Polish Journal of Veterinary Sciences Vol. 26, No. 4 (2023), 591–609

DOI 10.24425/pjvs.2023.148279

Original article

Teriflunomide induces Foxp3 expression in murine CD8$^+$ T cells while IL-27 and retinoic acid exert a synergistic effect on the induction of CD39 expression on these cells

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Abstract

The purpose of this study was to verify the possibility of pharmacological induction of Foxp3$^+$CD25$^+$CD8$^+$ and Foxp3$^-$CD103$^+$CD8$^+$ T regulatory cells 'armed' with immunosuppressive molecules, i.e. CD39 and IL-10. To achieve this purpose, stimulated and unstimulated murine lymphocytes were exposed to IL-27, teriflunomide (TER) and all trans retinoic acid (ATRA). The study found that: (a) IL-27 induced CD39 expression on Foxp3$^+$CD25$^+$CD8$^+$ T cells and the ability of CD103$^+$Foxp3$^-$CD8$^+$ T cells to produce IL-10 as well as increasing the absolute number of IL-10$^+$CD103$^+$Foxp3$^-$CD8$^+$ T cells; (b) TER induced Foxp3 expression in CD25$^+$CD8$^+$ T cells and CD103 expression on Foxp3$^-$CD8$^+$ T cells as well as increasing the absolute number of Foxp3$^+$CD25$^+$CD8$^+$ T cells; (c) ATRA induced the capacity of Foxp3$^+$CD25$^+$CD8$^+$ T cells to produce IL-10. The following desired interactions were demonstrated between IL-27 and ATRA: (a) a strong synergistic effect with respect to increasing CD39 expression and the ability to produce IL-10 by Foxp3$^+$CD25$^+$CD8$^+$ T cells; (b) a synergistic effect with respect to increasing the absolute count of CD39$^+$Foxp3$^+$CD25$^+$CD8$^+$ T cells. The study revealed that TER abolished all these effects. Therefore, a combination of the tested agents did not induce the generation of Foxp3$^+$CD25$^+$CD8$^+$ and Foxp3$^-$CD103$^+$CD8$^+$ T cells characterized by extensive CD39 expression and IL-10 production. Thus, in the context of the pharmacological induction of IL-10$^+$CD39$^+$Foxp3$^+$CD25$^+$CD8$^+$ and IL-10$^+$CD103$^+$Foxp3$^-$CD8$^+$ T cells, these findings strongly suggest that a combination of TER with IL-27 and/or ATRA does not provide any benefits over TER alone; moreover, such a combination may result in abolishing the desired effects exerted by IL-27 and/or ATRA.

Keywords: CD8$^+$ T cells, Treg cells, Foxp3, IL-27, ATRA, teriflunomide

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Introduction

It is well known that regulatory T cells (Tregs) play a key role in inducing and maintaining immune tolerance, preventing autoimmunity, limiting chronic inflammatory diseases, and regulating abnormal immune responses (Tang and Bluestone 2008). Although forkhead box transcription factor (Foxp3)$^+$CD25$^+$CD4$^+$ cells are the Treg cells that have been most thoroughly studied and characterized, substantial progress has been made in recent years in the phenotypic and functional characterization of their CD8$^+$ counterparts. Similarly to Foxp3$^+$CD25$^+$CD4$^+$ Treg cells, Foxp3$^+$CD25$^+$CD8$^+$ Treg cells can be divided into two principal subsets, namely naturally occurring (Correale and Villa 2010, Ménoret et al. 2023) and induced/inducible (Mahic et al. 2008, Suzuki et al 2008). There is a growing body of evidence indicating that Foxp3$^+$CD25$^+$CD8$^+$ Treg cells play a role in maintaining immune tolerance (Churlaud et al. 2015, Lin et al. 2021). Foxp3 is not only a marker of Treg cells, but it also confers a suppressive capacity to conventional T cells, ensuring the production of immunosuppressive molecules, including IL-10 (Wing et al. 2019). CD39 (ectonucleoside triphosphate diphosphohydrolase-1) is another immunosuppressive molecule which is crucial for the Treg cell function. The production of adenosine via the CD39/CD73 pathway is recognized as a major mechanism responsible for the immunosuppressive function of Treg cells (Bastid et al. 2015). Quantitative and/or functional deficiency of Foxp3$^+$CD25$^+$CD8$^+$ Treg cells has been implicated in the pathogenesis of different immune-mediated diseases, including multiple sclerosis (Correale and Villa 2010), allergic asthma (Eusebio et al. 2012), and giant cell arteritis (Wen et al. 2016). Reduced CD39 expression has been also associated with certain autoimmune diseases (Friedman et al. 2009, Loza et al. 2011).

Taking into consideration the association of the deficiency of Foxp3$^+$CD25$^+$CD8$^+$ Treg cells and CD39 expression with the development of autoimmune or allergic diseases, it is unsurprising that the generation of inducible Foxp3$^+$CD25$^+$CD8$^+$ Treg (iTreg) cells and/or reinforcement of their function may – similarly to their CD4$^+$ analogue – constitute an attractive and promising therapeutic approach to treatment of such diseases. Therefore, the principal purpose of this study was to verify the concept of the pharmacological induction of Foxp3$^+$CD25$^+$CD8$^+$ iTreg cells via induction of the Foxp3 expression in Foxp3-negative CD8$^+$ T cells, which will be additionally 'armed' with immunosuppressive molecules, i.e. extensive CD39 expression and IL-10 production. It is unlikely that a single agent could simultaneously induce Foxp3, CD39 and IL-10 expression. Therefore, a concept pursued in this study was to generate CD8$^+$ iTreg cells with a highly suppressive phenotype (i.e. CD39$^+$IL-10$^+$Foxp3$^+$CD25$^+$CD8$^+$) through a combined treatment with IL-27, teriflunomide (TER) and all trans retinoic acid (ATRA). The essence of this concept is that individual compounds: (a) will complement each other, i.e. they will upregulate different immunosuppressive molecules of Treg cells; (b) together will exert a superadditive or at least an additive effect with respect to the upregulation of particular immunosuppressive molecules and the number of Treg cells. The selection of the agents was substantiated by the studies cited further in this paper as well as our preliminary and prior studies (Maślanka 2022). Based on the rationales derived from these studies, the following hypotheses were formulated and tested: (a) TER and/or ATRA may induce Foxp3 expression in CD8$^+$ T cells (Benson et al. 2007, Xiao et al. 2008, Jasiecka-Mikołajczyk and Socha 2020); (b) IL-27 and/or ATRA may induce CD39 expression on Foxp3$^+$CD25$^+$CD8$^+$ T cells (Maślanka 2022); (c) IL-27 may induce IL-10 production by Foxp3$^+$CD25$^+$CD8$^+$ T cells (Batten et al. 2008).

In 2014 Liu et al. described the phenotypic and functional characteristic of inducible CD8$^+$Foxp3$^-$CD103$^+$ regulatory T cells. These investigators identified CD103 expression and IL-10 production particularly crucial for the function of this subset. In a separate study (Zhong et al. 2018), the same research team found that inducible CD8$^+$CD103$^+$ regulatory T cells showed a therapeutic effect on the chronic graft-versus-host disease lupus nephritis mice. Therefore, these results strongly suggests that these cells may represent a potential promise for the treatment of lupus nephritis and other autoimmune and inflammatory diseases. Hence, another aim of this study was to determine whether the studied agents and their combination have a potential to induce these iTreg cells.

Materials and Methods

Animals

The experiments were carried out on female 8-week-old Balb/c mice. The mice were bred and maintained under standard laboratory conditions (12/12 h light cycle, controlled temperature (21±2°C) and humidity (55±5%)) with ad libitum access to food and water, in the Animal Facility of the Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn. The mice were euthanized by asphyxiation with CO_2. The law in Poland (Act of 15 January 2015 on the Protection of Animals Used for Scientific or Educational Purposes) does not require a permit from

Table 1. Staining combinations, characteristics of monoclonal antibodies and evaluated parameters.

Marker	Fluorochrome	Clone	Isotype	Evaluated parameters
CD8	APC-Cy7	53-6.7	IgG2a, κ	Percentage and absolute count of: • $CD8^+$ T cells • $Foxp3^+CD25^+CD8^+$ T cells • CD39-expressing $Foxp3^+CD25^+CD8^+$ T cells • $CD103^+Foxp3^-CD8^+$ T cells
CD25	PE-Cy7	PC61	IgG1, λ	
Foxp3	Alexa Fluor 488	MF23	IgG2b	
CD39	PE	Duha59	IgG2a, κ	
CD103	APC-R700	M290	IgG2a, κ	
CD8	APC-Cy7	53-6.7	IgG2a, κ	Percentage and absolute count of: • IL-10-producing $Foxp3^+CD25^+CD8^+$ T cells • IL-10-producing $CD103^+Foxp3^-CD8^+$ T cells
CD25	PE-Cy7	PC61	IgG1, λ	
Foxp3	Alexa Fluor 488	MF23	IgG2b	
CD103	APC-R700	M290	IgG2a, κ	
IL-10	APC	JES5-16E3	IgG2b	
CD8	APC-Cy7	53-6.7	IgG2a, κ	Percentage of BrdU-incorporating (i.e. proliferating) $CD8^+$ T cells
BrdU	APC	IgG2a	BU1/75	

an ethics commission to conduct experiments in which samples for research are obtained post mortem from animals not submitted to any procedure while alive.

Isolation of lymphocytes

The submandibular gland, parotid gland, deep cervical, axillary and mesenteric lymph nodes were harvested and subjected to dounce homogenization. The resulting cell suspensions were filtered through a 70 μm cell strainer (BD Biosciences, NJ, USA) and washed (300 × g for 5 min. at 5°C; the same parameters were used for all cell-washing procedures) and re-suspended in complete medium [CM; RPMI 1640, 10% FBS, 10 mM HEPES buffer, 10 mM nonessential amino acids, 10 mM sodium pyruvate and 10 U/ml penicillin/streptomycin (all from Sigma-Aldrich Munich, Germany)].

In vitro stimulation and culture conditions

The cells were adjusted to a final concentration of 4 x 10^6 cells/mL in CM and seeded in 24-well plates in 1 mL aliquots and incubated for 72 (experiments performed under stimulation conditions) and 96 h (experiments performed under unstimulated conditions) in the absence (CONTROL) or presence of IL-27 (Recombinant mouse IL-27; BioLegend, San Diego, USA), TER and ATRA (both from Tocris Bioscience, Bristol, UK) and their combinations. In each experiment, cells were exposed to TER and ATRA in concentrations reflecting their plasma levels achievable *in vivo*, i.e. 10^{-4} (Aubagio, assessment report, 2013) and 10^{-6} M (Ponthan et al. 2001, Jing et al. 2017), respectively. As regards IL-27, the concentration (i.e. 100 ng/mL) was chosen on the basis of relevant published reports (Matsui et al. 2009, Murugaiyan et al. 2010) and preliminary experiments. TER and ATRA were dissolved in DMSO; therefore, the same amount of DMSO was added to the control and IL-27 treated cells. IL-27 was diluted in 1% bovine serum albumin (BSA; carrier protein; Sigma-Aldrich) in phosphate buffered saline (PBS; Dulbecco's PBS devoid of Ca^{2+} and Mg^{2+}; Sigma-Aldrich); therefore, the same amount of BSA in PBS was added to the control and TER and ATRA treated cells. In experiments performed under activation conditions, cells were stimulated with IL-2, anti-CD3, and anti-CD28 and re-stimulated with PMA and ionomycin as previously described (Maślanka 2022). Brefeldin A (Protein transport inhibitor, 1 μl/mL; BD Biosciences) was added to cultures designed to evaluate intracellular IL-10 production for the final 4 h to inhibit release of cytokines into the media. In turn, cell proliferation was evaluated in the presence of 5-bromo-2'-deoxyuridine (BrdU; APC BrdU Flow Kit, BD Biosciences) at a final concentration of 100 μM in cell culture medium during the last 12 h. Each experiment included 4-5 wells of lymphocytes (obtained from individual mice) for each condition tested. All experiments were repeated independently two or three times.

Extracellular staining

The cells were stained for surface antigens with fluorochrome conjugated monoclonal antibodies (mAb) specific to mouse CD8, CD25, CD103 (all from BD Biosciences, NJ, USA) and CD39 (BioLegend), as previously described (Maślanka 2022, Jasiecka-Mikołajczyk and Maślanka 2023). The staining combinations, properties of antibodies used in the experiments and evaluated parameters are summarized in Table 1.

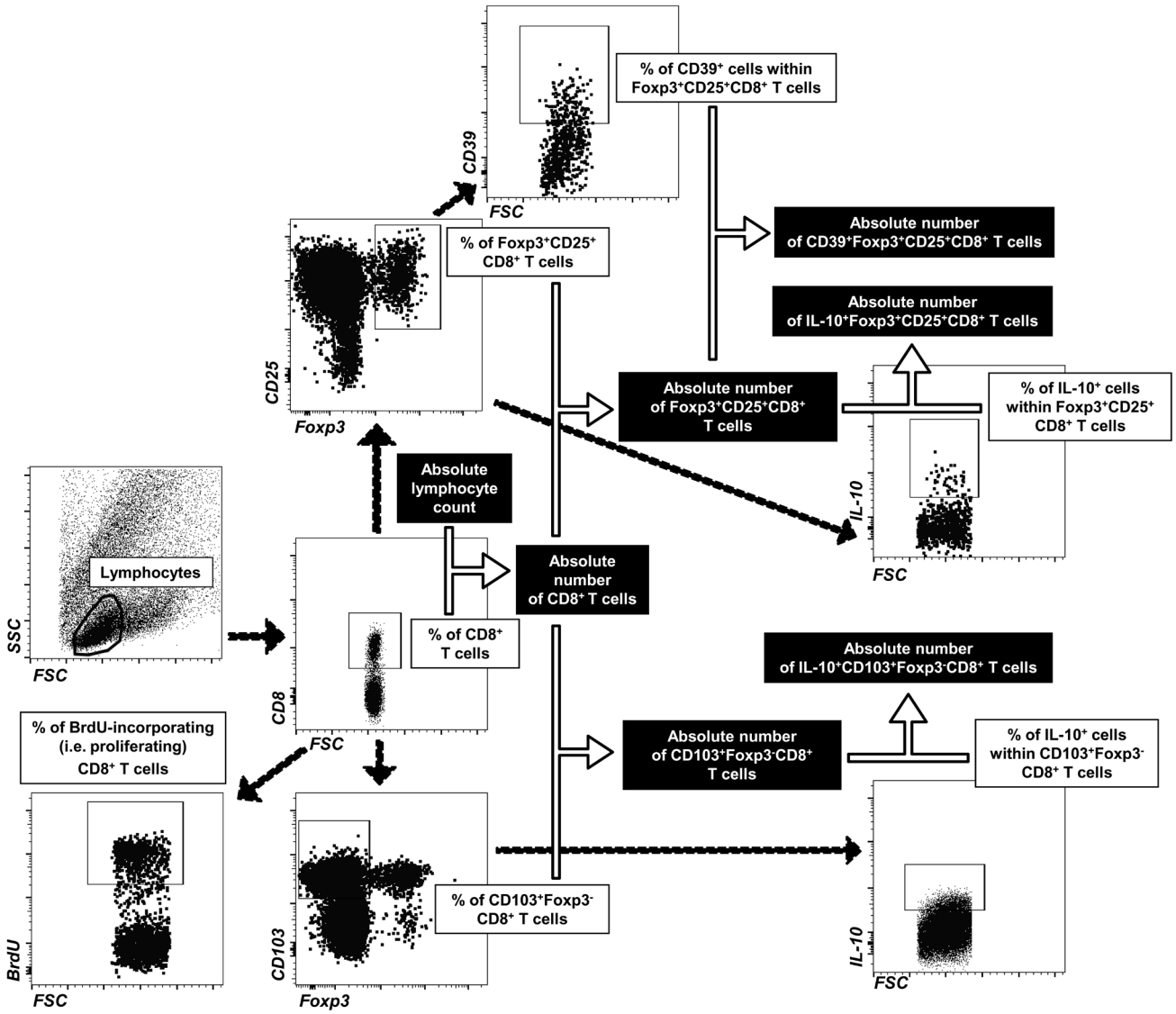

Fig. 1. Gating strategy for flow cytometric data analysis and calculation of the absolute cell counts of murine lymphocyte subsets. Lymphocytes were identified based on forward and side scatter (FSC/SSC) properties, and then gated for expression of CD8 surface receptor. $CD8^+$ T cells were analyzed for expression/co-expression of CD25 and Foxp3 as well as CD103 and Foxp3. On this basis, $Foxp3^+CD25^+CD8^+$ and $CD103^+Foxp3^-CD8^+$ regulatory T cells were distinguished. Subsequently, IL-10-producing cells were identified within these cell subsets. Moreover, 5-bromo-2-deoxyuridine(BrdU)-incorporating (i.e. proliferating) cells and CD39-expressing cells were identified within $CD8^+$ and $Foxp3^+CD25^+CD8^+$ T cell subsets, respectively. Absolute cell counts of lymphocyte subsets (i.e. number of cells from particular subpopulations per sample well) were calculated using the dual platform method, as shown above.

Intracellular staining for determination of Foxp3-expressing cells, IL-10-producing cells and of proliferating cells

Cells stained for surface markers as described above were washed, fixed, permeabilized and labelled as previously described (Maślanka 2022).

FACS acquisition and data analysis

Flow cytometry analysis was performed using a FACSCelesta cytometer (BD Biosci-ences). The data were acquired using FACSDiva version 9.0 software (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc., Stanford, USA). Absolute cell counts (i.e. number of cells from a particular subpopulation per sample well) were calculated using the dual platform method, i.e. an absolute cell count was determined by calculating the data obtained from a cell counting chamber by the percentage of particular cell subsets, as illustrated in Fig. 1.

Statistical analysis

Results were expressed as the mean (± S.D.) of two or three independent experiments. Statistical analysis

was performed using one-way analysis of variance followed by Bonferroni's post hoc test. Differences were deemed significant when the p values were <0.05. SigmaPlot Software Version 12.0 (Systat Software Inc., San Jose, USA) was used for statistical analysis and the plotting of graphs.

Approach to interpreting the absence/presence of interactions between the evaluated agents and types of these interactions

Traditionally, pharmacodynamic interactions fall into a few types (i.e. superadditivity, additivity and antagonism), and are assigned simple definitions, whereas in reality they are a more complex phenomenon. This matter has been widely discussed in a previous paper (Maślanka 2022). The present study has adopted the same method for interpreting the results as was followed in the cited research (Maślanka 2022).

Results

TER depletes CD8⁺ T cells and suppresses their proliferation

The study demonstrated that exposure of cells to TER alone caused a significant reduction in the absolute count of CD8⁺ T cells, both under stimulated and unstimulated conditions (Fig. 2A and B). IL-27 and ATRA did not affect this parameter under stimulated conditions. However, under unstimulated conditions, the exposure to ATRA alone caused a significant reduction in the absolute number of CD8⁺ T cells; in contrast, the exposure of cells to IL-27 alone resulted in a high increase in the value of this parameter (Fig. 2A and B). As expected, the treatment with TER alone induced a significant decrease in the percentage of BrdU⁺CD8⁺ T cells (Fig. 2C and D). IL-27 and ATRA did not affect this parameter (Fig. 2C and D).

The research found that under unstimulated conditions TER as well as ATRA antagonized the IL-27-induced increase in the absolute count of CD8⁺ T cells. On the other hand, IL-27 antagonized both TER-induced and ATRA-induced depletion of CD8⁺ T cells (Fig. 2B). This is evidenced by the fact that the absolute count of these cells in the culture co-exposed to IL-27 + TER and IL-27 + ATRA did not differ significantly from the control values. In addition, the absolute count of CD8⁺ T cells in these cultures was lower than that in the cultures treated with IL-27 alone, but higher compared to the cultures co-treated with IL-27 + TER and IL-27 + ATRA (Fig. 2B). Thus, these results indicate antagonistic interactions between IL-27 and TER as well as between IL-27 and ATRA with respect to their effect on the number of CD8⁺ T cells under unstimulated conditions. Interestingly, although the study did not find any interactions between TER and ATRA with respect to their effect on the absolute count of CD8⁺ T cells (under unstimulated conditions), the value of this parameter in the cultures treated with the triple combination was considerably lower than that in the cultures exposed to the combinations of IL-27 + TER and IL-27 + ATRA (Fig. 2B). What is more, the absolute count of CD8⁺ T cells in the culture exposed to the triple combination was several times lower than the value of this parameter calculated by summing up the means of single agents alone, i.e. the "theoretically expected means" (Table 2). These findings indicate the occurrence of a synergistic (i.e. superadditive) effect between TER and ATRA with respect to their decreasing effect on the IL-27-induced increase in the absolute number of CD8⁺ T cells. On the other hand, the depletion of these cells induced by the co-treatment with TER and ATRA was significantly greater than that observed in the culture treated with the triple combination (Fig 2B). These results confirm the antagonistic action of IL-27 on the depletive effect on CD8⁺ T cells caused by TER and ATRA.

Under stimulated, but not unstimulated, conditions TER increases the percentage and absolute count of Foxp3⁺CD25⁺CD8⁺ T cells

Treatment with TER alone under stimulated conditions led to a significant increase in the percentage and absolute count of Foxp3⁺CD25⁺CD8⁺ T cells, while TER and ATRA did not affect these parameters (Fig. 3A, B and C). This effect was extremely strongly expressed as the percentage and absolute count of Foxp3⁺CD25⁺CD8⁺ T cells in the culture exposed to TER alone were 5- and 3.3-times greater, respectively, than the corresponding control values. An additional analysis found that TER increased the percentage of Foxp3⁺ cells within CD8⁺ T cells but did not affect the CD25 expression on these cells (data not shown). Therefore, it should be clarified that the TER-induced increase in the number of Foxp3⁺CD25⁺CD8⁺ T regulatory cells was triggered by increasing the expression of Foxp3, but not CD25, in CD8⁺ T cells.

The percentage and absolute count of Foxp3⁺CD25⁺CD8⁺ T cells in the culture co-treated with TER and IL-27 were lower than in the culture exposed to TER alone, but still 4.1- and 2.3-times higher, respectively, than the control values (Fig. 3A, B and C). Therefore, it can be concluded that IL-27 antagonized, but only to a very limited degree, the TER-induced increase in the percentage and absolute count of Foxp3⁺CD25⁺CD8⁺ T cells under activation condi-

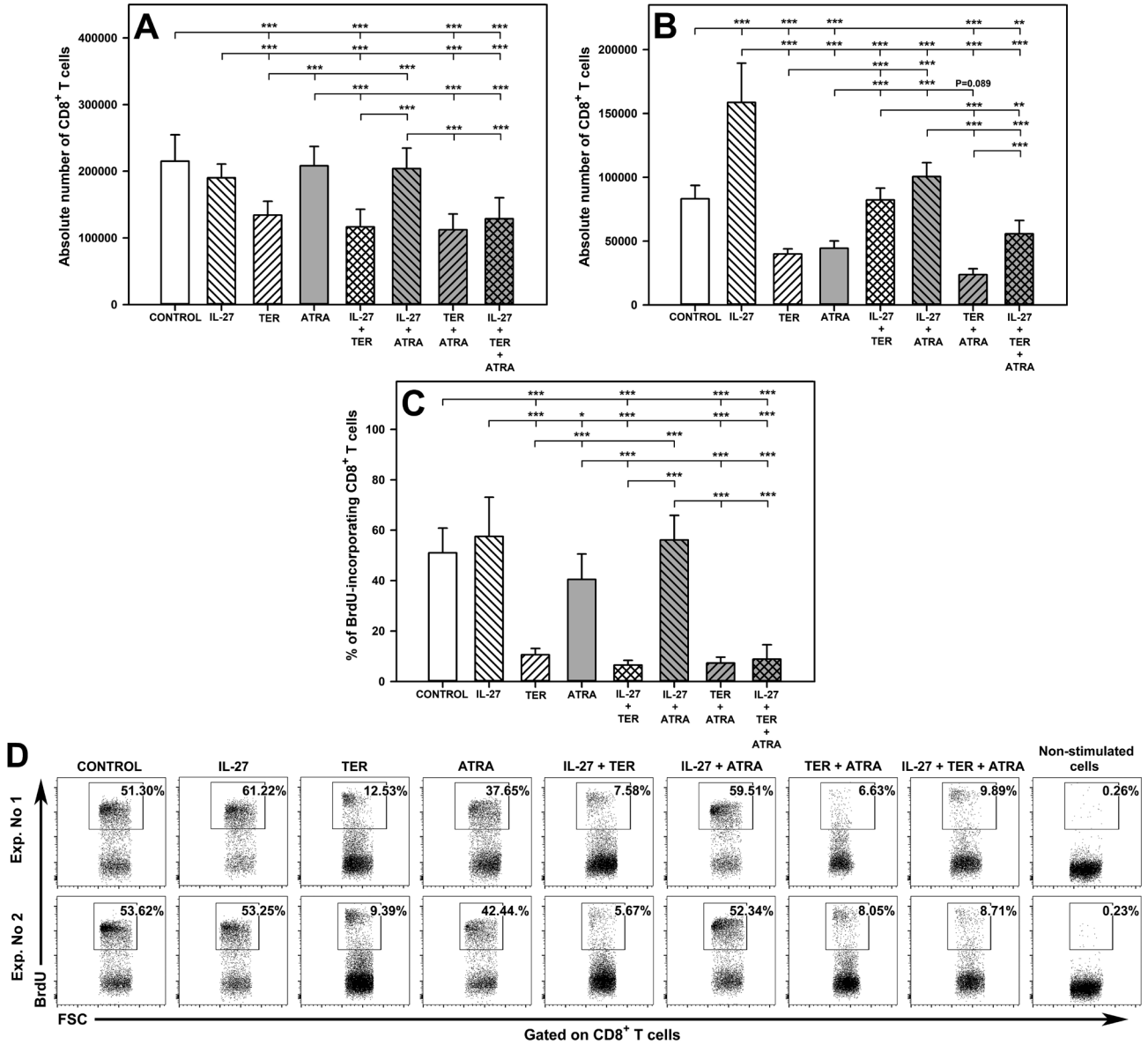

Fig. 2. Effect of IL-27, teriflunomide (TER), all-trans-retinoic acid (ATRA) and their combinations on the absolute number of murine $CD8^+$ T cells under stimulated (A) and unstimulated (B) conditions and on proliferation (C) of these cells. The absolute count (A and B) represents the number of $CD8^+$ T cells per sample well. These results are expressed as the mean (± S.D.) of three independent experiments with 4 mice per experiment (n = 12). The percentage of 5-bromo-2-deoxyuridine(BrdU)-incorporating $CD8^+$ T cells (C) was determined in stimulated cell cultures in the presence of BrdU during the final 12 h. These results are expressed as the mean (± S.D.) of two independent experiments with 4 mice per experiment (n = 8). *p<0.05, **p<0.01, ***p<0.001. Examples of dot plot cytograms for all treatments show the distribution of BrdU-incorporating cells within the $CD8^+$ T cell subset (D).

tions. The results indicate that ATRA did not affect this TER-induced effect. The percentage and absolute count of $Foxp3^+CD25^+CD8^+$ T cells under activation conditions in the culture treated with the triple combination did not differ from those in the cultures exposed to TER alone and to its dual combinations (Fig. 3A, B and C).

The research demonstrated that, under unstimulated conditions, the treatment with IL-27 alone significantly reduced the percentage of $Foxp3^+CD25^+CD8^+$ T cells, while TER and ATRA did not affect this parameter (Fig. 3D and F). The research did not provide evidence for the existence of interactions between any treatment with respect to the discussed parameter. In contrast, the exposure of cells to all the studied agents alone and their combinations considerably decreased the absolute count of $Foxp3^+CD25^+CD8^+$ T cells (Fig. 3E). The statistical analysis did not find any significant differences in the magnitude of the effect between particular agents applied alone. However, the study revealed a subadditive effect between IL-27 and ATRA with respect to decreasing the absolute count of $Foxp3^+CD25^+CD8^+$ T cells. This is indicated by the fact that the value of this

Table 2. Summary table showing the treatment means obtained in the experiments and calculated by summing up the means of single agents alone.

Parameter	Experimental means obtained after subtracting the control values							Means of treatment combinations calculated by summing up the means of single agents alone ("Theoretically expected means")			
	IL-27	TER	ATRA	IL-27 + TER	IL-27 + ATRA	TER + ATRA	IL-27 + TER + ATRA	IL-27 + TER	IL-27 + ATRA	TER + ATRA	IL-27 + TER + ATRA
Absolute number of $CD8^+$ T cells – stimulated cultures	-24793	-80733	-6762	-98616	-11149	-102964	-86231	-105527	-31555	-87495	-112289
Absolute number of $CD8^+$ T cells – unstimulated cultures	75469	-43185	-38663	-875	17400	-59354	-27415	32284	36806	-81848	-6379
% of BrdU-incorporating $CD8^+$ T cells	6.48	-40.42	-10.55	-44.54	5.12	-43.73	-42.22	-33.94	-4.07	-50.97	-44.49
% of $Foxp3^+CD25^+$ cells within $CD8^+$ T cells – stimulated cultures	-0.22	3.11	-0.31	2.39	-0.37	3.24	2.94	2.90	-0.53	2.80	2.59
Absolute number of $Foxp3^+CD25^+CD8^+$ T cells – stimulated cultures	-558	3630	-672	2118	-776	2913	3240	3072	-1230	2959	2401
% of $Foxp3^+CD25^+$ cells within $CD8^+$ T cells – unstimulated cultures	-0.41	0.11	0.03	-0.38	-0.48	0.12	-0.40	-0.30	-0.38	0.14	-0.27
Absolute number of $Foxp3^+CD25^+CD8^+$ cells – unstimulated cultures	-227	-202	-209	-318	-377	-314	-381	-430	-436	-411	-639
% of $CD39^+$ cells within $Foxp3^+CD25^+CD8^+$ T cells	2.87	-2.60	1.62	-2.05	15.14	-2.51	-1.52	0.27	4.49	-0.98	1.89
Absolute number of $CD39^+Foxp3^+CD25^+CD8^+$ T cells	8	-7	-14	1	84	-6	39	0	-6	-21	-13
% of $CD103^+Foxp3^-$ cells within $CD8^+$ T cells – stimulated cultures	-0.52	9.08	2.19	10.89	1.20	14.33	17.60	8.56	1.67	11.27	10.75
Absolute number of $CD103^+Foxp3^-CD8^+$ T cells – stimulated cultures	-8132	-11265	2722	-15888	-559	-14221	-1600	-19397	-5409	-8543	-16675
% of $CD103^+Foxp3^-$ cells within $CD8^+$ T cells – unstimulated cultures	-13.46	11.80	7.99	-7.58	-7.47	12.11	-6.46	-1.66	-5.48	19.79	6.33
Absolute number of $CD103^+Foxp3^-CD8^+$ T cells – unstimulated cultures	-6840	-3641	-4064	-6390	-4183	-8893	-9249	-10481	-10904	-7705	-14545
% IL-10-producing cells within $Foxp3^+CD25^+CD8^+$ T cells	0.99	-2.24	2.65	-1.40	4.93	-2.00	-1.58	-1.25	3.64	0.41	1.40
Absolute number of $IL\text{-}10^+Foxp3^+CD25^+CD8^+$ T cells	-29	-36	-21	-14	-20	-32	-29	-65	-50	-58	-86
% IL-10-producing cells within $CD103^+Foxp3^-CD8^+$ T cells	4.98	-0.46	-0.74	0.14	3.90	0.00	0.47	4.52	4.24	-1.20	3.79
Absolute number of $IL\text{-}10^+CD103^+Foxp3^-CD8^+$ T cells	6358	-2098	-1454	-1720	7709	-1842	-1399	4260	4903	-3552	2806

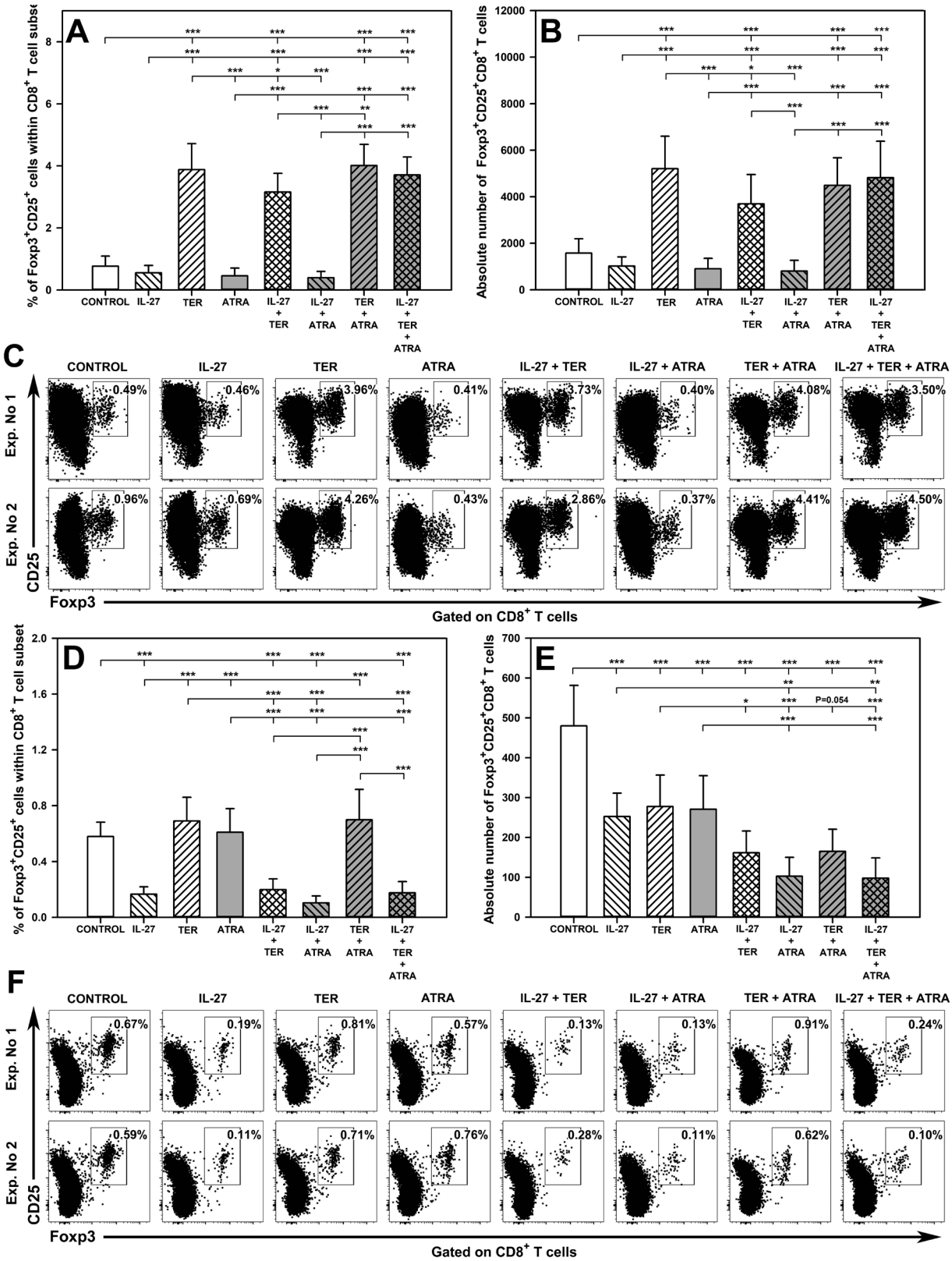

Fig. 3. Effect of IL-27, teriflunomide (TER), all-trans-retinoic acid (ATRA) and their combinations on the number of murine Foxp3$^+$CD25$^+$CD8$^+$ regulatory T cells under stimulated (A and B) and unstimulated (D and E) conditions. The relative count is expressed as a percentage of Foxp3$^+$CD25$^+$ cells within the CD8$^+$ T cell subset (A and D). The absolute count (B and E) represents the number of Foxp3$^+$CD25$^+$CD8$^+$ T cells per sample well. Results are expressed as the mean (± S.D.) of three independent experiments with 4 mice per experiment (n = 12, *p<0.05, **p<0.01, ***p<0.001). Examples of dot plot cytograms for all treatments show the distribution of Foxp3$^+$CD25$^+$ cells within the CD8$^+$ T cell subset under stimulated (C) and unstimulated (F) conditions.

parameter in samples from the culture treated with the combination of IL-27 and ATRA was lower compared to the cultures treated with IL-27 alone and ATRA alone (Fig. 3E).

IL-27 and ATRA exert a synergistic effect on induction of CD39 expression on Foxp3⁺CD25⁺CD8⁺ T cells

The study showed that exposure of cells to IL-27 alone, but not to ATRA alone, increased the percentage of CD39-expressing cells within the Foxp3⁺CD25⁺CD8⁺ T cell subset (Fig. 4A and C). After subtracting the control values, the mean percentage of these cells induced by the exposure to IL-27 alone was 2.87 (Table 2, Fig. 4D). Co-exposure to IL-27 and ATRA induced a considerably greater increase in the percentage of CD39-expressing Foxp3⁺CD25⁺CD8⁺ T cells compared to that produced by IL-27 alone. This combined effect was exceptionally powerful considering that the mean percentage of CD39-expressing Foxp3⁺CD25⁺CD8⁺ T cells after the subtraction of control values was found to be 15.24, i.e. over 5-times greater than that induced by IL-27 alone (Table 2, Fig. 4D). These results indicate the presence of a superadditive effect between both IL-27 and ATRA with respect to the induction of CD39 expression on Foxp3⁺CD25⁺CD8⁺ T cells under activation conditions. Taking into consideration the previously given definitions of subtypes of such an interaction (Maślanka 2022), the discussed effect should be subcategorized as 'enhancement'.

In conrast, exposure of cells to TER alone significantly reduced the percentage of CD39-expressing Foxp3⁺CD25⁺CD8⁺ T cells (Fig. 4A and C). The percentage of CD39-expressing Foxp3⁺CD25⁺CD8⁺ T cells in the cultures exposed to the combinations of IL-27 + TER was not significantly different from that in the control culture and in the culture treated with TER alone, and was lower than in the culture treated with IL-27 alone; the value of this parameter in the culture co-exposed to TER + ATRA was also not different from that in the culture treated with TER alone, but was lower compared to the control values (Fig. 4A and C). The percentage of CD39-expressing Foxp3⁺CD25⁺CD8⁺ T cells in the culture treated with the triple combination was considerably lower than in the cultures exposed to the combinations of IL-27 + ATRA, and did not differ from that in the cultures exposed to TER alone and to the combinations of IL-27 + TER and TER + ATRA (Fig. 4A and C). Taking the above into consideration, it can be stated that: (a) TER fully antagonized the IL-27-mediated induction of CD39 expression on Foxp3⁺CD25⁺CD8⁺ T cells as well as the synergistic effect exerted by IL-27 + ATRA in this respect; (b) IL-27 antagonized, but only partially, the TER-mediated decrease in the induction of CD39 expression on Foxp3⁺CD25⁺CD8⁺ T cells; (c) ATRA did not interact with TER as regards its impact on CD39-expressing Foxp3⁺CD25⁺CD8⁺ T cells. Finally, the study revealed the existence of an antagonistic effect between IL-27 and TER (with predominance of TER) on CD39 expression on Foxp3⁺CD25⁺CD8⁺ T cells.

Treatment with each of the studied agents alone as well as with their combinations, except for the combination of IL-27 and ATRA, did not significantly affect the absolute count of CD39⁺Foxp3⁺CD25⁺CD8⁺ T cells (Fig. 4B). The research found that although the treatments with IL-27 alone and ATRA alone did not influence the absolute count of CD39⁺Foxp3⁺CD25⁺CD8⁺ T cells, the exposure of cells to their combination induced a significant increase in the absolute count of this cell subset (Fig. 4B and E). Thus, this effect represents the type of interaction here referred to as 'induction of an effect' (Maślanka 2022); nevertheless, it could be also considered as a type of superadditive interaction. The absolute count of CD39⁺Foxp3⁺CD25⁺CD8⁺ T cells in the culture treated with the triple combination was considerably lower than that in the cultures exposed to the combination of IL-27 + ATRA (Fig. 4B). These findings indicate that TER antagonized the IL-27 + ATRA-induced increase in the absolute count of CD39-expressing Foxp3⁺CD25⁺CD8⁺ T cells.

Under unstimulated conditions, IL-27 and TER exert antagonistic effect on the percentage of CD103-expressing Foxp3⁻CD8⁺ T cells

The study found that the treatment with TER alone under stimulated conditions led to a significant increase in the percentage of CD103⁺Foxp3⁻ cells within the CD8⁺ T cell subset, although the magnitude of this effect was relatively modest (Fig. 5A and C). The exposure of cells to IL-27 alone and ATRA alone did not affect the percentage of CD103⁺Foxp3⁻CD8⁺ T cells (Fig. 5A and C). The percentage of these cells in the culture treated with TER alone did not differ from that in the cultures exposed to the combinations of IL-27 + TER and TER + ATRA (Fig. 5A and C). Therefore, it can be concluded that IL-27 and ATRA did not interact with TER with respect to its increasing action on the percentage of CD103⁺Foxp3⁻ cells within the CD8⁺ T cell subset under stimulated conditions. Treatment with each of the studied agents alone as well as with their combinations did not affect significantly the absolute count of CD103⁺Foxp3⁻CD8⁺ T cells (Fig. 5B).

The research demonstrated that under unstimulated

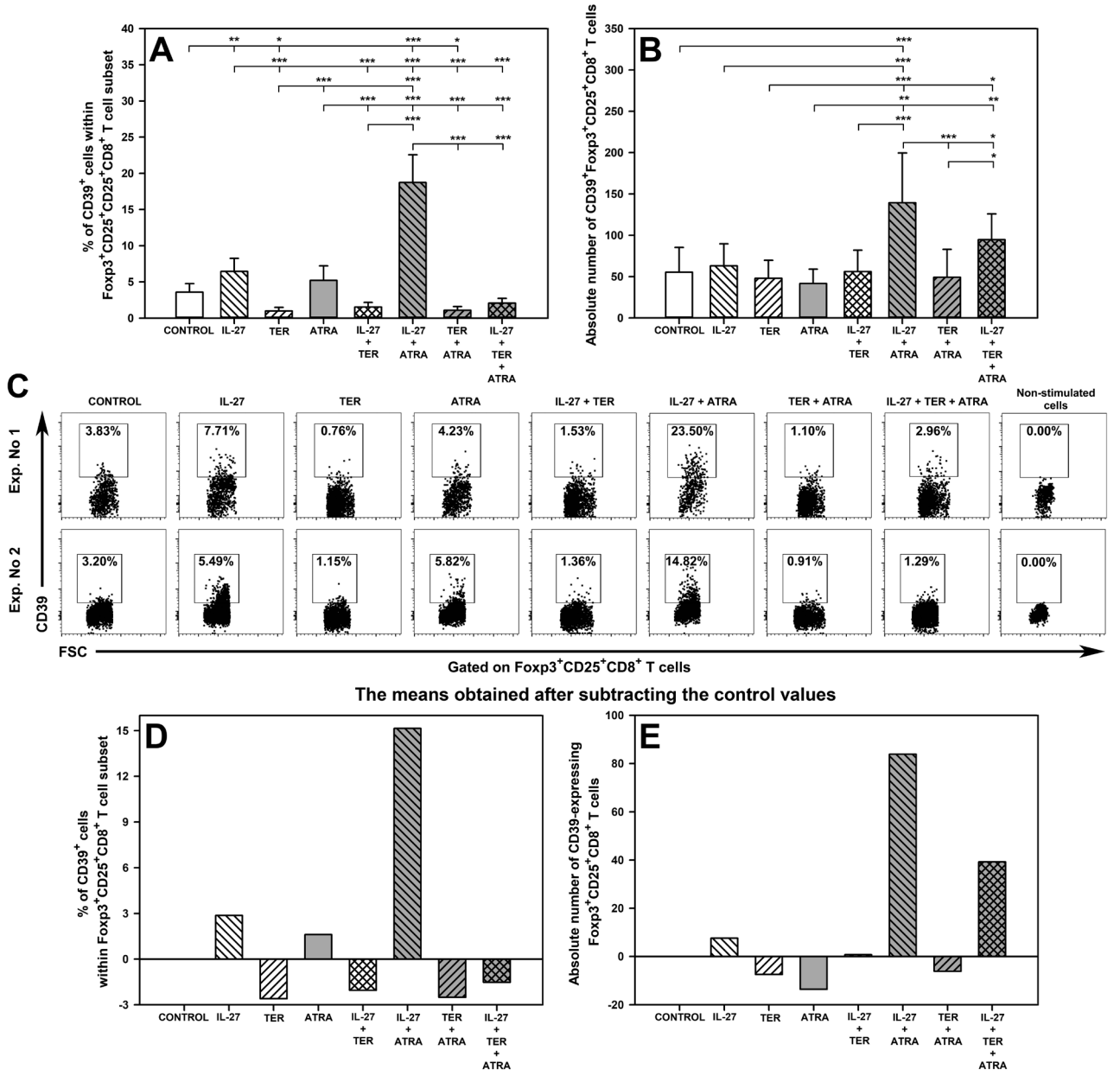

Fig. 4. Effect of IL-27, teriflunomide (TER), all-trans-retinoic acid (ATRA) and their combinations on the number of murine CD39-expressing Foxp3$^+$CD25$^+$CD8$^+$ regulatory T cells under stimulated conditions. The relative count is expressed as a percentage of CD39-expressing cells within the Foxp3$^+$CD25$^+$CD8$^+$ T cell subset (A). The absolute count represents the number of CD39$^+$Foxp3$^+$CD25$^+$CD8$^+$ T cells per sample well (B). Results are expressed as the mean (± S.D.) of three independent experiments with 4 mice per experiment (n = 12, *p<0.05, **p<0.01, ***p<0.001). Examples of dot plot cytograms for all treatments show the distribution of CD39-expressing cells among Foxp3$^+$CD25$^+$CD8$^+$ T cells (C). Additionally, in order to better visualize the superadditive effect between IL-27 and ATRA in the induction of CD39 expression on Foxp3$^+$CD25$^+$CD8$^+$ T cells, the mean of the percentage (D) and absolute count (E) of CD39-expressing Foxp3$^+$CD25$^+$CD8$^+$ T cells are shown after subtracting the control values.

conditions the treatment with IL-27 alone significantly decreased the percentage of CD103$^+$Foxp3$^-$CD8$^+$ T cells, while the exposure to TER induced an increase in the value of this parameter (Fig. 5D and F). In turn, the treatment with ATRA alone did not affect the percentage of CD103$^+$Foxp3$^-$CD8$^+$ T cells, although a certain trend (p=0.079) toward increasing this parameter appeared (Fig. 5D and F). The study did not detect any influence of ATRA on the TER-induced increase in the percentage of CD103$^+$Foxp3$^-$CD8$^+$ T cells (Fig. 5D and F). In contrast, it was found that IL-27 antagonized the TER-induced increase in the percentage of CD103$^+$Foxp3$^-$CD8$^+$ T cells, while TER antagonized the IL-27-mediated decrease in the value of this parameter (Fig. 5D and F). This is evidenced by the fact that the percentage of CD103$^+$Foxp3$^-$CD8$^+$ T cells in the cultures treated with IL-27 + TER was not different from the control values (Fig. 5D and F). Thus, the results

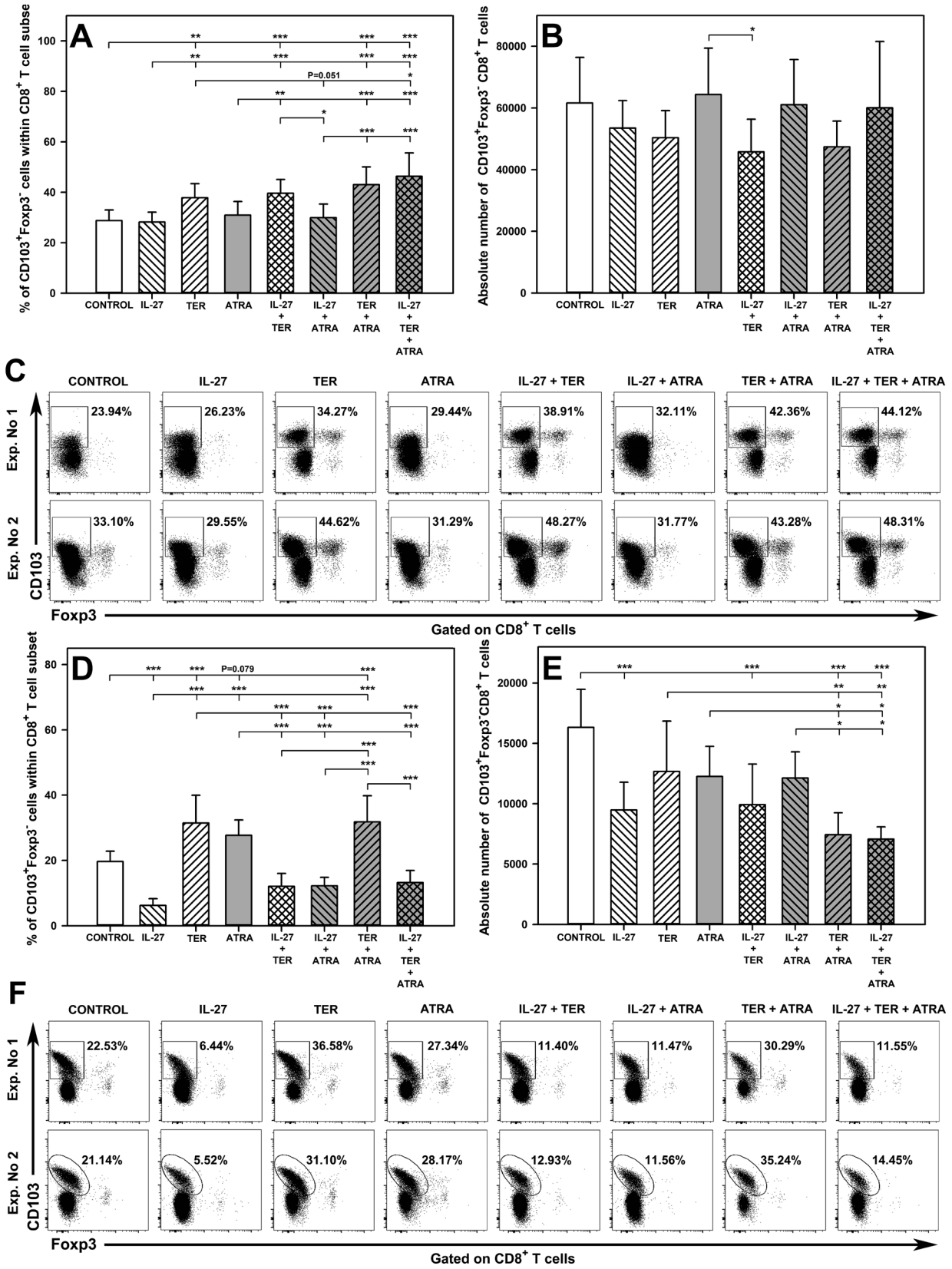

Fig. 5. Effect of IL-27, teriflunomide (TER), all-trans-retinoic acid (ATRA) and their combinations on the number of murine $CD103^{+}$-$Foxp3^{-}CD8^{+}$ regulatory T cells under stimulated (A and B) and unstimulated (D and E) conditions. The relative count is expressed as a percentage of $CD103^{+}Foxp3^{-}$ cells within the $CD8^{+}$ T cell subset (A and D). The absolute count (B and E) represents the number of $CD103^{+}Foxp3^{-}CD8^{+}$ T cells per sample well. Results are expressed as the mean (± S.D.) of three independent experiments with 4 mice per experiment (n = 12, *p<0.05, **p<0.01, ***p<0.001). Examples of dot plot cytograms for all treatments show the distribution of $CD103^{+}Foxp3^{-}$ cells within the $CD8^{+}$ T cell subset under stimulated (C) and unstimulated (F) conditions.

indicate the existence of antagonism between IL-27 and TER with respect to their effect on CD103 expression on Foxp3$^-$CD8$^+$ T cells. As the percentage of CD103^{+-}Foxp3$^-$CD8$^+$ T cells in the cultures co-treated with IL-27 and ATRA was also not different from the control values, it might be suspected that ATRA antagonized the IL-27-induced decrease in the value of this parameter (Fig. 5D). However, this conclusion may be questionable because ATRA alone did not exert any effect on the percentage of CD103$^+$Foxp3$^-$CD8$^+$ T cells.

The study found that under unstimulated conditions IL-27 alone induced a significant reduction in the absolute count of CD103$^+$Foxp3$^-$CD8$^+$ T cells (Fig. 5E). TER alone and ATRA alone did not affect this parameter nor did they affect the IL-27-mediated depletion of these cells (Fig. 5E). Despite this, however, the exposure of cells to the combination of TER and ATRA caused a decrease in the absolute count of CD103^{+-}Foxp3$^-$CD8$^+$ T cells (Fig. 5E). Thus, these results strongly suggest the existence of an interaction between TER and ATRA classified here as 'induction of an effect' (Maślanka 2022). The present study did not find any interactions between the triple combination and dual combinations with respect to the percentage (Fig. 5A, C, D and F) and absolute count (Fig. 5B and E) of CD103$^+$Foxp3$^-$CD8$^+$ T cells under stimulated and unstimulated conditions.

IL-27 enhances ATRA-induced increase in the percentage of IL-10-producing Foxp3$^+$CD25$^+$CD8$^+$ T cells & TER antagonizes IL-27-mediated increase in the percentage of IL-10-producing CD103$^+$Foxp3$^-$CD8$^+$ T cells

The study demonstrated that the exposure of cells to TER alone led to a significant decrease in the percentage of IL-10-producing Foxp3$^+$CD25$^+$CD8$^+$ T cells (Fig. 6A and C). In contrast, the treatment with ATRA alone significantly increased the percentage of IL-10-producing Foxp3$^+$CD25$^+$CD8$^+$ T cells, while IL-27 alone did not alter this parameter (Fig. 6A and C). The combination of IL-27 and ATRA triggered a significantly greater increase in the percentage of IL-10-producing Foxp3$^-$CD25$^+$CD8$^+$ T cells compared to that mediated by ATRA alone (Fig. 6A and C). In turn, the percentage of IL-10-producing Foxp3$^+$CD25$^+$CD8$^+$ T cells in the culture treated with the combination of TER and ATRA was lower compared to the control values, and did not differ significantly from that in the cultures treated with TER alone (Fig. 6A and C). The percentage of IL-10-producing Foxp3$^+$CD25$^+$CD8$^+$ T cells in the culture treated with the triple combination was considerably lower than in the cultures exposed to the combinations of IL-27 + ATRA, and was not different from the corresponding values in the cultures co-treated with IL-27 + TER and TER + ATRA (Fig. 6A and C). These results indicate the following interactions between the studied agents with respect to the percentage of IL-10-producing Foxp3$^+$CD25$^+$CD8$^+$ T cells: a) IL-27 enhanced the ATRA-induced increase in the value of this parameter; b) TER fully antagonized the ATRA-induced increase in the value of this parameter; c) TER fully antagonized the IL-27 + ATRA-induced enhancement effect on the value of this parameter.

Exposure to TER alone led to a significant reduction in the absolute count of IL-10-producing Foxp3$^+$CD25$^+$CD8$^+$ T cells; however, the magnitude of this effect should be assessed as relatively small (Fig. 6B). The study did not reveal any influence of IL-27 alone and ATRA alone on this parameter (Fig. 6B). Similarly, the exposure of cells to all combinations of the studied agents did not significantly affect the absolute count of IL-10-producing Foxp3$^+$CD25$^+$CD8$^+$ T cells (Fig. 6B).

The research demonstrated that the treatment with IL-27 alone induced a significant increase in the percentage and absolute number of IL-10-producing CD103$^+$Foxp3$^-$CD8$^+$ T cells, while TER alone and ATRA alone did not affect these parameters (Fig. 6D-F); these increases represented a relatively large scale effect as the percentage and absolute number of IL-10-producing CD103$^+$Foxp3$^-$CD8$^+$ T cells were approximately 4- and 3-times greater, respectively, than the control values. The results demonstrated that ATRA did not interact with IL-27 with regard to its increasing effect on the percentage and absolute number of IL-10-producing CD103$^+$Foxp3$^-$CD8$^+$ T cells (Fig. 6D-F). In contrast, TER fully antagonized the IL-27-induced increase in these parameters. This is evidenced by the fact that the percentage and absolute number of IL-10-producing CD103$^+$Foxp3$^-$CD8$^+$ T cells in the culture co-exposed to IL-27 + TER did not differ from the control values, and in addition were significantly lower than those in the culture exposed to IL-27 alone (Fig. 6D-F). The study did not find any interactions between the triple combination and dual combinations with respect to the above parameters (Fig. 6D and E).

Overview tables summarizing the effects of single agents and their combinations on the evaluated parameters and the presence or absence of interactions between these agents were compiled in order to provide a bigger picture of this research (Table 3).

Discussion

Under unstimulated conditions, IL-27 alone increased the absolute number of CD8$^+$ T cells cultured, while TER alone and ATRA alone induced their depletion. Under such conditions, T cells practically do not proli-

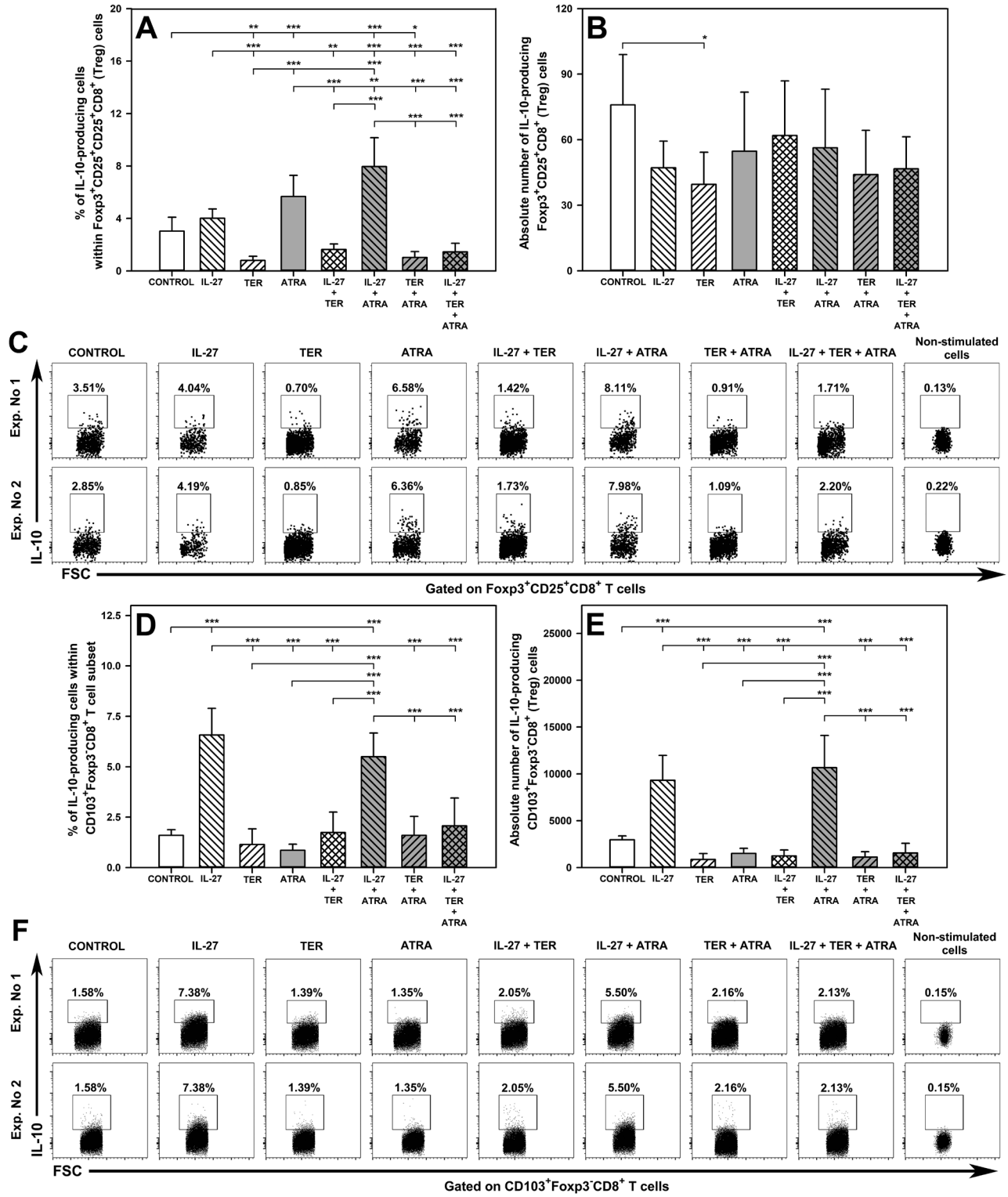

Fig. 6. Effect of IL-27, teriflunomide (TER), all-trans-retinoic acid (ATRA) and their combinations on the number of murine IL-10-producing $Foxp3^+CD25^+CD8^+$ and $CD103^+Foxp3^-CD8^+$ T regulatory cells. The relative count is expressed as a percentage of IL-10-producing cells within the $Foxp3^+CD25^+CD8^+$ (A) and $CD103^+Foxp3^-CD8^+$ (D) T cell subsets. The absolute count represents the number of $IL\text{-}10^+Foxp3^+CD25^+CD8^+$ (B) and $IL\text{-}10^+CD103^+Foxp3^-CD8^+$ (E) T cells per sample well. Results are expressed as the mean (± S.D.) of two independent experiments with 4 mice per experiment (n=8, * $p<0.05$, ** $p<0.01$, *** $p<0.001$). Examples of dot plot cytograms for all treatments show distribution of IL-10-producing cells within the $Foxp3^+CD25^+CD8^+$ (C) and $CD103^+Foxp3^-CD8^+$ (F) T cell subsets.

Table 3. Overview table summarizing the effects of single agents and their combinations on the evaluated parameters and the presence or absence of interactions between theses agents.

Parameter	Effect of single agents on evaluated parameter			Effect of combinations of agents on evaluated parameter/Presence or absence of interactions			
				Identification of the interactions: dual combinations compared to single agents alone or control			Identification of the interactions: triple combination compared to the dual combinations
	IL-27	TER	ATRA	IL-27 + TER	IL-27 + ATRA	TER + ATRA	IL-27 + TER + ATRA
Absolute number of $CD8^+$ T cells – stimulated cultures	NSS effect	Decrease	NSS effect	Decrease/Absence of interaction	NSS effect/Absence of interaction	Decrease/Absence of interaction	Decrease/Absence of interaction
Absolute number of $CD8^+$ T cells - unstimulated cultures	Increase	Decrease	Decrease	NSS effect/Antagonism of IL-27-induced increase by TER & Antagonism of TER-induced decrease by IL-27	NSS effect/Antagonism of IL-27-induced increase by ATRA & Antagonism of ATRA-induced decrease by IL-27	Decrease/Absence of interaction	Decrease/Superadditivity: synergism between TER and ATRA with respect to their decreasing effect on IL-27 induced increase in the absolute number of $CD8^+$ T cells & Antagonism of TER + ATRA-induced decrease by IL-27
% of BrdU-incorporating $CD8^+$ T cells	NSS effect	Decrease	NSS effect	Decrease/Absence of interaction	NSS effect/Absence of interaction	Decrease/Absence of interaction	Decrease/Absence of interaction
% of $Foxp3^+CD25^+$ cells within $CD8^+$ T cells – stimulated cultures	NSS effect	Increase	NSS effect	Increase/Antagonism of TER-induced increase by IL-27	NSS effect/Absence of interaction	Increase/Absence of interaction	Increase/Absence of interaction
Absolute number of $Foxp3^+CD25^+CD8^+$ T cells – stimulated cultures	NSS effect	Increase	NSS effect	Increase/Antagonism of TER-induced increase by IL-27	NSS effect/Absence of interaction	Increase/Absence of interaction	Increase/Absence of interaction
% of $Foxp3^+CD25^+$ cells within $CD8^+$ T cells – unstimulated cultures	Decrease	NSS effect	NSS effect	Decrease/Absence of interaction	Decrease/Absence of interaction	NSS effect/Absence of interaction	Decrease/Absence of interaction
Absolute number of $Foxp3^+CD25^+CD8^+$ cells – unstimulated cultures	Decrease	Decrease	Decrease	Decrease/Absence of interaction	Decrease/Subadditivity	Decrease/Absence of interaction	Decrease/Absence of interaction
% of $CD39^+$ cells within $Foxp3^+CD25^+CD8^+$ T cells	Increase	Decrease	NSS effect	NSS effect/Antagonism of IL-27-induced increase by TER & Antagonism of TER-induced decrease by IL-27	Increase/Superadditivity: enhancement → ATRA enhances the IL-27-induced increase	Decrease/Absence of interaction	NSS effect/Antagonism of IL-27 + ATRA-induced enhancement by TER
Absolute number of $CD39^+Foxp3^+CD25^+CD8^+$ T cells	NSS effect	NSS effect	NSS effect	NSS effect/Absence of interaction	Increase/Induction of an effect → combination of IL-27 + ATRA induces an increase	NSS effect/Absence of interaction	NSS effect/Antagonism of IL-27 + ATRA-induced increase by TER

cont. Table 3.

% of $CD103^+Foxp3^-$ cells within $CD8^+$ T cells – stimulated cultures	NSS effect	Increase	NSS effect	Increase/Absence of interaction	NSS effect/Absence of interaction	Increase/Absence of interaction	Increase/Absence of interaction
Absolute number of $CD103^+Foxp3^-CD8^+$ T cells – stimulated cultures	NSS effect	NSS effect	NSS effect	NSS effect/Absence of interaction	NSS effect/Absence of interaction	NSS effect/Absence of interaction	NSS effect/Absence of interaction
% of $CD103^+Foxp3^-$ cells within $CD8^+$ T cells – unstimulated cultures	Decrease	Increase	NSS effect	NSS effect/ Antagonism of IL-27-induced decrease by TER & Antagonism of TER-induced increase by IL-27	NSS effect/Absence of interaction	Increase/Absence of interaction	NSS effect/Absence of interaction
Absolute number of $CD103^+Foxp3^-CD8^+$ T cells – unstimulated cultures	Decrease	NSS effect	NSS effect	Decrease/Absence of interaction	NSS effect/Absence of interaction	Decrease/Induction of an effect → combination of TER + ATRA induces a decrease	Decrease/Absence of interaction
% IL-10-producing cells within $Foxp3^+CD25^+CD8^+$ T cells	NSS effect	Decrease	Increase	Decrease/Absence of interaction	Increase/Superadditivity: enhancement → IL-27 enhances the ATRA-induced increase	Decrease/Antagonism of ATRA-induced increase by TER	NSS effect/Antagonism of IL-27 + ATRA-induced enhancement by TER
Absolute number of $IL\text{-}10^+Foxp3^+CD25^+CD8^+$ T cells	NSS effect	Decrease	NSS effect	NSS effect/Absence of interaction	NSS effect/Absence of interaction	NSS effect/Absence of interaction	NSS effect/Absence of interaction
% IL-10-producing cells within $CD103^+Foxp3^-CD8^+$ T cells	Increase	NSS effect	NSS effect	NSS effect/Antagonism of IL-27-induced increase by TER	Increase/Absence of interaction	NSS effect/Absence of interaction	NSS effect/Absence of interaction
Absolute number of $IL\text{-}10^+CD103^+Foxp3^-CD8^+$ T cells	Increase	NSS effect	NSS effect	NSS effect/Antagonism of IL-27-induced increase by TER	Increase/Absence of interaction	NSS effect/Absence of interaction	NSS effect/Absence of interaction

NSS: not statistically significant

ferate but, with time, their number gradually decreases as a result of normal cell death in culture. The results strongly suggest that under unstimulated conditions IL-27 can induce an anti-apoptotic/pro-survival effect on $CD8^+$ T cells, while TER and ATRA can exert a pro-apoptotic effect on these cells. This claim is supported by results indicating that these agents may affect T cells in this manner (Szondy et al. 1998, Ringshausen et al. 2008, Kim et al. 2013, Liu et al. 2013). Predictably, TER exerted an antiproliferative effect on $CD8^+$ T cells, and induced their depletion under stimulated conditions. Therefore, it can be concluded that this depletive effect of TER was largely caused by its direct antiproliferative action on $CD8^+$ T cells; this is in agreement with the fact that the antiproliferative effect of TER on T and B cells constitutes the main mechanism responsible for its immunosuppressive action (Nwankwo et al. 2012). To gain a more complete picture, a decision was made in the present study to present both percentage and absolute values. Percentage data better capture effects of given substances on a cell's active processes (e.g. on expression/production of some molecules and proliferation), whereas absolute data are better at capturing losses or increases in the number of cells, thereby demonstrating consequences of the effects of these substances on apoptosis and proliferation. Because of the depletive and antiproliferative actions described above, the TER- or ATRA-induced increases in the percentage values of some parameters were not reflected in the corresponding increases in the absolute values. Therefore, the above considerations are important for interpreting and understanding properly the results regarding absolute values.

The results of this study pertaining to the effect of IL-27, TER and ATRA on $Foxp3^+CD25^+CD8^+$ Treg cells are similar to those we obtained in our previous studies concerning the effect of these compounds on $Foxp3^+CD25^+CD4^+$ Treg cells (Maślanka 2022). Consequently, the interpretation of the results provided in this paper, as well as the method for drawing conclusions, are similar to those found in the cited article. The principal purpose of this study was to assess the possibility of pharmacological induction of $Foxp3^+CD25^+CD8^+$ and $CD103^+Foxp3^-CD8^+$ iTreg cells 'armed' with immunosuppressive molecules, i.e. CD39 (with respect to the first subset) and IL-10, through combined treatment with IL-27, TER and ATRA. Whenever referring to a desired or undesired effect or interaction in this paper, the authors mean effects and interactions that are desired or undesired considering the purpose of this research. To recapitulate the desired effects produced by each analyzed agent, the following need to be brought to our attention: (a) IL-27 induced CD39 expression on $Foxp3^+CD25^+CD8^+$ and the ability of $CD103^+Foxp3^-CD8^+$ T cells to produce IL-10 as well as increasing the absolute number of $IL\text{-}10^+CD103^+Foxp3^-CD8^+$ T cells; (b) TER induced Foxp3 expression in Foxp3-negative $CD8^+$ T cells and CD103 expression on $Foxp3^-CD8^+$ T cells as well as increasing the absolute number of $Foxp3^+CD25^+CD8^+$ T cells; (c) ATRA induced the capacity of $Foxp3^+CD25^+CD8^+$ T cells to produce IL-10. Moreover, such interactions as 'enhancement' and 'induction of an effect' either enhanced or induced some desired effects. The following desired interactions were found between IL-27 and ATRA: (a) a strong synergistic effect with respect to increasing CD39 expression and the ability to produce IL-10 by $Foxp3^+CD25^+CD8^+$ T cells; (b) a synergistic effect with respect to increasing the absolute count of $CD39^+Foxp3^+CD25^+CD8^+$ T cells (although these agents alone did not affect it). On the other hand, the study revealed the following undesired effects induced by the tested agents: (a) IL-27 down-regulated Foxp3 expression in $Foxp3^+CD25^+CD8^+$ T cells under unstimulated conditions; (b) TER down-regulated CD39 expression on $Foxp3^+CD25^+CD8^+$ T cells and suppressed the capacity of these cells to produce IL-10. At this point, it is worth noting that these undesired effects can represent desired ones in the context of treatment of neoplastic diseases. The infiltration of $Foxp3^+CD25^+CD8^+$ Treg cells into the tumor microenvironment has been reported in various tumors (Liston and Aloulou 2022) including colorectal (Chaput et al. 2009) and prostate cancer (Kiniwa et al. 2007). The results of these and other studies (e.g. Canale et al. 2018) indicate that apart from $CD4^+$ Treg cells, $Foxp3^+CD25^+CD8^+$ Treg also contribute to immune response evasion against the tumor and consequently progression of the disease. In the light of these studies, the depletion of $Foxp3^+CD25^+CD8^+$ Treg cells and/or inhibition of their function may augment antitumor immune responses, which has led to the development of a new immunotherapeutic strategy in the treatment of cancer. The down-regulation or blockade of CD39 is an especially promising therapeutic strategy in oncology (Bastid et al. 2013, Canale et al. 2018).

The study demonstrated that IL-27 down-regulated CD103 expression under unstimulated, but not stimulated, conditions. However, this action is most likely related to effector $CD8^+$ T cells, but not to $CD103^+Foxp3^-CD8^+$ iTreg cells, since these latter cells do not occur naturally as such, but constitute only inducible Treg cells. Although CD103 is involved in conferring the regulatory properties of $CD103^+Foxp3^-CD8^+$ T cells (Liu et al. 2014, Zhong et al. 2018), the expression of this molecule is not specific to $CD8^+$ Treg cells; it is also expressed, among others, by tissue-resident memory $CD8^+$ T cells (Molodtsov and Turk 2018).

Taking into consideration only the desired effects listed above, the combination of studied agents theoretically should induce the generation of Foxp3⁺CD25⁺CD8⁺ T cells characterized by increased CD39 expression and IL-10 production, and CD103⁺Foxp3⁻CD8⁺ T cells characterized by increased IL-10 production. However, the study revealed that the combination of IL-27, TER and ATRA did not induce the generation of a cell subset with all the aforementioned attributes. Due to the antagonistic interactions between the tested agents, certain desired effects were either abolished or reduced. The study revealed the following significant undesired interactions: (a) TER fully antagonized the IL-27-mediated induction of CD39 expression on Foxp3⁺CD25⁺CD8⁺ T cells and the increment of CD39-expressing Foxp3⁺CD25⁺CD8⁺ T cells, as well as the synergistic effect exerted by IL-27 + + ATRA in these respects; (b) TER fully antagonized the ATRA-induced increase in the ability of Foxp3⁺CD25⁺CD8⁺ T cells to produce IL-10 as well as the synergistic effect exerted by IL-27 + ATRA in this respect; (c) TER fully antagonized the IL-27-induced increase in the ability of CD103⁺Foxp3⁻CD8⁺ T cells to produce IL-10 and the increment of IL-10-producing CD103⁺Foxp3⁻CD8⁺ T cells; (d) technically, IL-27 decreased TER-induced up-regulation of Foxp3 expression and increment of Foxp3⁺CD25⁺CD8⁺ T cells; however, the magnitude of this antagonism was extremely small.

The study found that TER induced Foxp3 expression in Foxp3-negative CD8⁺ T cells and increased the absolute number of Foxp3⁺CD25⁺CD8⁺ T cells. Foxp3 is not only a marker of Treg cells, but it also confers suppressive capacity to conventional T cells (Wing et al. 2019). On the other hand, TER down-regulated CD39 expression on Foxp3⁺CD25⁺CD8⁺ T cells and suppressed their capacity to produce IL-10. The absolute number of CD39⁺Foxp3⁺CD25⁺CD8⁺ and IL-10⁺Foxp3⁺CD25⁺CD8⁺ T cells in the culture treated with TER did not differ or was even lower, respectively, than the control values. Thus, assuming that Foxp3 confers a regulatory function to non-regulatory T cells, the results strongly suggest that TER may induce the generation of Foxp3⁺CD25⁺CD8⁺ iTreg cells; however, these cells are apparently devoid of CD39 expression and the ability to produce IL-10, i.e. important immunosuppressive molecules. Therefore, the suppressive function of TER-induced Foxp3⁺CD25⁺CD8⁺ T cells remains in question. And, importantly, TER induced Foxp3 expression only under conditions mimicking T cell activation, which suggests that TER may induce the generation of Foxp3⁺CD25⁺CD8⁺ iTreg cells in the course of allergic and autoimmune disorders rather than in the steady state.

The findings of this study indicate that treatment with a combination of IL-27 and ATRA will not result in the induction of Foxp3⁺CD25⁺CD8⁺ T cells, but may enhance the suppressive function of existing, i.e. naturally occurring, Foxp3⁺CD25⁺CD8⁺ Treg cells via up-regulation of CD39 expression and IL-10 production. It should be pointed out that these effects need not necessarily be associated with combined administration of IL-27 and ATRA to patients since IL-27 occurs in the body naturally. Therefore, it is highly likely that the synergistic interactions observed in this study between IL-27 and ATRA may take place in the body as a consequence of the administration of ATRA alone. The study found that TER fully abolished all the previously listed desired effects induced by IL-27 and ATRA and their combination. Thus, in the context of the pharmacological induction of IL-10⁺CD39⁺Foxp3⁺CD25⁺CD8⁺ and IL-10⁺CD103⁺Foxp3⁻CD8⁺ T cells, these findings strongly suggest that a combination of TER with IL-27 and/or ATRA does not provide any benefits over TER alone; moreover, such a combination may result in abolishing the desired effects exerted by IL-27 and/or ATRA. However, the results obtained in this study do not totally rule out the possibility of using TER with IL-27 and/or ATRA for generation of these cells, although the concept of applying these agents together should be modified in order to avoid undesired interactions. This means applying an appropriate sequential treatment with TER, IL-27 and ATRA instead of concurrent treatment with these agents, as was proposed in our previous paper (Maślanka 2022). Namely, the initial treatment with TER could be applied to induce Foxp3 expression in Foxp3-negative CD8⁺ T cells, which would be followed by treatment with ATRA and IL-27 (after discontinuation of TER) to induce CD39 expression and IL-10 production in existing and TER-induced Foxp3⁺CD25⁺CD8⁺ T cells. It is highly likely that such an approach could allow the discussed undesired interactions to be avoided. The verification of this concept should be the subject of future research.

Acknowledgements

The study was supported by a grant from the National Science Center (NCN), No. DEC-2017/01/X/NZ7/01082.

References

Aubagio®, assessment report. International non-proprietary name: Teriflunomide. Procedure No. EMEA/H/C/002514/0000. 27 June 2013 EMA/529295/2013. Available from here: https://www.ema.europa.eu/en/documents/assessment-report/aubagio-epar-public-assessment-report_en.pdf

Bastid J, Cottalorda-Regairaz A, Alberici G, Bonnefoy N, Eliaou JF, Bensussan A (**2013**) ENTPD1/CD39 is a promising therapeutic target in oncology. Oncogene 32: 1743-1751.

Bastid J, Regairaz A, Bonnefoy N, Déjou C, Giustiniani J, Laheurte C, Cochaud S, Laprevotte E, Funck-Brentano E, Hemon P, Gros L, Bec N, Larroque C, Alberici G, Bensussan A, Eliaou JF (**2015**) Inhibition of CD39 enzymatic function at the surface of tumor cells alleviates their immunosuppressive activity. Cancer Immunol Res 3: 254-265.

Batten M, Kljavin NM, Li J, Walter MJ, de Sauvage FJ, Ghilardi N (**2008**) Cutting edge: IL-27 is a potent inducer of IL-10 but not FoxP3 in murine T cells. J Immunol 180: 2752-2756.

Benson MJ, Pino-Lagos K, Rosemblatt M, Noelle RJ (**2007**) All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. J Exp Med 204: 1765-1774.

Canale FP, Ramello MC, Núñez N, Araujo Furlan CL, Bossio SN, Gorosito Serrán M, Tosello Boari J, Del Castillo A, Ledesma M, Sedlik C, Piaggio E, Gruppi A, Acosta Rodríguez EA, Montes CL (**2018**) CD39 Expression Defines Cell Exhaustion in Tumor-Infiltrating $CD8^+$ T Cells. Cancer Res 78: 115-128.

Chaput N, Louafi S, Bardier A, Charlotte F, Vaillant JC, Ménégaux F, Rosenzwajg M, Lemoine F, Klatzmann D, Taieb J (**2009**) Identification of $CD8^+CD25^+Foxp3^+$ suppressive T cells in colorectal cancer tissue. Gut 58: 520-529.

Churlaud G, Pitoiset, F, Jebbawi, F, Lorenzon R, Bellier B, Rosenzwajg M, Klatzmann D (**2015**) Human and Mouse CD8+CD25+FOXP3+ Regulatory T Cells at Steady State and during Interleukin-2 Therapy. Front Immunol 6: 171.

Correale J, Villa A (**2010**) Role of $CD8^+$ $CD25^+$ $Foxp3^+$ regulatory T cells in multiple sclerosis. Ann Neurol 67: 625-638.

Eusebio M, Kraszula L, Kupczyk M, Kuna P, Pietruczuk M (**2012**) Low frequency of CD8+CD25+FOXP3(BRIGHT) T cells and FOXP3 mRNA expression in the peripheral blood of allergic asthma patients. J Biol Regul Homeost Agents 26: 211-220.

Friedman DJ, Künzli BM, A-Rahim YI, Sevigny J, Berberat PO, Enjyoji K, Csizmadia E, Friess H, Robson SC (**2009**) From the Cover: CD39 deletion exacerbates experimental murine colitis and human polymorphisms increase susceptibility to inflammatory bowel disease. Proc Natl Acad Sci USA 106: 16788-16793.

Jasiecka-Mikołajczyk A, Maślanka T (**2023**) Depletion of T and B cells in lymphoid tissues of mice induced by oclacitinib, a Janus kinase inhibitor. Pol J Vet Sci 26: 431-440.

Jasiecka-Mikołajczyk A, Socha P (**2020**) Teriflunomide inhibits activation-induced CD25 expression on T cells and may affect Foxp3-expressing regulatory T cells. Res Vet Sci 132: 17-27.

Jing J, Nelson C, Paik J, Shirasaka Y, Amory JK, Isoherranen N (**2017**) Physiologically Based Pharmacokinetic Model of All- trans-Retinoic Acid with Application to Cancer Populations and Drug Interactions. J Pharmacol Exp Ther 361: 246-258.

Kim G, Shinnakasu R, Saris CJ, Cheroutre H, Kronenberg M (**2013**) A novel role for IL-27 in mediating the survival of activated mouse CD4 T lymphocytes. J Immunol 190: 1510-1518.

Kiniwa Y, Miyahara Y, Wang HY, Peng W, Peng G, Wheeler TM, Thompson TC, Old LJ, Wang RF (**2007**) CD8+ Foxp3+ regulatory T cells mediate immunosuppression in prostate cancer. Clin Cancer Res 13: 6947-6958.

Lin L, Dai F, Wei J, Chen Z (**2021**) $CD8^+$ Tregs ameliorate inflammatory reactions in a murine model of allergic rhinitis. Allergy Asthma Clin Immunol 17: 74.

Liston A, Aloulou M (**2022**) A fresh look at a neglected regulatory lineage: $CD8^+Foxp3^+$ Regulatory T cells. Immunol Lett 247: 22-26.

Liu Y, Lan Q, Lu L, Chen M, Xia Z, Ma J, Wang J, Fan H, Shen Y, Ryffel B, Brand D, Quismorio F, Liu Z, Horwitz DA, Xu A, Zheng SG (**2014**) Phenotypic and functional characteristic of a newly identified $CD8^+$ $Foxp3^-$ $CD103^+$ regulatory T cells. J Mol Cell Biol 6: 81-92.

Liu Z, Liu JQ, Talebian F, Wu LC, Li S, Bai XF (**2013**) IL-27 enhances the survival of tumor antigen-specific $CD8^+$ T cells and programs them into IL-10-producing, memory precursor-like effector cells. Eur J Immunol 43: 468-479.

Loza MJ, Anderson AS, O'Rourke KS, Wood J, Khan IU (**2011**) T-cell specific defect in expression of the NTPDase CD39 as a biomarker for lupus. Cell Immunol 271: 110-117.

Mahic M, Henjum K, Yaqub S, Bjørnbeth BA, Torgersen KM, Taskén K, Aandahl EM (**2008**) Generation of highly suppressive adaptive CD8+CD25+FOXP3+ regulatory T cells by continuous antigen stimulation. Eur J Immunol 38: 640-646.

Maślanka T (**2022**) Effect of IL-27, teriflunomide and retinoic acid and their combinations on $CD4^+$ T regulatory T cells – an in vitro study. Molecules 27: 8471.

Matsui M, Kishida T, Nakano H, Yoshimoto K, Shin-Ya M, Shimada T, Nakai S, Imanishi J, Yoshimoto T, Hisa Y, Mazda O (**2009**) Interleukin-27 activates natural killer cells and suppresses NK-resistant head and neck squamous cell carcinoma through inducing antibody-dependent cellular cytotoxicity. Cancer Res 69: 2523-2530.

Ménoret S, Tesson L, Remy S, Gourain V, Sérazin C, Usal C, Guiffes A, Chenouard V, Ouisse LH, Gantier M, Heslan JM, Fourgeux C, Poschmann J, Guillonneau C, Anegon I (**2023**) $CD4^+$ and $CD8^+$ regulatory T cell characterization in the rat using a unique transgenic Foxp3-EGFP model. BMC Biol 21: 8.

Molodtsov A, Turk MJ (**2018**) Tissue Resident CD8 Memory T Cell Responses in Cancer and Autoimmunity. Front Immunol 9: 2810.

Murugaiyan G, Mittal A, Weiner HL (**2010**) Identification of an IL-27/osteopontin axis in dendritic cells and its modulation by IFN-gamma limits IL-17-mediated autoimmune inflammation. Proc Natl Acad Sci USA 107: 11495-11500.

Nwankwo E, Allington DR, Rivey MP (**2012**) Emerging oral immunomodulating agents – focus on teriflunomide for the treatment of multiple sclerosis. Degener Neurol Neuromuscul Dis 2: 15-28.

Ponthan F, Kogner P, Bjellerup P, Klevenvall L, Hassan M (**2001**) Bioavailability and dose-dependent anti-tumour effects of 9-cis retinoic acid on human neuroblastoma xenografts in rat. Br J Cancer 85: 2004-2009.

Ringshausen I, Oelsner M, Bogner C, Peschel C, Decker T (**2008**) The immunomodulatory drug Leflunomide inhibits cell cycle progression of B-CLL cells. Leukemia 22: 635-638.

Suzuki M, Konya C, Goronzy JJ, Weyand CM (**2008**) Inhibitory CD8+ T cells in autoimmune disease. Hum Immunol 69: 781-789.

Szondy Z, Reichert U, Fésüs L (**1998**) Retinoic acids regulate apoptosis of T lymphocytes through an interplay between RAR and RXR receptors. Cell Death Differ 5: 4-10.

Tang Q, Bluestone JA (**2008**) The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. Nat Immunol 9: 239-244.

Wing JB, Tanaka A, Sakaguchi S (**2019**) Human FOXP3$^+$ regulatory T cell heterogeneity and function in autoimmunity and cancer. Immunity 50: 302-316.

Wen Z, Shimojima Y, Shirai T, Li Y, Ju J, Yang Z, Tian L, Goronzy JJ, Weyand CM (**2016**) NADPH oxidase deficiency underlies dysfunction of aged CD8+ Tregs. J Clin Invest 126: 1953-1967.

Xiao S, Jin H, Korn T, Liu SM, Oukka M, Lim B, Kuchroo VK (**2008**) Retinoic acid increases Foxp3+ regulatory T cells and inhibits development of Th17 cells by enhancing TGF-beta-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression. J Immunol 181: 2277-2284.

Zhong H, Liu Y, Xu Z, Liang P, Yang H, Zhang X, Zhao J, Chen J, Fu S, Tang Y, Lv J, Wang J, Olsen N, Xu A, Zheng SG (**2018**) TGF-β-Induced CD8$^+$CD103$^+$ regulatory T cells show potent therapeutic effect on chronic graft-versus-host disease lupus by suppressing B cells. Front Immunol 9: 35.