SUPPLEMENTAL DATA

P2X7R mutation disrupts the NLRP3-mediated Th program and predicts poor cardiac allograft outcomes

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Materials and Methods

Study cohorts

In order to study the effects of mutation in P2X7R, we evaluated a large international cohort of cardiac-transplanted patients, which includes the US-based Clinical Trials in Organ Translantation-05 (*CTOT-05*) cohort, the Northern Italian Transplant Group (*NIT*, Bergamo, Italy) and the Inter Regional Transplant Association (*AIRT*, Bologna, Italy), with 747 patients in total receiving their first cardiac transplantation. The *CTOT-05*, an observational study designed to understand the natural history of cardiac allograft vasculopathy (CAV), was used to assess the presence of early CAV through intravascular ultrasound (IVUS) exam at 1 year. The *CTOT-05* cohort consisted of 200 cardiac-transplanted patients enrolled in the US from June 2007 to July 2011. Primary outcome measures were a composite of incidence of death, re-transplantation or re-listing for transplantation, biopsy-proven acute rejection (BPAR) of > 2R, episode of rejection associated with hemodynamic compromise, and coronary artery vasculopathy defined by a change in Maximal Intimal Thickness (MIT) of ≥0.5 mm between study entry and 12 months. At 1 year post-transplantation, 102 out of the 148 genotyped patients were examined through IVUS for early rejection/CAV to determine whether the mutated P2X7R allele was associated with development of early CAV. The *NIT*-Bergamo cohort consisted of 187 patients who underwent cardiac transplantation at Ospedali Riuniti in Bergamo from 1985 to 2012. The number of clinically-assessed and/or biopsy-proven acute rejection episodes as well as the requirement for medical intervention in at least 3 episodes per year (n > 3/year) in the first year was assessed as clinical outcome in this cohort. 181 out of 187

patients were evaluated. The *AIRT*-Bologna cohort consisted of 360 patients who underwent heart transplantation at S. Orsola Hospital, Bologna, Italy between 1985 and 2013. The occurrence of major adverse cardiac events (MACE), which included non-fatal myocardial infarction, coronary revascularization, or hospitalization for graft failure at 10 years of follow-up was assessed as clinical outcome in this cohort. Among 359 patients genotyped, 130 were eligible for this analysis.

Immunosuppressive treatment at blood collection is described in Supplemental Table S1. Immunosuppression was not standardized; doses and levels of immunosuppressive drugs were defined and maintained within therapeutic ranges as per local practice.

Blood was collected at 1.1±0.4 years of follow-up in the *CTOT-05* for genotyping, at 5±1.2 years of follow-up in the *NIT*-Bergamo cohort and at 15±0.8 years of follow-up in the *AIRT*-Bologna cohort for genotyping and immunological studies (Supplemental Table S2). As proof-of-concept of the role of the P2X7R mutation in affecting T cell activation, we enrolled 50 healthy volunteers matched for age and gender and collected blood for genotyping and for immunological studies (Supplemental Table S4). The frequency of the P2X7R mutation was also assessed in the NHLBI GO Exome Sequencing Project (ESP) cohort, which includes genome sequencing of a large and well-phenotyped population in the United States (n=4300, http://evs.gs.washington.edu/EVS).

Clinical endpoints

In the *CTOT-05* cohort, intracoronary ultrasonography (IVUS) was performed at study entry and at 12 months after transplantation to detect early CAV. Early CAV was defined by a change ≥ 0.5mm in Maximal Intimal Thickness (MIT)(1) within one year after transplantation. In the *NIT*-Bergamo cohort, the number of clinically-assessed and/or

biopsy-proven acute rejection events was assessed and used as a clinical endpoint. Requirement of medical interventions in a number of episodes higher than 3 was also assessed by reviewing hospitalization records. In the *AIRT*-Bologna cohort, Major Adverse Cardiac Events (MACE) were evaluated and verified through a review of hospital admissions and diagnosis. MACE was defined as a combined occurrence of non-fatal myocardial infarction, coronary revascularization, and hospitalization for graft failure.

P2X7R protein quantification

P2X7R protein was quantified for ubiquitination studies, subcellular localization, and surface expression analysis using the Human P2RX7 ELISA Kit (LS-F12239, LSBio, Seattle, WA) according to the manufacturer's instructions.

NLRP3/P2X7R DNA methylation

Methylation of the *NLRP3* promoter was assessed in $CD4^+$ T cells isolated from carriers and non-carriers at baseline conditions and in non-carrier $CD4^+$ T cells upon exposure to the P2X7R inhibitor CE-224,535 as described in the Methods section. Genomic DNA was extracted, and sodium bisulfate sequencing was performed using the EZ DNA Methylation Gold Kit (D5005, Enzo Life Sciences, Farmingdale, NY). Briefly, 2 μg of genomic DNA was treated according to the manufacturer's instructions. The modified DNA was amplified with primers that were specific for the *NLRP3* promoter region and that have been previously described(2). A similar approach was employed for detecting methylation of the *P2X7R* promoter(3), in $CD4^+$ T cells of carriers and non-carriers.

mRNA decay assay

$CD4^+$ T cells were plated and treated with 5 μg/mL actinomycin D (A1410-2MG, Sigma-Aldrich, St. Louis, MO) for 4 hours and then harvested by TRIzol LS (15596026, Ambion,

Austin, TX) extraction according to the manufacturer's instructions. qRT-PCR was used to assess the half-lives of *P2X7R* or *NLRP3* transcripts and the mean mRNA level at each time point was normalized to the mean mRNA level of β-actin remaining at each time point.

Anti-vimentin antibody levels

Plasma collected from carrier and non-carrier patients was tested for antibodies against vimentin using the Human Anti-VIM (Anti-Vimentin Antibody) ELISA Kit (MBS2533441, MyBiosource, San Diego, CA) according to the manufacturer's instructions.

Assessment of P2X7R DNA binding using modified ChIP

We performed chromatin immunoprecipitation using an Agarose ChIP Kit (26156, Thermo Fisher Scientific, Waltham, MA). Briefly, $CD4^+$ T cells were isolated as above, and protein-DNA complexes were stabilized and then extracted. *In vivo* crosslinking was achieved with formaldehyde. To lyse, extract and solubilize the crosslinked complexes, the Thermo Fisher Scientific Chromatin Prep Module was used (included in the kit), thus reducing contamination of the chromatin-bound DNA from other cellular compartments and enriching samples for P2X7R. Once chromatin was digested, immunoprecipitation of P2X7R was performed as described in the main text (see *Immunoprecipitation*). To analyze protein-binding sequences, genomic DNA was sheared into small pieces with enzymatic digestion as per the manufacturer's instructions. After proteinase digestion and crosslink reversal, DNA was purified, and expression of genes of interest (*NLRP3, STAT5A, MYD88, NFKB1, IL10*) was assessed using a TaqMan® Copy Number Assay (4400291, Applied Biosystems, Beverly, MA; Hs03362171_cn; Hs06421032_cn; Hs01838632_cn;

Hs01130065_cn; Hs00323100_cn). Expression of genes was normalized to that of total non-immunoprecipitated DNA extracted from the same number of cells.

Animal studies

Mice

C57BL/6, B6.129P2-*P2rx7tm1Gab*/J (*P2X7R*$^{-/-}$) and B6.C-H2^{bm12} (bm12) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were cared for and used in accordance with institutional guidelines. *P2X7R*$^{-/-}$ mice were generated by disrupting the last exon coding for the long C–terminal cytoplasmic tail, thus paralleling the mutation observed in humans(4).

Heart transplantation

Vascularized cardiac allografts were transplanted intra-abdominally using microsurgical techniques as described by Corry *et al*.(5). Rejection was determined as complete cessation of cardiac contractility and was confirmed by direct visualization. Animals were sacrificed at day 40 after transplantation for *in vitro* studies and for pathology.

***Interventional studies* in vivo**

Anti-IL-17 (BE0173, BioxCell, West Lebanon, NH) was administered to C57BL6/J (B6) and to *P2X7R*$^{-/-}$ recipients of bm12 heart transplants intraperitoneally (i.p.) at the following dose: 0.1 mg at days 0, 1, 2, 3, 5, 7, 9, 11, and 13.

Histology and immunohistochemistry

Immunohistochemistry was performed with 5-μm-thick formalin-fixed, paraffin-embedded tissue section. Photomicrographs (400×) were taken using an Olympus BX41 microscope (Center Valley, PA). Graft histology was evaluated by a pathologist and was

quantified as follows: (i) heart coronary vasculopathy: 0, normal arteries; 1, mild arterial wall infiltration; 2, heavy arterial wall infiltration with partial luminal occlusion; 3, complete luminal occlusion; and (ii) heart cell infiltrate: 0, no cell infiltrate; 1, mild cell infiltrate; 2, medium cell infiltrate; 3, heavy cell infiltrate.

Immunological studies

Isolation of splenocytes and CD4$^+$ cells

Recipient lymphocytes were isolated from spleens at 40 days after transplantation as previously described(6). CD4$^+$ cells were obtained by magnetic microbead isolation (130-049-201, Miltenyi Biotec, Auburn, CA).

Flow cytometry and intracellular cytokine staining

Anti-mouse CD4 (100538), CD8 (553031), CD25 (553866), CD44 (553134), CD62L (553152), IFN-γ (12731182), IL-17 (506904), IL-10 (554467), IL-4 (554435) and FoxP3 (17577382) were purchased from Biolegend (San Diego, CA), BD Biosciences (San Jose, CA) and eBioscience (San Diego, CA). Anti-mouse P2X7R (APR-008F) was purchased from Alomone Labs (Jerusalem, Isarael), and anti-mouse NLRP3 (IC7578A) was purchased from R&D Systems. Intracellular staining for cytokines was performed as described above using the Cytofix/Cytoperm kit (544722, BD Biosciences) and following the manufacturer's instructions(7). Th1 cells were defined as producing IFN-γ, Th2 cells were defined as producing either IL-10 or IL-4, and Th17 cells were defined as producing IL-17. Flow cytometry was performed using a FACSCalibur™ flow cytometry system (Becton Dickinson, Franklin Lakes, NJ) and analyzed using Flowjo software (version 6, Treestar, Ashland, OR). Results are representative of at least 3 independent experiments.

Enzyme-linked immunoSpot assay (ELISPOT)

An ELISPOT assay was used to measure the number of IFN-γ-producing and IL-4-producing cells (551881 and 551878, BD Biosciences). Allogeneic donor splenocytes (bm12) or anti-CD3- and anti- CD28-Ig (553058 and 553295 BD Biosciences, 0.5 μg/ml each) were used to stimulate $1x10^6$ responder splenocytes (C57BL/6 or *P2X7R*$^{-/-}$). Spots were counted using an Immunospot analyzer (Cellular Technology Ltd., Cleveland, OH) as previously described(8).

Serum cytokine assessment (Luminex)

Treated and untreated transplanted P2X7R$^{-/-}$ and B6 recipient mice were bled via tail vein, and serum samples were collected. The Bio-Plex Pro™ Mouse Cytokine 8-plex (m60000007a), and the Bio-Plex Pro™ Mouse Cytokine IL-6 and IL-17A Sets (171g5007m and 171g5013m, Bio-Rad, Hercules, CA), were used according to the manufacturer's protocol to determine cytokine levels. After the addition of stop solution, sample cytokine levels were calculated from a standard curve using a Luminex 200 reader (Bio-Rad).

Statistical analysis

Continuous variables are presented as means and standard errors, and categorical variables are presented as proportions. We used independent sample t-tests to compare continuous variables and chi-square test/Fisher's exact test to compare categorical variables. For multiple comparisons, one-way or two-way ANOVA followed by Bonferroni's *post hoc* test between the group of interests and all other groups was used. Genotype variable was dichotomized as carrier or non-carrier of the mutated P2X7R allele in the analysis of clinical outcomes to assess odds ratio (OR). The number of clinically-determined and/or

biopsy-proven acute rejection episodes within the first year in the *NIT*-Bergamo cohort was dichotomized as > 3 or ≤ 3 in order to perform the OR analysis. The association between immune events and carrier/non-carrier status in each cohort was analyzed in 413 total participants who had follow-up events recorded. A multivariable logistic regression analysis was performed by including age, gender and relevant parameters related to the outcome. The multivariable logit of age was linear as detected by using multivariable fractional polynomials. The multivariable logistic regression analysis was performed in 412 total participants (missing therapy data in one patient in the *CTOT-O5* cohort). Analysis of Th17 proportion/levels was adjusted for all relevant parameters.

Kaplan-Meier analysis (log-rank test) was performed for survival studies in preclinical models. A P value of < 0.05 (by two-tailed testing) was considered an indicator of statistical significance. All analyses were performed with the use of STATA 12 and Prism Graphpad (La Jolla, CA) 6 software.

SUPPLEMENTAL TABLES

Supplemental Table S1. Clinical characteristics of the cohorts examined.

Characteristics	*CTOT-05* (n=200)	*NIT*-Bergamo (n=187)	*AIRT*-Bologna (n=360)
Males, Number (%)	85 (80.2)	133 (71.5)	291 (80.8)
Age at transplantation, median (IQR), y	55 (46 - 63)	47 (29 - 56)	54 (43 - 60)
Ethnicity, Number (%)			
Caucasian	149 (74.5)	185 (99.0)	355 (98.6)
African-American	32 (16.0)	-	-
Asian	7 (3.5)	-	-
Other	12 (6.0)	2 (1.0)	5 (0.4)
Cardiomyopathy, Number (%)			
Idiopathic	76 (38.0)	95 (50.8)	126 (35.1)
Ischemic	70 (35.0)	45 (24.1)	121 (33.4)
Other	54 (27.0)	47 (25.1)	113 (31.5)
P2X7R genotype, Number (%)			
A/A	90 (60.8)	111 (61.3)	236 (65.7)
A/C	46 (31.0)	60 (33.2)	93 (25.9)
C/C	12 (8.1)	10 (5.5)	30 (8.4)
Immunosuppression*, Number (%)			
Steroids	172 (97.7)	21 (11.2)	222 (63.4)
CNIs	173 (98.3)	166 (88.8)	358 (100.0)
mTOR inhibitor	-	24 (12.8)	82 (23.4)
Micophenolate mofetil	173 (98.3)	23 (12.3)	198 (55.1)
Azathioprine	-	89 (47.6)	35 (10.0)

Abbreviations: *CTOT-05*, Clinical Trial in Organ Transplantation-05; NIT, Northern Italian Transplant Group; AIRT, Inter Regional Transplant Association; *P2X7R*, Purinergic receptor-7; y, years; IQR, interquartile range; CNIs, calcineurin inhibitors; *Immunosuppression was recorded at the time of blood collection.

Supplemental Table S2. List of characteristics of samples collected from cardiac-transplanted patients for mechanistic studies.

See uploaded Excel file.

Abbreviations: Tx, transplantation; CsA, cyclosporine A; FK, Tacrolimus; ST, steroids; MMF, mycophenolate; mTOR, mTOR inhibitors; Aza, azathioprine; PBMCs, peripheral blood mononuclear cells. *Immunosuppression was recorded at the time of blood collection.

Supplemental Table S3. List of upregulated and downregulated inflammation- and T cell activation-related genes identified by transcriptome profiling in $CD4^+$ cells obtained from carrier as compared to non-carrier patients. Genes with statistically significant differences ($p<0.05$) are in bold.

Upregulated genes	Downregulated genes
AIM2	*MAP3K7*
BCL2L1	*MAPK3*
BIRC2	***NLRP3***
BIRC3	***P2RX7***
CASP1	
CASP5	
CASP8	
CCL2	
CD40LG	
CTSB	
CXCL1	
HSP90AA1	
HSP90AB1	
HSP90B1	
IKBKB	
IKBKG	
IL12A	
IL6	
IRF1	
MAPK1	
MAPK13	
MEFV	
MYD88	
NLRC4	
NLRP1	
NLRP6	
PANX1	
PSTPIP1	
PYCARD	
RELA	
RIPK2	
TNF	
TXNIP	

Supplemental Table S4. Clinical characteristics of healthy volunteers included in the study.

Characteristics	*Healthy Volunteers* (n=50)
Males, Number (%)	24 (48.0)
Age, median (IQR), y	25 (26 - 46)
Ethnicity, Number (%)	
Caucasian	50 (100.0)
P2X7R genotype, Number (%)	
A/A	31 (62.0)
A/C	18 (36.0)
C/C	1 (2.0)

Abbreviations: *P2X7R*, Purinergic receptor-7; y, years; IQR, interquartile range.

Supplemental Table S5. Head-to-head comparison between human P2X7R mutation carriers and murine *P2X7R*$^{-/-}$ with respect to immunological features.

	Human Carriers	**Murine *P2X7R* $^{-/-}$**
Peripheral Th17 cells (%)	↑↑	↑↑
Peripheral IL-17 level	↑↑	↑
Peripheral Th2 cells (%)	↓↓↓	↓↓
Peripheral IL-4 level (ng/ml)	→	↓
Peripheral Th1 cells (%)	↑	↑↑
Peripheral INF-γ level (ng/ml)	→	→
Peripheral CD4$^+$ T eff/mem cells (%)	↑↑	↑↑
Peripheral CD8$^+$ T eff/mem cells (%)	↑	↑
Peripheral Treg cells (%)	→	→
NLRP3 expression on CD4$^+$ T cells (%)	↓	↓↓
Signs of CAV	↑↑	↑↑

Abbreviations: *P2X7R*$^{-/-}$, mice in which P2X7R has been genetically deleted; CAV, chronic allograft vasculopathy; Th17, T-helper 17 cells; Th1, T-helper 1 cells; Th2, T-helper 2 cells; IL-17, interleukin 17; IL-4, interleukin 4; IFN-γ, interferon gamma; eff/mem, effector memory; Treg, regulatory T cells; NLRP3, NOD-like receptor P3.

Supplemental Table S6. *P2X7R* genotype prevalence in cardiac-transplanted patients included in the study and in the general population (Exome Sequencing Project).

Group	***P2X7R***		
	AA	AC	CC
Transplanted patients (n=688)	437 (63.5%)	199 (28.9%)	52 (7.6%)
Ischemic (n=217)	145 (66.8%)	64 (29.5%)	8 (3.7%)
Non-ischemic (n=471)	292 (70.0%)	135 (28.7%)	44 (9.3%)
Exome Sequencing Project (n=4300)	2860 (66.5%)	1290 (30.0%)	185 (3.5%)

Abbreviations: *P2X7R*, Purinergic receptor-7; AA, homozygous non-mutated patients; AC, heterozygous patients; CC, homozygous mutated patients; n, number.

Supplemental Table S7. Multivariable analysis of factors associated with development of rapidly progressive cardiac allograft vasculopathy defined by evaluating IVUS-measured coronary artery maximal intimal thickness (MIT) > 5 mm in the *CTOT-05* cohort (1 year follow-up).

	M1	M2	M3
P2X7R mutation (carrier/non-carrier)	3.24* [1.15 to 9.15]	3.22* [1.14 to 9.10]	2.99* [1.04 to 8.59]
Gender (Male)	8.73* [1.05 to 72.96]	9.12* [1.09 to 76.43]	8.94* [1.06 to 75.26]
Age (y) / 10	0.82 [0.53 to 1.25]	0.84 [0.54 to 1.29]	0.86 [0.56 to 1.32]
Pre-tx ischemic cardiomyopathy	—	0.67 [0.19 to 2.40]	0.63 [0.17 to 2.30]
Induction Therapy	—	—	0.62 [0.20 to 1.90]
N of patients	101	101	101

Odds ratios and 95% confidence intervals in brackets.
*$p < 0.05$

Abbreviations: *CTOT-05*, Clinical Trial in Organ Transplantation-05; M, model; M1-M3, multivariable logistic regression; P2X7R, Purinergic receptor-7; Tx, transplant; N, number; y, years.

Supplemental Table S8. Multivariable analysis of factors associated with development of clinically-assessed and/or biopsy-proven acute rejection episodes in the *NIT*-Bergamo cohort (1 year follow-up).

	M1	M2	M3
P2X7R mutation (carrier/non-carrier)	3.12 [0.99 to 9.86]	3.07 [0.97 to 9.69]	3.26* [1.01 to 10.48]
Gender (Male)	1.06 [0.38 to 2.96]	1.14 [0.40 to 3.23]	1.16 [0.40 to 3.32]
Age (y) / 10	1.34* [1.03 to 1.75]	1.44* [1.07 to 1.94]	1.42* [1.05 to 1.92]
Pre-tx ischemic cardiomyopathy	—	0.50 [0.15 to 1.63]	0.51 [0.16 to 1.68]
Use of Azathioprine	—	—	1.46 [0.49 to 4.37]
Use of mTOR inhibitors	—	—	1.00 [0.23 to 4.40]
N of patients	181	181	181

Odds ratios and 95% confidence intervals in brackets.
*$p < 0.05$

Abbreviations: NIT, Northern Italian Transplant Group; M, model; M1-M3, multivariable logistic regression; P2X7R, Purinergic receptor-7; Tx, transplant; N, number; y, years.

Supplemental Table S9. Multivariable analysis of factors associated with development of long-term major cardiac adverse events (MACE) in the *AIRT*-Bologna cohort (10 years follow-up).

	M1	M2	M3
P2X7R mutation (carrier/non-carrier)	2.89* [1.29 to 6.47]	2.87* [1.28 to 6.45]	2.83* [1.26 to 6.38]
Gender (Male)	3.25* [1.07 to 9.81]	3.19* [1.04 to 9.74]	3.01 [0.98 to 9.22]
Age (y) / 10	1.48* [1.04 to 2.10]	1.46* [1.02 to 2.11]	1.48* [1.02 to 2.14]
Pre-tx ischemic cardiomyopathy	—	1.10 [0.46 to 2.62]	1.13 [0.47 to 2.69]
Use of Azathioprine	—	—	1.12 [0.38 to 3.31]
Use of mTOR inhibitors	—	—	1.60 [0.60 to 4.29]
N of patients	130	130	130

Odds ratios and 95% confidence intervals in brackets
*$p < 0.05$

Abbreviations: AIRT, Inter Regional Transplant Association; M, model; M1-M3, multivariable logistic regression; P2X7R, Purinergic receptor-7; Tx, transplant; N number; y, years.

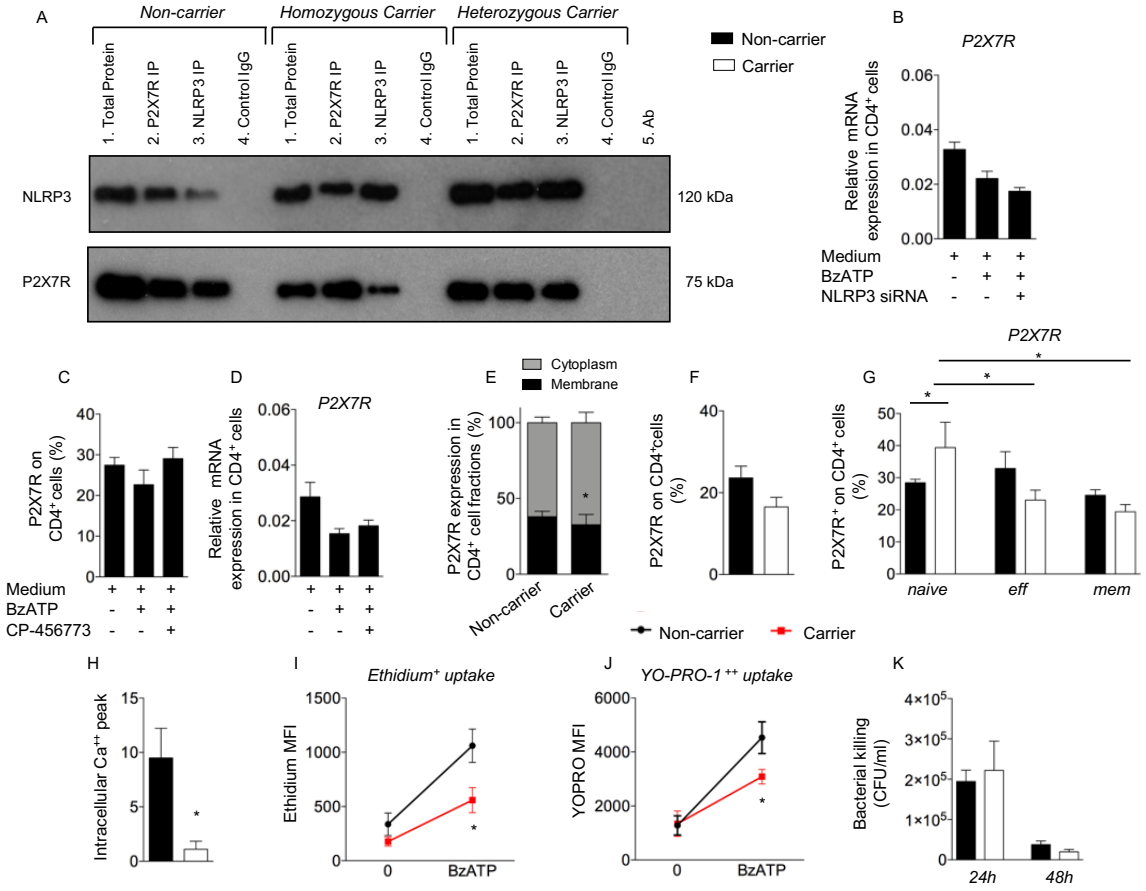
Supplemental Table S10. Main characteristics of primers used in qRT-PCR analysis.

Gene Symbol:	**UniGene #:**	**Refseq Accession #:**	**Band Size (bp)**:	**Reference Position**:
P2X7R	Hs.729169	NM_002562.5	89	1329
P2X4R	Hs.321709	NM_001256796.1	91	638
P2X1R	Hs.41735	NM_002549.1	73	1188
NLRP3	Hs.159483	NM_001079821.2	84	421
RORc	Hs.256022	NM_001001523.1	62	1480
GATA3	Hs.524134	NM_001002295.1	80	802
IL4	Hs.73917	NM_000589.3	70	427
IL10	Hs.193717	NM_000572.2	74	510
CASP1	Hs.2490	NM_001223.4	76	1099
PYCARD	Hs.499094	NM_013258.4	61	422
ACTB	Hs.520640	NM_001101	174	730

Abbreviations: Refseq, reference sequence.

SUPPLEMENTAL FIGURES

Supplemental Figure S1. NLRP3 inhibition does not alter P2X7R expression, while the P2X7R loss-of-function mutation slightly alters ion influx and bacterial killing capability.



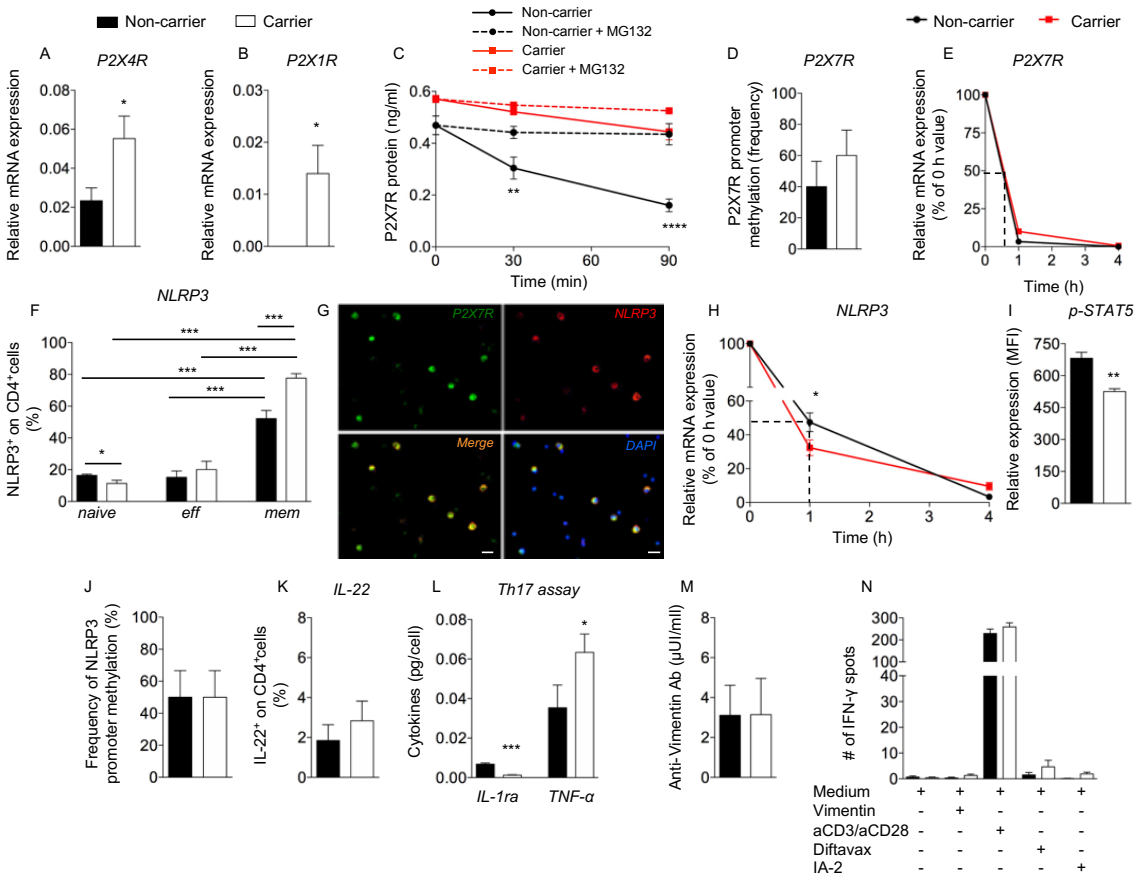
A. P2X7R and NLRP3 immunoprecipitation in human CD4$^+$ T cells obtained from homozygous non-carrier patients, and from homozygous and heterozygous carriers of the mutation. Expression of NLRP3 and P2X7R are shown in the upper and lower blot respectively. (Upper blot). Lane 1: total protein; Lane 2: immunoprecipitation of sample with P2X7R Ab; Lane 3: immunoprecipitation of sample with NLRP3 Ab. Lane 4: immunoprecipitation with Control IgG; Lane 5: immunoprecipitation with Abs alone (NLRP3 Ab). (Lower blot). Lane 1: total protein; Lane 2: immunoprecipitation of sample

with P2X7R Ab; Lane 3: immunoprecipitation of sample with NLRP3 Ab. Lane 4: immunoprecipitation with Control IgG; Lane 5: immunoprecipitation with Abs alone (P2X7R Ab). Experiment was run in triplicate; representative blots shown. **B**. Bar graph representing normalized mRNA expression of *P2X7R* measured in $CD4^+$ T cells obtained from non-carrier patients and exposed to transient knockdown of NLRP3 using siRNA, at baseline and upon BzATP stimulation (n=4 samples per group). mRNA expression was normalized to *ACTB*. **C**, **D**. Bar graphs representing P2X7R protein (C) and *P2X7R* mRNA (D) expression evaluated by flow cytometry and qRT-PCR respectively, in $CD4^+$ T cells obtained from non-carrier patients and treated with the inflammasome inhibitor CP-456773 at baseline and upon BzATP exposure (n=3 samples per group). mRNA expression was normalized to *ACTB*. All samples were run in duplicate. **E**. Grouped graph representing the subcellular localization of P2X7R (membrane and cytoplasm) in $CD4^+$ T cells obtained from patients carrying the mutant P2X7R allele and from non-carrier patients (n=3 samples per group). Data are expressed as percentage of P2X7R measured in each cell compartment out of total P2X7R. All samples were run in duplicate. **F**. Bar graph representing percentage of P2X7R surface expression on total $CD4^+$ T cells obtained from carrier and non-carrier patients measured by flow cytometry (n=5 samples per group). **G.** Bar graph representing percentage of $P2X7R^+$ cells within $CD4^+CD45RO^-CCR7^+$ (naïve), $CD4^+CD45RO^-CCR7^-$ (effector), and $CD4^+CD45RO^+CCR7^-$ (memory) T cells obtained from carrier and non-carrier patients measured by flow cytometry (n=5 samples per group). **H.** Bar graph representing the intracellular calcium peak measured by fluorimetry upon P2X7R stimulation with BzATP in $CD4^+$ T cells of carrier and non-carrier cardiac-transplanted patients. Results are representative of at least 3 independent experiments (n=8 samples per

group). **I**, **J**. Line graphs representing the mean fluorescence intensity (MFI) of large cations ethidium^{+} (I) and YO-PRO-1^{++} (J) measured by flow cytometry following gating on live cells within CD4^{+} T cells obtained from carrier (red line) and non-carrier (black line) patients at baseline and after BzATP stimulation (n=8 samples per group). **K**. Bar graph representing viable bacteria (CFU/ml) counted at 24 and 48 hours in differentiated macrophages, obtained from CD14^{+} cells of carrier and non-carrier patients, previously incubated with equal numbers of bacteria (*E. coli*). All samples were run in triplicate (n=5 samples per group). Results are expressed as mean ± SEM. All parameters examined were significantly statistically different when comparing different groups by using Student's t test or 1-way ANOVA with *post hoc* Bonferroni's test as follows: $^{*}p<0.05$.

Abbreviations: P2X7R, purinergic receptor-7; NLRP3, NOD-like receptor (NLR)P3; qRT-PCR, quantitative real-time polymerase chain reaction; SEM, standard error of mean; BzATP, Benzoyl ATP; Ab, antibody; eff, effector cells; mem, memory cells; CFU, colony forming unit; MFI, mean fluorescence intensity; IP, immunoprecipitation, IgG, immunoglobulin; *ACTB*, beta actin; siRNA, silencing RNA.

Supplemental Figure S2. Expression of other P2X receptors, role of P2X7R within the P2X7R/NLRP3 pathway in CD4$^+$ T cells of carrier and non-carrier patients, Th17 skewing, and the anti-vimentin autoimmune response.



A, **B**. Bar graphs representing mRNA expression of *P2X4R* and *P2X1R* on CD4$^+$ T cells obtained from carrier and non-carrier patients measured by qRT-PCR. All samples were run in triplicate and normalized to *ACTB* relative expression level (n=5 samples per group). **C**. Line graph representing the percentage of remaining P2X7R protein observed at different timepoints in CD4$^+$ T cells of carrier and non-carrier patients treated with ubiquitin/protease inhibitor MG132 (dashed lines) or left untreated (solid lines), (n=3 samples per group). All samples were run in duplicate**. D**. Bar graph representing the frequency of *P2X7R* promoter methylation detected in CD4$^+$ T cells of carrier and non-

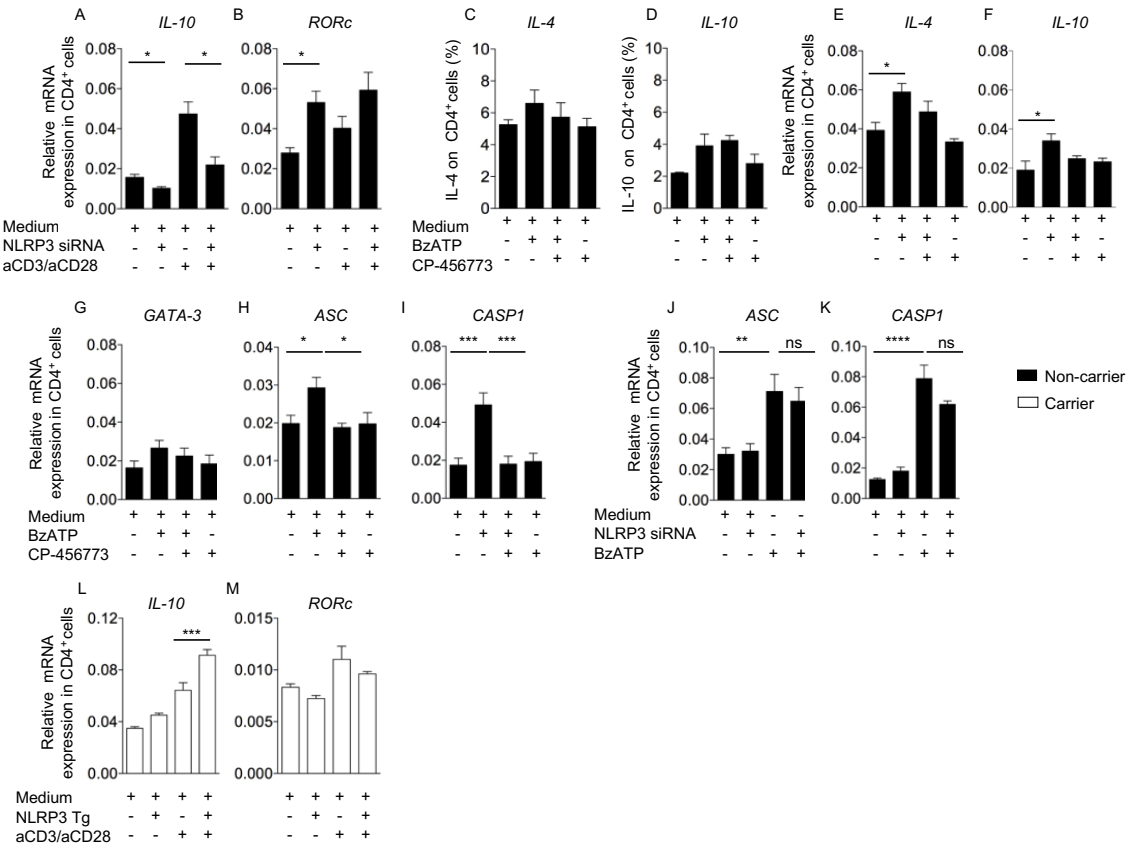
carrier patients, (n=10 samples per group). Data are expressed as percentage of carrier vs. non-carrier DNA samples with methylation of the *P2X7R* promoter. All samples were run in triplicate**. E**. Actinomycin D (5 μg/mL) was added to $CD4^+$ T cells obtained from carrier and non-carrier patients to block transcription, and qRT-PCR was performed for *P2X7R* at baseline before treatment (0 hours) and after 1 and 4 hours of treatment. Each point shows the mean percentage of mRNA remaining relative to the 0 h control ± SEM. All samples were run in triplicate (n=3 samples per group). **F**. Bar graph representing percentage of NLRP3 expression on $CD4^+CD45RO^-CCR7^+$ (naïve), $CD4^+CD45RO^-CCR7^-$ (effector), and $CD4^+CD45RO^+CCR7^-$ (memory) T cells obtained from carrier and non-carrier patients measured by flow cytometry (n=5 samples per group). **G**. Confocal analysis (40X original magnification, scale bar 20 μm) of P2X7R (green) and NLRP3 (red) co-expression in $CD4^+$ T cells obtained from patients carrying the mutant P2X7R allele (n=3 samples analyzed). Cells were stained with DAPI (blue) and immunolabeled with anti-P2X7R (green) and anti-NLRP3 Abs (red). Merge, third column (n=3 samples analyzed). **H**. Actinomycin D (5 μg/mL) was added to $CD4^+$ T cells obtained from carrier and non-carrier patients to block transcription, and qRT-PCR was performed for *NLRP3* at baseline before treatment (0 hours) and after 1 and 4 hours of treatment. Each point shows the mean percentage of mRNA remaining relative to the 0 h control ± SEM. All samples were run in triplicate (n=3 samples per group). **I**. Bar graph representing relative mean fluorescence intensity (MFI) of phosphorylated STAT5 (Tyr694) detected by flow cytometry in $CD4^+$ T cells of carrier and non-carrier cardiac-transplanted patients (n=3 samples per group). **J**. Bar graph representing the frequency of *NLRP3* promoter methylation detected in $CD4^+$ T cells of carrier and non-carrier patients, (n=10 samples per group). Data are expressed as

percentage of carrier vs. non-carrier DNA samples with methylation of the *NLRP3* promoter. All samples were run in triplicate. **K**. Bar graph depicts the percentage of IL-22 expression on *in vitro*-generated Th17 cells in patients carrying the mutant allele as compared to non-carrier patients (n=3 samples per group). **L**. Bar graph representing levels of the pro-inflammatory cytokine TNF-α and the anti-inflammatory cytokine IL-1ra, measured by Luminex using the culturing supernatant of Th17-polarized cells obtained from patients carrying the mutant allele as compared to non-carrier patients (n=3 samples per group). All samples were run in duplicate. **M**. Bar graph representing levels of anti-vimentin autoantibodies measured in plasma samples of carrier and non-carrier patients, (n=10 samples per group). All samples were run in triplicate. **N**. Bar graph representing IFN-γ-producing cells in an autoimmune ELISPOT assay in which PBMCs of carrier and non-carrier patients were previously exposed to the self-peptide vimentin, and to other self-peptides such as IA-2 or control conditions (difteric-toxoid vaccine and anti-CD3-Ig/anti-CD28-Ig stimulation). Number of IFN-γ spots are reported, n=3 samples per group. All samples were run in triplicate. Results are expressed as mean ± SEM. All parameters examined were significantly statistically different (Student's *t* test, 1-way or 2-way ANOVA with *post hoc* Bonferroni's test) when comparing different groups by using as follows: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

Abbreviations: P2X7R, purinergic receptor-7; P2X1R, purinergic receptor-1; P2X4R, purinergic receptor-4; qRT-PCR, quantitative real-time polymerase chain reaction; SEM, standard error of mean; BzATP, Benzoyl ATP; eff, effector cells; mem, memory cells; NLRP3, NOD-like receptor (NLR)P3; h, hours; Ab, antibodies; *ACTB*, beta actin; MFI, mean fluorescence intensity; IL-1ra, interleukin 1 receptor antagonist; TNF-α, tumor

necrosis factor alpha; IL-22,interleukin 22; Th17, T-helper 17 cells; IFN-γ, interferon gamma; PBMCs, peripheral blood mononuclear cells.

Supplemental Figure S3. **NLRP3 silencing/overexpression is associated with altered T-helper cell mRNA profile**.

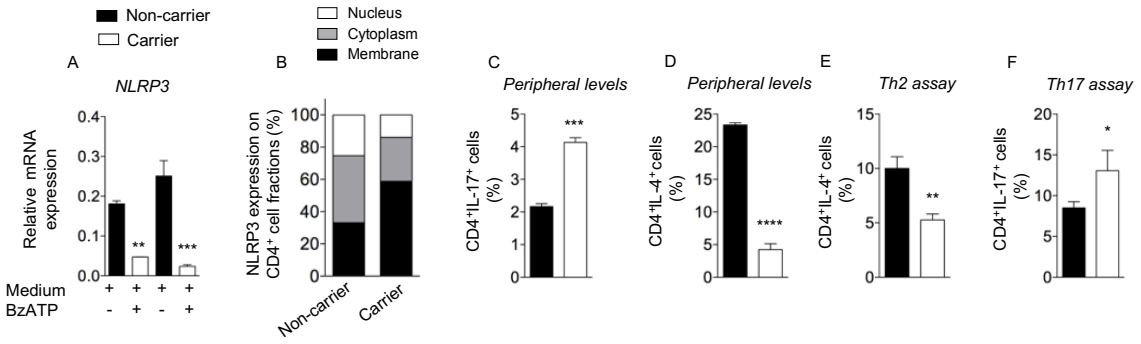


A, **B**. Bar graphs representing normalized mRNA expression of the Th2-related factor *IL-10* (A) and the Th17-related factor *RORc* (B) measured in CD4$^+$ T cells obtained from non-carrier patients with transient knockdown of *NLRP3* using siRNA, at baseline and after T cell stimulation (n=3 samples per group). mRNA expression was normalized to *ACTB*. All samples were run in duplicate. **C**, **D**. Bar graphs representing percentage of IL-4 (C) and IL-10 (D) expression evaluated by flow cytometry in CD4$^+$ T cells obtained from non-carrier patients and treated with the inflammasome inhibitor CP-456773 at baseline and upon BzATP exposure (n=3 samples per group). **E**, **F G**, **H**, **I**. Bar graphs representing normalized mRNA expression of the Th2-related factors *IL-4* (E), *IL-10* (F), *GATA-3* (G) and of the inflammasome components *ASC* (H) and *CASP1* (I) measured in CD4$^+$ T cells

obtained from non-carrier patients and treated with the inflammasome inhibitor CP-456773 at baseline and upon BzATP exposure (n=4 samples per group). mRNA expression was normalized to *ACTB*. All samples were run in duplicate. **J**, **K**. Bar graphs representing normalized mRNA expression of *ASC* (J) and *CASP1* (K) measured in $CD4^+$ T cells obtained from non-carrier patients and exposed to transient knockdown of NLRP3 using siRNA, at baseline and upon BzATP stimulation (n=4 samples per group). mRNA expression was normalized to *ACTB*. All samples were run in triplicate. **L**, **M**. Bar graphs representing normalized mRNA expression of the Th2-related factors *IL-10* (L), and of the Th17-related factor *RORc* (M) measured in $CD4^+$ T cells obtained from carrier patients, in which NLRP3 was overexpressed, at baseline and after T cell stimulation (n=3 samples per group). mRNA expression was normalized to *ACTB*. All samples were run in duplicate. Results are expressed as mean ± SEM. All parameters examined were significantly statistically different when comparing different groups by using Student's *t* test or 1-way ANOVA with *post hoc* Bonferroni's test as follows: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

Abbreviations: NLRP3, NOD-like receptor (NLR)P3; P2X7R, purinergic receptor-7; SEM, standard error of mean; BzATP, Benzoyl ATP; IL-10, interleukin 10; NLRP3 siRNA, *NLRP3* silencing mRNA; NLRP3 Tg, overexpression of *NLRP3*; aCD3, antiCD3-Ig stimulation; aCD28, antiCD28-Ig stimulation; *ACTB*, beta actin; Th2, T-helper 2 cells; Th17, T-helper 17 cells; *CASP1*, caspase 1; *IL-4*, interleukin 4; *ASC*, *PYCARD* gene; *RORc*, RAR Related Orphan Receptor C gene.

Supplemental Figure S4. **The P2X7R/NLRP3 pathway is similarly altered in untransplanted patients carrying the mutated P2X7R allele.**



A. Bar graphs comparing expression of *NLRP3* mRNA using qRT-PCR in $CD4^+$ T cells obtained from untransplanted patients carrying the mutated P2X7R allele with those of non-carrier patients with/without BzATP. Experiments were run in triplicate. mRNA expression was normalized to *ACTB*. **B**. Grouped graph representing the subcellular localization of NLRP3 (membrane, cytoplasm and nucleus) in $CD4^+$ T cells obtained from patients carrying the mutated P2X7R allele and from non-carrier patients. Experiments were run in triplicate. **C**, **D**. Bar graphs representing the percentage of peripheral $IL\text{-}17^+$ (C) and $IL\text{-}4^+$ (D) $CD4^+$ T cells of carrier and non-carrier patients. Experiments were run in triplicate. **E**, **F**. Bar graph representing the percentage of *in vitro*-generated Th2 (E) and Th17 (F) cells obtained from naïve $CD4^+$ T cells of carrier and non-carrier patients. Experiments were run in triplicate. Results are expressed as mean ± SEM. All parameters examined were significantly statistically different when comparing different groups by using Student's *t* test or 1-way ANOVA with *post hoc* Bonferroni's test as follows: $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

Abbreviations: NLRP3, NOD-like receptor (NLR)P3; P2X7R, purinergic receptor-7; qRT-PCR, quantitative real-time polymerase chain reaction; SEM, standard error of mean,

Th2, T-helper 2 cells; Th17, T-helper 17 cells, IL-4, interleukin 4; IL-17, interleukin 17; BzATP, Benzoyl ATP; *ACTB*, beta actin.

References

1. Starling RC, Stehlik J, Baran DA, Armstrong B, Stone JR, Ikle D, et al. Multicenter Analysis of Immune Biomarkers and Heart Transplant Outcomes: Results of the Clinical Trials in Organ Transplantation-05 Study. *Am J Transplant.* 2016;16(1):121-36.
2. Wei M, Wang L, Wu T, Xi J, Han Y, Yang X, et al. NLRP3 Activation Was Regulated by DNA Methylation Modification during Mycobacterium tuberculosis Infection. *Biomed Res Int.* 2016;2016:4323281.
3. Shin YH, Kim M, Kim N, Choi SK, Namkoong E, Choi SY, et al. Epigenetic alteration of the purinergic type 7 receptor in salivary epithelial cells. *Biochem Biophys Res Commun.* 2015;466(4):704-10.
4. Solle M, Labasi J, Perregaux DG, Stam E, Petrushova N, Koller BH, et al. Altered cytokine production in mice lacking P2X(7) receptors. *J Biol Chem.* 2001;276(1):125-32.
5. Corry RJ, Winn HJ, and Russell PS. Primarily vascularized allografts of hearts in mice. The role of H-2D, H-2K, and non-H-2 antigens in rejection. *Transplantation.* 1973;16(4):343-50.
6. D'Addio F, Yuan X, Habicht A, Williams J, Ruzek M, Iacomini J, et al. A novel clinically relevant approach to tip the balance toward regulation in stringent transplant model. *Transplantation.* 2010;90(3):260-9.
7. D'Addio F, Riella LV, Mfarrej BG, Chabtini L, Adams LT, Yeung M, et al. The link between the PDL1 costimulatory pathway and Th17 in fetomaternal tolerance. *J Immunol.* 2011;187(9):4530-41.

8. Vergani A, Tezza S, D'Addio F, Fotino C, Liu K, Niewczas M, et al. Long-term heart transplant survival by targeting the ionotropic purinergic receptor P2X7. *Circulation.* 2013;127(4):463-75.