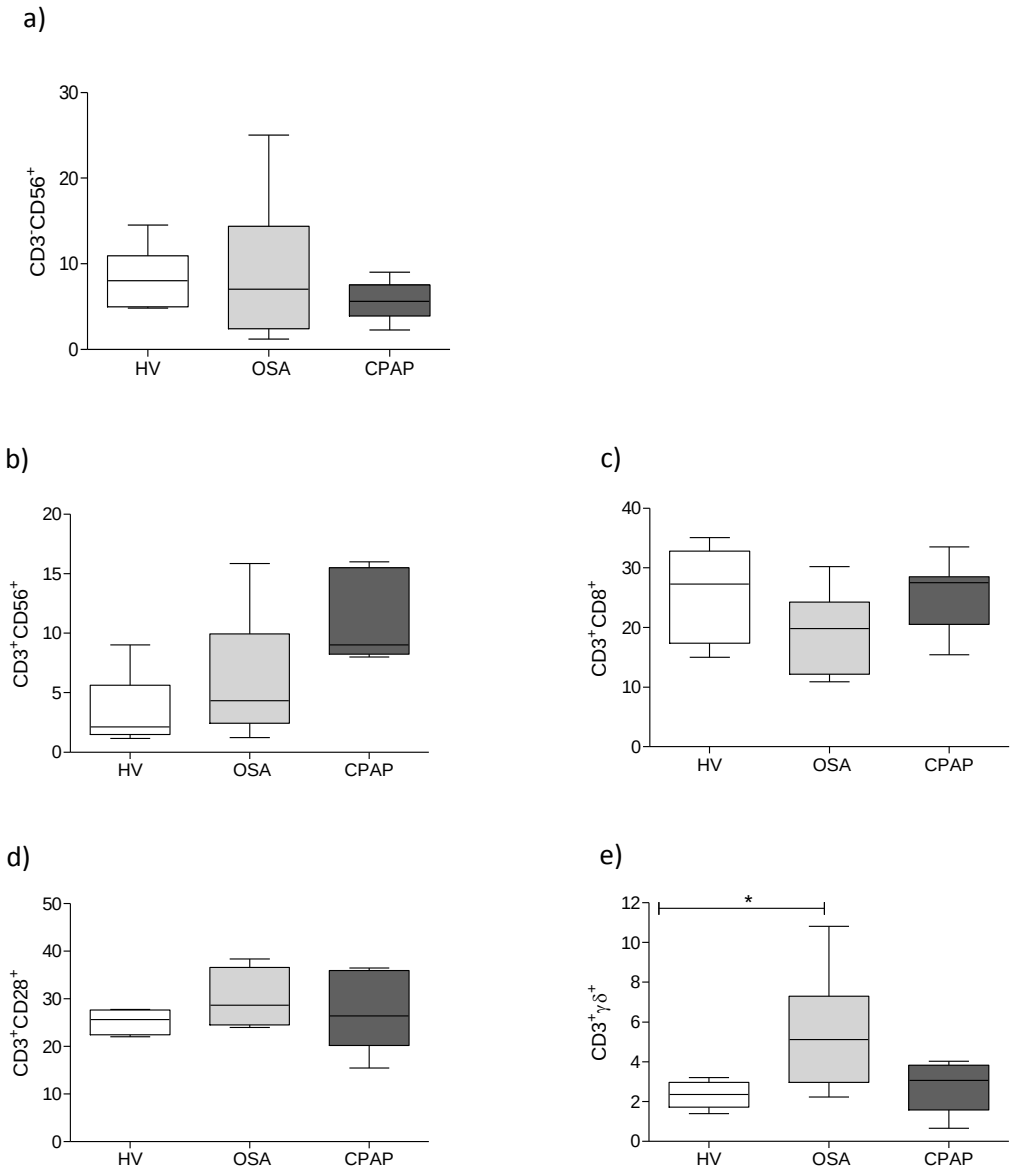

Figure E2. Lymphocyte distribution and phenotype.

Isolated PBMCs from HV (open boxes, n=20 randomly selected), OSA (light grey boxes, n=20 randomly selected), CPAP (dark grey boxes, n=20 randomly selected) were analysed by flow cytometry. The percentage distribution of a) $CD3^{-}CD56^{+}$ b) $CD3^{+}CD56^{+}$, c) $CD3^{+}CD8^{+}$, d) $CD3^{+}CD28^{+}$ and e) $CD3^{+}CD\gamma\delta^{+}$ on total gated lymphocytes are shown. *p<0.05, using Kruskal-Wallis test with a Dunn's multiple comparison.

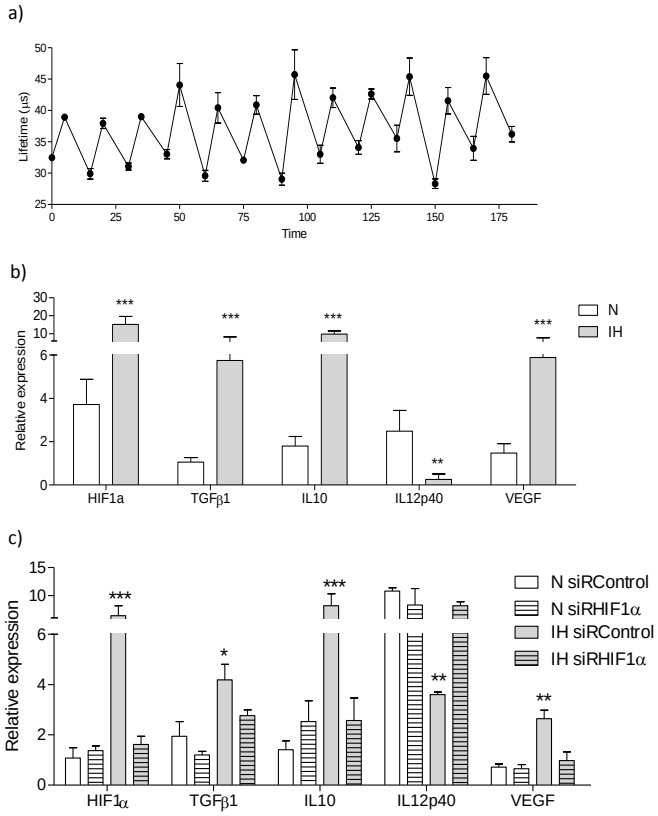

Figure E3. Monocytes exhibit an immunosuppressive phenotype after intermittent hypoxia.

$CD14^+$ monocytes were isolated from HV and cultured under normoxia (N, open bars, n=7) or intermittent hypoxia (IH, light gray bars, n=7) conditions. a) The effect of intermittent hypoxia on intracellular oxygen concentration in $CD14^+$ monocytes. Lifetimes of phosphorescent probe are shown. b) Expression analysis by qPCR of HIF1α, TGFβ1, IL10, IL12p40, and VEGF mRNA in $CD14^+$ monocytes. Relative expression is shown. *p<.05; **p<.01; ***p<.001, using a paired t-test.

$CD14^+$ monocytes isolated from HV were nucleofected with siRNAControl (30 nM, empty bars, n=6) or siRNAHIF1α (30 nM, striped bars, n=6). After 16 hours, the monocytes were cultured under normoxia (N, open bars, n=6) or intermittent hypoxia (IH, light gray bars, n=6) conditions. c) Expression analysis by qPCR of HIF1α, TGFβ1, IL10, IL12p40, and VEGF mRNA in $CD14^+$ monocytes. *p<.05; **p<.01; ***p<.001, siRControl compared with siRHIF1α using paired t-test.

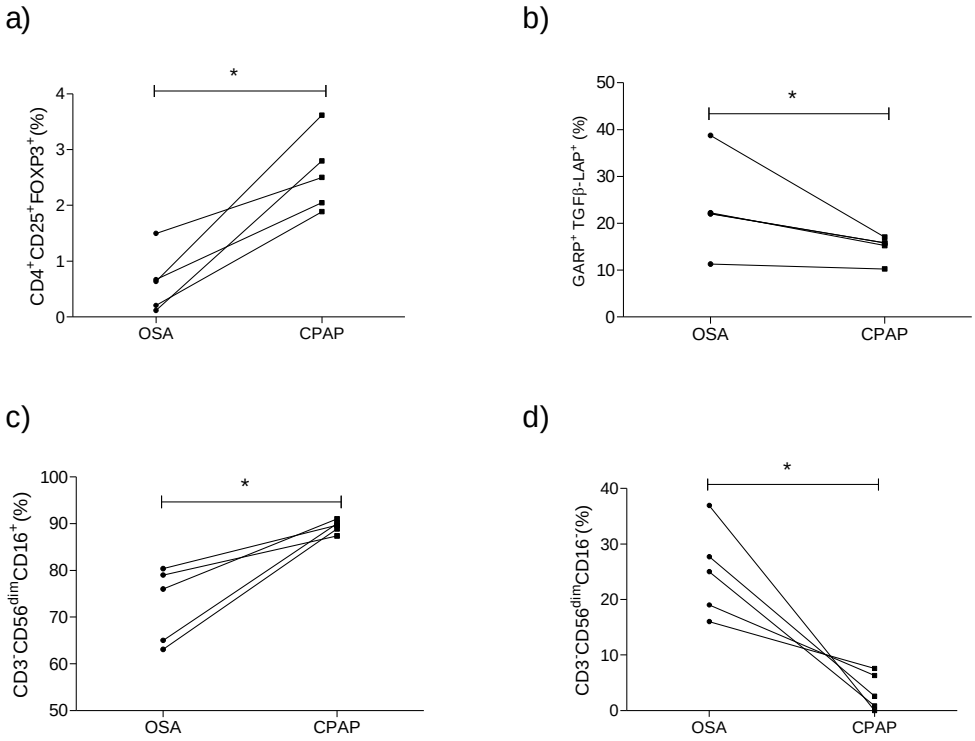

Fig. E4. OSA recovers the healthy phenotype after more than six months of CPAP treatment.
PBMCs isolated from patients with OSA (n= 5) were analyzed before and after 6 months of treatment (OSA and CPAP, respectively). a) The percentage distribution of Tregs ($CD4^+CD25^+FOXP3^+$) on total gated T-lymphocytes. b) The percentage distribution of $GARP^+TGF\beta\text{-}LAP^+$ on total gated $CD14^+$ cells is shown. The percentage distribution of c) $CD3^-CD56^{dim}CD16^+$ and d) $CD3^-CD56^{dim}CD16^-$ on total gated NK cells. *p<.05; **p<.01, using a Wilcoxon test.