Cytokine 113 (2019) 50–60

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Cyclophosphamide with or without fluorouracil followed by subcutaneous or intravenous interleukin-2 use in solid tumors: A feasibility off-label experience

Giovanni Lo Re[a,*], Francesco Lo Re[b], Paolo Doretto[c], Alessandro Del Conte[a], Maria Amadio[d], Cinzia Cozzi[e], Maria Maddalena Casarotto[f], Daniele Maruzzi[g], Wally Marus[h], Paolo Ubiali[i], Paolo Sandri[a]

[a] CRO Pordenone-S. Vito Oncology, Pordenone, Italy
[b] Study University, Milan, Italy
[c] Clinical Patholgy, AAS5 Pordenonese, Pordenone, Italy
[d] Medical Direction, AAS5 Pordenonese, Pordenone, Italy
[e] Pharmacy, AAS5 Pordenonese, Pordenone, Italy
[f] Obstetrics and Gynecology Unit, AAS5 Pordenonese, Pordenone, Italy
[g] Urology Unit, AAS5 Pordenonese, Pordenone, Italy
[h] Pathology Unit, AAS5 Pordenonese, Pordenone, Italy
[i] Surgery Unit, AAS5 Pordenonese, Pordenone, Italy

ARTICLE INFO

Keywords:
Cyclophosphamide
Fluorouracil
Interleukin-2
Cancer

ABSTRACT

Background: Immune tolerance seems to correlate with disease progression and T regulatory cells (Tregs) and myeloid-derived suppressor cells play a relevant role in immunosuppression. Cyclophosphamide (Cyt) and Fluorouracil (FU) seem to reduce these cell populations.

Methods and objective: Establishing safety, feasibility, activity and impact on the immune system (neutrophil/lymphocyte [N/L], platelet/L [Plt/L], monocyte [M] and lymphocyte subpopulation (CD3, CD4, CD8, CD16, HLADR/CD3, Tregs, cells count), CD8/Treg and C-reactive protein (CRP). Treatment: 1) Cyt 300 mg/sqm ± FU 500 mg/sqm day (d) 1 and interleukin 2 (IL-2) 18 MUI/sqm intravenous (I.V.) d 4–6, 18–20 or 2) Cyt 300 mg/sqm + FU 500 mg/sqm day d 1, IL-2 4.5 MUI subcutaneous (S.C.) d 3–6, 17–20. The cycle was repeated every four weeks for 2 cycles. Stable or responding patients (pts) continued therapy for 3 cycles.

Results: From February 2014 to December 2016, 13/14 pre-treated pts (mean 3 lines) with solid tumors were enrolled. Male/Female: 1/1. The median age and Eastern Cooperative Oncology Group Performance Status (ECOG PS) was 68 years and 1 respectively. Mean 2 cycles of therapy were administered. G3-4 toxicities presented as diarrhea and bleeding anemia in 2 pts and proteinuria and erhytroderma in 1pt, respectively. Regarding the hematological profile, a more reduction in Plt, less decrease of Plt/Ly, and less increase of Treg with I.V. than S.C. IL-2 administration was observed. However a transient decrease of Treg on day 7 of first cycle in the I.V. IL-2 was reported.

Response: PR 3 (23%), SD 3 (23%), PD 7 (54%). The response duration was 2+ and 3 months in 2 HCC and 18+ months in the pancreatic cancer (PC). Pathological CR was reported in one HCC treated with I.V. IL-2. The

Abbreviations: ADM, adriamycin; α-FTP, alfa-fetoprotein; β-IFN, beta-interferon; Cyt, cyclophosphamide; FU, fluorouracil; CRC, colorectal cancer; DDP, *cis*-diamine-diclhoroplatinum; GEM, gemcitabine; IL-2, interleukin-2; sIL-2R, soluble interleukin-2 receptor; TACE, *trans*-arterial chemoembolization; Ly, Lymphocytes; MLT, melatonine; S, surgery; w, week; PR, partial remission; SD, stable disease; PD, progressive disease; MRD, median response duration; R, responder; DCR, disease control rate; s.c., subcutaneous; i.a., intra-arterial; ICC, intrahepatic cholangiocarcinoma; WNV, within the normal values; DE, escalating doses; CT, chemotherapy; Cy, cycles; D, dead; A, alive; sc, subcutaneous; iv, intravenous; GIST, gastrointestinal stromal tumor; HCC, hepatocarcinoma; NEC, neuroendocrine carcinoma; PC, pancreatic carcinoma; OS, overall survival; mos, months; Bas, basal; Post, post therapy; M, monocytes; N, neutrophil; P, platelet; Treg, T regulator cell; CTLA4 ligands, Cytotoxic T-Lymphocyte Antigen 4 ligands; IDO, indoleamine dioxygenase; PGE2, Prostaglandin E2; TGFb, transforming growth factor beta-2; IL10, interleukin 10; FasL, Fas ligand; PDL1, PD-L2, programmed death-ligand 1,2; MDSCs, myeloid-derived suppressor cells; FDA, Food and Drug Administration; NK, natural killer; RCC, renal cell carcinoma; GITR, glucocorticoid-induced TNFR family related gene; ALT, alanine amino transferase; AST, aspartate transaminase; LVEF, left ventricular ejection fraction; TAMs, tumor-associated macrophages; IMCs, immature myeloid cells

* Corresponding author.
E-mail addresses: giovanni.lore@cro.it (G. Lo Re), francesco.lore@hotmail.com (F. Lo Re), paolo.doretto@aas5.sanita.fvg.it (P. Doretto), alessandro.delconte@cro.it (A. Del Conte), maria.amadio@aas5.sanita.fvg.it (M. Amadio), cinzia.cozzi.farm@aas5.sanita.fvg.it (C. Cozzi), maddalena.casarotto@aas5.sanita.fvg.it (M.M. Casarotto), daniele.maruzzi@aas5.sanita.fvg.it (D. Maruzzi), wally.marus@aas5.sanita.fvg.it (W. Marus), paolo.ubiali@aas5.sanita.fvg.it (P. Ubiali), paolo.sandri@cro.it (P. Sandri).

https://doi.org/10.1016/j.cyto.2018.06.005
Received 23 September 2017; Received in revised form 13 May 2018; Accepted 5 June 2018
Available online 27 June 2018

median progression-free-survival (PFS) and overall survival (OS) were 1 and 7 months.
Conclusion: Cyt-FU-IL-2 can be considered safe, feasible and moderately active in heavily pre-treated pts. Plt, Plt/Ly, CD8/Treg and a transient Tregs reduction were observed without significative difference on survival.

1. Introduction

The progression of disease during or after systemic treatment in solid tumors may be related to both primary and acquired resistance phenomena. The major known mechanisms found in 50% of tumors are p53 [1] and p-glycoprotein [2] mutations, responsible for the multi-drug-resistant phenotype.

Recently, the important role of the immune system in growth cell control and neoplastic progression has been identified. The mechanisms triggering the immune escape can be of vascular character for endothelial damage or due to inhibition of immune activation by galectin [3] or CTLA4 ligands [4–7]. Furthermore, the metabolic damage resulting in amino acid depletion, such as tryptophan and arginine, in the microenvironment due to indoleamine dioxygenase (IDO) [8–10] and arginase, and the secretion of suppressive mediators, such as in the microenvironment due to indoleamine dioxygenase (IDO) [8–10] and arginase, and the secretion of suppressive mediators, such as PGE2, TGFb, IL10 and adenosine [11], disrupt lymphocyte proliferation. Finally, this can be linked to a functional impairment of the immune response through apoptotic depletion by FasL, PD-L1 or PD-L2 [12–17]. These immunosuppressive mechanisms involve different types of cells such as tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), type 2 natural killer T (NKT) cells [18], and regulatory T cells (Tregs) [19–22], but Tregs and MDSCs are the most studied ones.

CD4 + FOXP3 + CD25 high Tregs seem to influence the local (intra tumor and stromal) recruitment of immunosuppressive cells [19–22]. This occurrence becomes relevant in presence of Tregs increase with consequent negative prognostic impact, probably due to the negative influence on antitumor immune activation [23–25]. This is applicable to both immunogenic tumors such as kidney cancer [26] and melanoma [27], as well as, to other cancers [20,23,25,28].

The myeloid-derived suppressor cells (MDSCs) [18,29], a CD11b + CD33 + CD14-HLA-DRhuman subpopulation cell, in basal

Table 1
Results of therapeutic studies concerning immunotherapy alone or associated to other drugs in solid tumors.

Reference	Organ	Line	N° patients	Therapy	Resp	Overall survival (months)	Notes
Cappa [42]	HCC	**I**	9	Megestrol Thalidomide (50 mg/day to MTD) IL-2 (1MUI/day × 3w/4w) s.c (4pt)	SD 1 PD 8	9.9 (2.6–18.6)	α-FTP ↑ 6 stable 2; ↓1
Hayakawa [43]	CRC ICC	**I**	9	(rIL-2) i.a rIL-2 + low dose Cy	PD 9 PR 3	n.d	–
Onishi [44]	HCC	**I**	10	IL-2 15 μg for 14 to 64 days – 7 LAK 10^9-10^{10} for 1–2/week	PR 2 SD 8	n.d	> 35% ↓ α-FTP in 4/9 pts
Zhang [45]	HCC	**I**	24 24	IL-2 and sIL-2R after TACE plus Jinglong Capsule. TACE	sIL2R↓ (P < 0.05) ↑IL-2 sIL-2R ↑ IL-2↓ (P < 0.05).	n.d	Jinlong can significantly improve the ly function
Aldeghi [46]	HCC	**I**	14	IL-2 (3 MIU/day s.c for 6 days/wk × 4 wks) + MLT (50 mg/day)	36% MRD 7 + mos	n.d	
Uggeri [47]	Pancreas	**I**	31	9 pts IL-2 s.c9 MIU/day × 3 days pre-S 9 pts IL-2 at 12 MIU/day for 3 days pre-S; 13 pts S	Ly ↓ Ly WNVLy ↓	n.d	IL-2 12 MIU abrogate the S negative effects and recover a normal immune-function
Caprotti [48]	Pancreas		30	IL-2 (12 MIU/day SC x3 days → S S	Ly ↑ Ly ↓	27 13	–
Mayer [49]	Bladder	**I-II**	62	GEM + DDP plus ALT-801 (0.06 mg/kg/dose, d3, 5, 8, 12)/3wks for 3 cycles	3 CR, 9 PR (35) 6 SD, 12 PD	12.3	–
Wei [50]	Ovary	**II**	24	rIL-2 6 × 10^5 IU/m^2/wk × 16 w.	2 CR 4PR	R 5YS 70% No-R 15%	After IL-2 stop Treg more dropped in clinical R
Nicolini [51]	Breast	**II**	26	control β-IFN, IL-2	MDR 16 p: < 0.001 MDR 61	31p: < 0.000001 101	19 pts long-term surviving
Kimmick [52]	Breast	**II**	32	rIL-2 1.5 MU/m^2 × 5 days/wk × 3 wks, α-IFN 7.5 MU/m^2 s.c ×3 /wk	PR 1 (3%) SD 6 (19%)	8.9	Good performance status predictor of survival
Catania [53]	miscellanea	**II**	29	ED 5–25 mg/m^2 F16-IL2 days 1, 8, 15 ADM 20–25 mg/m^2	(Phase I-II) DCR (12 wk) 43%, 33% Phase II (9pts) PR1 (45 + wk) SD 3	9.8	–

ADM: adriamycin; α-FTP: alfa-fetoprotein; β-IFN: beta-interferon; Cyt: cyclophosphamide; CRC: colorectal cancer; DDP: *cis*-diamine-dichlore-platinum; GEM: gemcitabine; ICC: Intrahepatic Cholangiocarcinoma; IL-2: interleukin-2; sIL-2R: soluble interleukin-2 receptor; TACE: *trans*-arterial chemoembolization; Ly: Lymphocytes; MLT: melatonine; Resp: response; S: surgery; w: week; PR: partial remission; SD: stable disease; PD: progressive disease; MRD: median response duration; R: responder; DCR: disease control rate; s.c.: subcutaneous; i.a.: intra-arterial; WNV: within the normal values; ED: escalating doses.

Table 2
Characteristics of patients with advanced solid tumors treated with Cyclophosphamide-Fluorouracil-IL2 regimen.

Tumor	Age	Gender	ECOG PS	N°prev CT	Previous regimens
Ovary	75	F	1	8	C-P; CP; P; L; D; Cae-Bev; P; NVB metro
Breast	53	F	1	9	A-Cy; Ana; T-My; Exem; Fulv; EVE; Pal; Cape; Cy-MTX metro
Uterine tuba	75	F	2	4	C-P; C-Cae; E-Trab; C
HCC	51	M	2	1	Sor
Bladder	76	M	2	3	D-G; D-A i.a.; P-G
Prostate NEC	64	M	2	5	ADT; T; D-E; C-CPT11; ABI;
GIST	68	M	2	3	Gef; Sun; Regor
Melanoma	38	M	1	2	Adj IFN HD; Dabra-Trame
Pancreas	74	F	0	2	Nab-P-G; OHP-CPT11-Cape
NEC	35	M	1	2	DE; C-CPT11
Ovary NEC	67	F	0	3	C-P; PE; C-E
HCC	74	M	1	3	TACE; Sor; Cape;
Bladder	43	F	1	5	Neoadj. D-G; Nab-P; D-A i.a; Cae; Cyt-MTX metro

F: female; M: male; N° prev CT: number previous chemotherapy; GIST: gastrointestinal stromal tumor; HCC: hepatocarcinoma; NEC: neuroendocrine carcinoma; ECOG PS: Eastern Cooperative Oncology Group Performance Status; ABI: Abiraterone acetate; Adj IFN HD: adjuvant interferon high dose; Ana: Anastrazole; ADT: androgen deprivation therapy; Cae: Caelyx; C-P: Carboplatin-Paclitaxel; C-Cae: Carboplati-Caelyx; Cae-Bev: Caelyx-Bevacizumab; Cape: Capecitabine; Cyt-MTX: Cyclophosphamide- Methotrexate; C-CPT11: Carboplatin-Irinotecan; D: cisplatin; D-A i.a.: Cisplatin-Adriamycin intra-arterial; Dabra-Trame: Dabrafenib-Trametinib; D-E: Cisplatin-Etoposide; E-Trab: Epydoxorubicin-Trabectidine; Eve: Everolimus; Exem: Exemestane; Fulv: Fulvestrant; Gef: Gefitinib; L: Letrozole; nab-P: nab-Paclitaxel; NVB metro: Navelbine metronomic; OHP-CPT11-Cape: Oxaliplatin-Irinotecan-Capecitabine; P: paclitaxel; P-G: Paclitaxel-Gemcitabine; Pal: Palbociclib; Regor: Regorafenib; Sor: Sorafenib; Sun: Sunitinib; T-My: Docetaxel-Myocet ; TACE: *trans*-arterial chemoembolization.

conditions are poorly represented in the circulating stream and increase in presence of inflammatory, infectious, or neoplastic diseases. While in physiological conditions, the immature myeloid cells (IMCs) are stimulated to differentiation into mature macrophages, dendritic cells (DCs), or granulocytes, in the context of cancer, the release of mediators from the microenvironment and chemokines cause the formation of MDSC and their intratumor migration.

They block migration of naıve CD62LC (L-selectin) T cells to lymphoid organs and induce intracellular pathways that promote self-expansion. They induce cell cycle arrest and decrease of T cell receptor (TCR) functionality through the production of high levels of arginase 1 (ARG-1) that depletes T cells of L-arginine and reactive oxygen and nitrogen species (ROS, RNS) from Th17 cells, respectively.

MDSCs release factors that stimulate regulatory conversion and expansion of Tregs also through the production of TGF-β and retinoic acid leading trans differentiation of Foxp3 regulatory T cells from monocyte-induced Th17 cells [30]. Furthermore MDSCs correlate with the tumor stage, prognosis, and resistance to treatment [31]. This knowledge allowed to reconsider the use of first-generation cytokines such as interleukin-2 (IL-2) and the development of new agents, such as anti-PD1 and anti-PDL1, which have recently revolutionized tumor treatment, especially in kidney cancer [32] and melanoma [33], but also in lung [34], bladder [35], head and neck cancer [36], and in other neoplasms [37,38].

IL-2 is a cytokine produced by activated T cells increase the proliferation and activation of cytotoxic T cells, natural killer (NK) cells and monocytes [39]. IL-2 is essential for the maintenance and expansion of immunosuppressive CD4 + CD25 + Tregs, in which IL7 2Rαβγ is constitutively expressed, to reduce the risk of an uncontrolled

Table 3
Grade of toxicity and duration during and after treatment.

Tumor	Route IL-2 administration	Toxicity all Grades	Toxicity G3-4	Toxicity duration/cycle
Ovary	s.c	FLS G1, bleeding anemia G2, fever G1	–	FLS during therapy. bleeding anemia 1 day
Breast	s.c	FLS G1, fatigue G2 bleeding anemia G3	Bleeding anemia G3	FLS during therapy, fatigue improvement after therapy to G1, bleeding anemia G3 5 days
Uterine tuba	s.c	FLS G2, fever G1	–	FLS during therapy. fever during therapy
HCC	s.c	FLS G1, anemia G3, fatigue G2, lethargy, hypoalbuminemia G2	Bleeding anemia G3	FLS during therapy, fatigue improvement after therapy to G1, anemia 3 days, limbs oedema G2 14 days
Bladder	s.c	FLS G1, fever G1	–	FLS during therapy, fever G1 4 days
Prostate NEC	s.c	Fever G1, anemia G2	–	Fever 4 days, anemia 5 days
GIST	s.c	Fever G1, FLS G1	–	Fever and FLS during therapy
Melanoma	i.v	FLS G2, Fever G1, melanuria, fatigue G2	–	FLS for 8 days. fever G1 3 days, fatigue G2 to G1 7 days
Pancreas	i.v	Fever G1, ipotension G2, capillary leak syndrome G2, erhytroderma G3	Diarrhea G3, Erhytroderma G3	Fever 4 days, Ipotension 4 days. Capillary leak syndrome 4 days, Erhytroderma 14 days, hypereosinophilia. diarrhea G3 3 days hypothyroidism continuing
NEC	i.v	hypereosinophilia, hypothyroidism G2, diarrhea G3 Fever G1, ipotension G2, capillary leak syndrome G2, thrombocytopenia G2, hypothyroidism G2 pruritus G2, anemia G2, creatinine G2		Fever 4 days, thrombocytopenia 9 days, ipotension 4 days, anemia 2 days, hypothyroidism continuing, pruritus 10 days, creatinine 4 days
Ovary NEC	i.v	Fever G1, ipotension G1, diarrhea G3, FLS G1	Diarrhea G3	Fever 4 days, ipotension 3 days, diarrhea G3 4 days FLS during therapy
HCC	i.v	Fever G1, ipotension G1, FLS G1		FLS during therapy, fever 3 days, ipotension 3 days
Bladder	i.v	Fever G1, ipotension G1 proteinuria G3, FLS G1	Proteinuria G3	Fever 3 days, ipotension 3 days, proteinuria 6 days, FLS during therapy

NEC: Neuroendocrime carcinoma; GIST: Gastrointestinal stromal tumor; HCC: Hepatocarcinoma; S.C: subcutaneous; I.V: intravenous; FLS: Flu-like-syndrome.

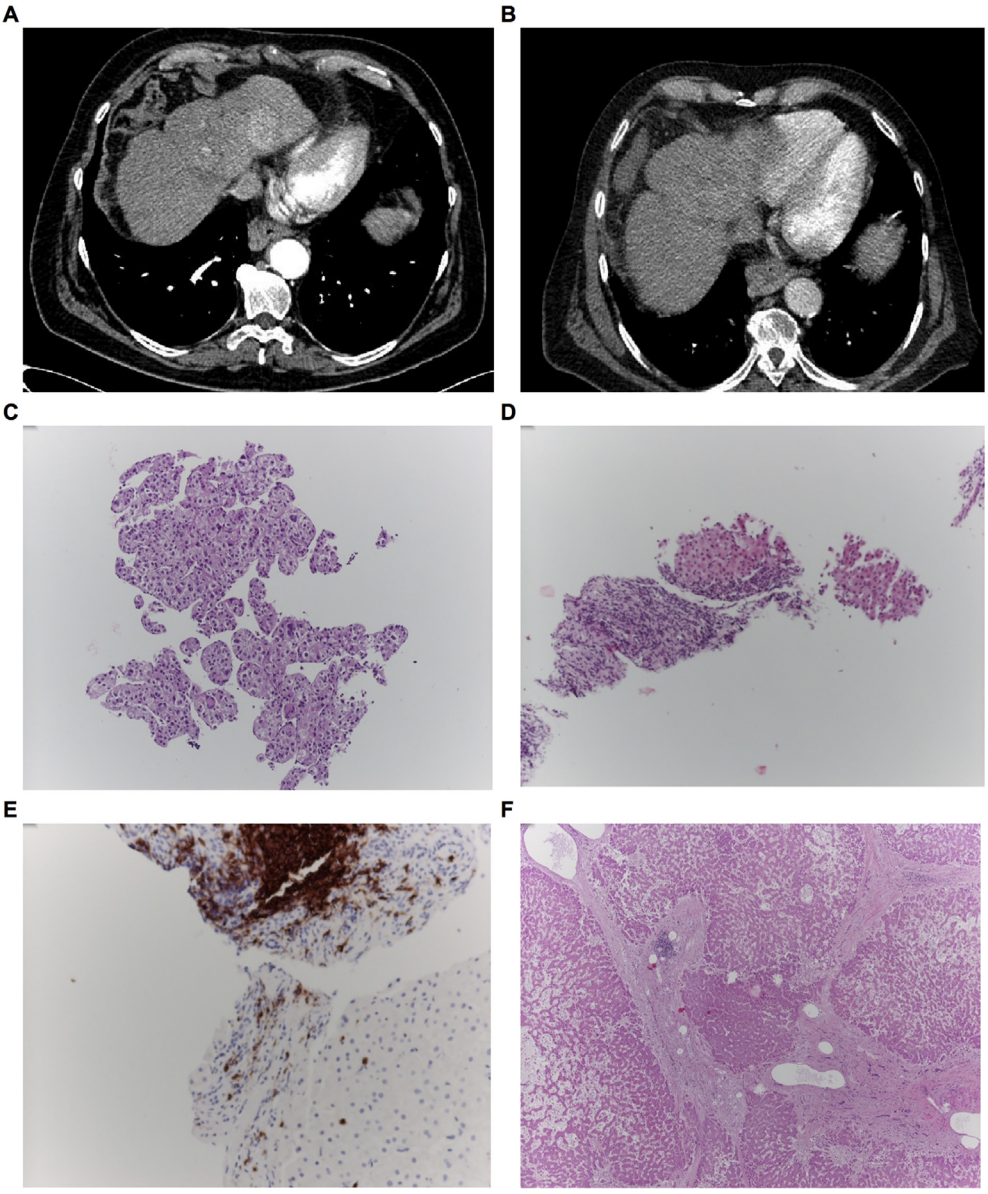

Fig. 1. Pre-treatment HCC CT scan shows (A) probable thrombosis of the left branch of the portal vein; areas of early enhancement with wash-out in subsequent post-contrasted phases in the 4° and 8° segments. Further pathological areas of 3 cm and 1 cm were detected to 2° and 3° segments respectively. Lymph node enlargement of 2 cm at the small curve of the stomach; post-treatment (B) shows no longer recognizable areas of rapid arterial wash-out described on 2°, 3°, 4° and 8° segments and reduction of lymph node size to the small curve of the stomach currently 1.6 cm. The basal anatomo-pathological specimen shows (C) moderately differentiated HCC with trabecular pattern and (D) micronodular cirrhosis with severe lymphoyd infiltrate only in the portal space, (E) with LCA positivity to immunohistochemistry. The post-treatment biopsy (F) shows the absence of neoplastic cells and presence of micronodular cirrhosis.

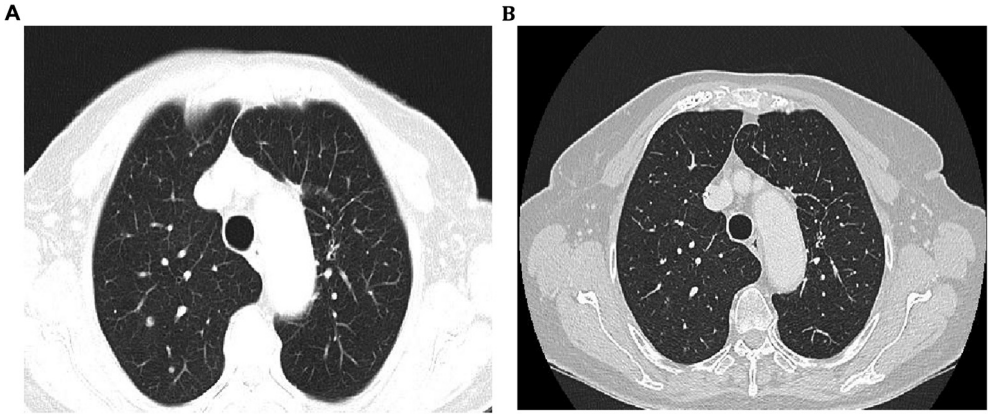

Fig. 2. Pre-treatment PC CT scan shows (A) presence of multiple bilateral pulmonary micronodules, greater than 0.7 cm to the upper right lobe and after 18 months of therapy (B) persistence of response to pulmonary nodules including that of the right upper lobe.

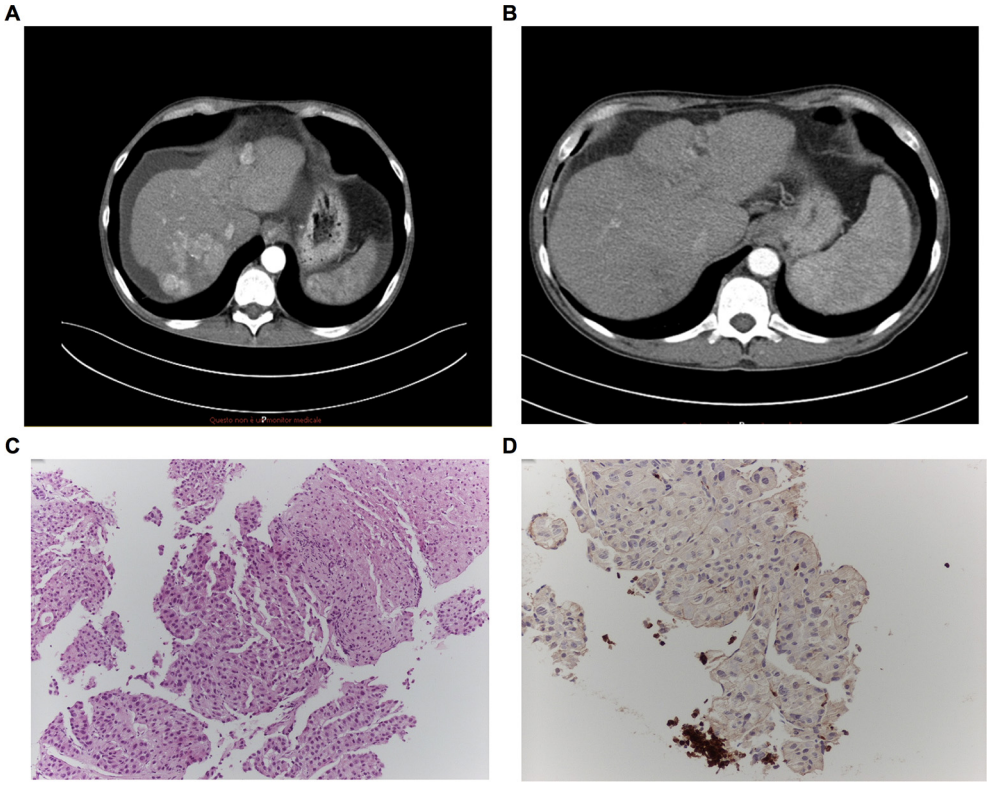

Fig. 3. Pre-treatment HCC CT scan shows (A) presence of hypervascular nodules in the 3°, 4°B and 5° hepatic segments of the left and right lobe with wash out in subsequent post-contrasted phase. The post-treatment CT scan (B) shows disappearance of hypervascular nodules to the 3° segment of left lobe and to 4°B and 5° segments of right lobe with wash out in subsequent post-contrasted phase. The basal anatomo-pathological specimen shows (C) well differentiated with scattered lymphocyte T (CD3) infiltration to immunohistochemistry (D).

Table 4
Relationship between route of IL-2 administration, response and overall survival of advanced solid tumors treated with Cyclophosphamide-Fluorouracil-IL2.

Tumor	RouteIL-2	N° cy	Resp (%)	OS (mos)	Status
Ovary	sc	3	PD (0.13)	4	D
Breast	sc	2	PD (0.13)	12+	A
Uterine tuba	sc	2	PD (0.13)	6	D
HCC	sc	1	PR (0.13)	5	D
Bladder	sc	3	SD (0.13)	6	D
Prostate NEC	sc	2	PD (0.13)	8	D
GIST	sc	4	SD (0.13)	5	D
Melanoma	iv	1	PD (0.13)	1	D
Pancreas	iv	2	PR (0.13)	18+	A
NEC	iv	1	PD (0.13)	1	D
Ovary NEC	iv	1	PD (0.13)	9	D
HCC	iv	2	PR (0.13)	11	D
Bladder	iv	2	SD (0.13)	20	D

D: Died; A: Alive; PD: Progressive disease; PR: Partial remission; SD: Stable disease; NEC: Neuroendocrime carcinoma; GIST: Gastrointestinal stromal tumor; HCC: Hepatocarcinoma S.C: subcutaneous; I.V: intravenous; Resp: Response.

immune responses and autoimmunity [40].

Food and Drug Administration (FDA) approval of IL-2 was obtained for treatment of patients with metastatic renal cancer (RCC) in 1992 and metastatic melanoma in 1998, after attaining an overall response of 16% in melanoma and 15% in RCC patients. It represents the only molecule capable of achieving long-term survival in < 10% of patients with melanoma and RCC. It can be used in different doses and modalities of administration but a high-dose bolus has been shown to be superior to other modalities only in patients attaining complete remission in renal cancer [41]. Furthermore, sporadic activity has been reported in other non immunogenic tumors [42–53] (Table 1).

It is therefore possible to hypothesize that agents capable of negatively modifying these two immunosuppressive cell populations can improve the antitumor immune activity.

Immunomodulatory and chemotherapeutic agents active on Tregs include anti-CD25 mAb [54], anti-CTLA-4 [55], anti-GITR mAb [56] antibodies, and Gemcitabine (GEM) [57], Cyclophosphamide (Cyt) [58] and Paclitaxel (P) respectively [59].

Chemotherapy [57,60] mostly Fluorouracil (FU) [60] and anti-angiogenetic molecules such as Sunitinib [61] and Bevacizumab [62] can reduce MDSCs, although it is difficult to measure this numerical and functional effect, considering also the rebound effect of MDSCs after Tregs reduction [63]. This phenomenon can be controlled by the simultaneous administration of Cyt and GEM [64], probably due also to the dual action of GEM.

In light of the major efficacy of combination drugs [64] on both immune-suppressive cells and of the superiority of FU, over other agents, on MDSC [60], we undertook a real-world experience with Cyt alone or in combination with FU, prior to the administration of IL-2, in heavily pre-treated solid tumor patients not susceptible to other potentially effective treatments.

The aim of this study was to evaluate the safety and feasibility of treatment, activity and the impact on the immune system (neutrophil/lymphocyte [N/Ly], platelet/L [Plt/Ly], monocyte [M] and lymphocyte subpopulation (CD3, CD4, CD8, CD16, HLA-DR/CD3, Tregs count), CD8/Treg and C-reactive protein (CRP).

2. Patients and methods

The study was made possible through the authorization of the competent institutions and after obtaining written informed consent from each patient in accordance with the Helsinki Declaration.

2.1. Eligibility criteria

The eligibility criteria required patients with advanced solid tumors, ECOG PS ≤ 1, previously treated, with adequate organ function (white blood cells ≥3 × 103/μL, neutrophil ≥1.5 × 103/μL, hemoglobin ≥10 g/dL, ALT/AST < 2.5 × UNV, bilirubin < 2.5 × UNV, left

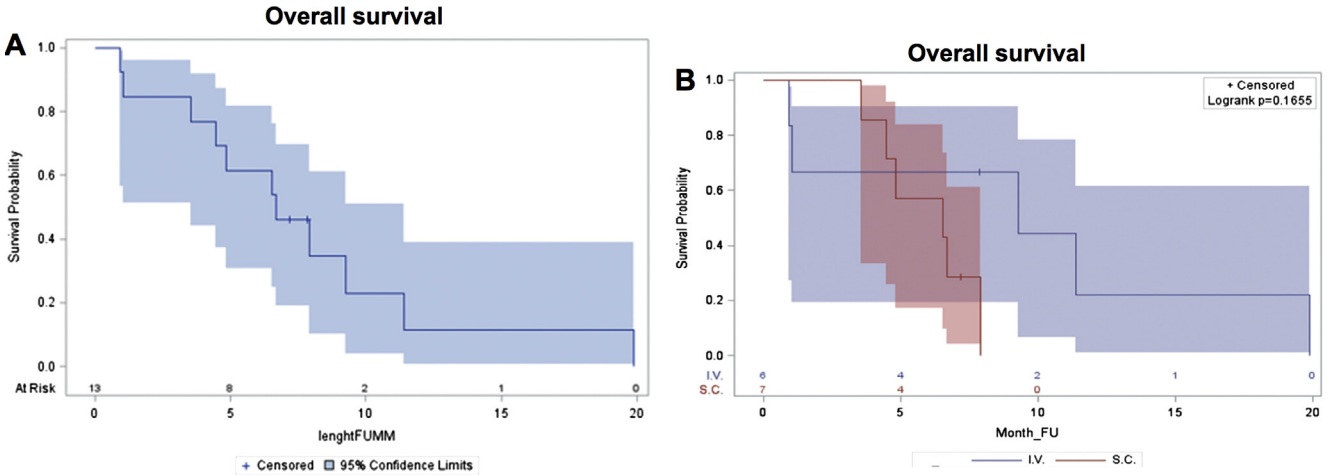

Fig. 4. Overall survival of advanced solid cancer cases treated with Cyt-FU and IL-2 I.V or S.C (A) and in relation to the route of administration of IL-2 i.v or s.c (B) (Kaplan-Meier estimates of overall survival (OS) for I.V (blue line) or S.C (red line) IL-2 administration. Log-rank test: p = 0.1655).

ventricular ejection fraction (LVEF) ≥50%, creatinine < 1.5 × UNV. Ineligibility criteria included ECOG PS > 1, the presence of clinically active brain metastases, recent cardiac illness, major infections, contraindications for the use of vasopressor drugs, and chronic steroid use.

2.2. Staging

Staging: brain/chest/abdominal computed tomography scan, electrocardiogram, echocardiography, hemochrome and chemistry, lymphocyte typing (CD3, CD4, CD8, Tregs, CD8/Treg, CD16, HLA-DR cells count), CRP, TSH, FT3, FT4. Lymphocyte typing was performed on a FACSCanto II system using FACSDiva software (BD Biosciences, Franklin Lakes, NJ, USA).

Before analysis, the flow cytometer settings were checked using compensation beads (CS&T beads, BD Bioscences) according to manufacturer's instructions. Multiparametric flow cytometry was performed on 50μL of sample stained for surface markers (10 μL for each monoclonal antibodies). Red blood cells were lysed with 2 mL of 1:5 diluted BD Pharm LyseTM lysing solution for 15 min at room temperature in darkness (10 mL of lysing solution in 40 mL of deionized water). The remaining cells were pelleted by centrifugation (1500 rpm for 5 min at room temperature), the supernatant was aspirated and the cell were resuspended in 500 μL of PBS. The following monoclonal antibodies (MoAb) were used: CD3 FITC, CD16 PE, CD4 PE-Cy7, CD8 APC-Cy7, CD45 PerCp, HLA-DR Horizon450, CD25 PE, CD127 PE-Cy7. All MoAb were purchased from BD Biosciences, excluding CD25 PE e CD127 PE-Cy7 (Beckman Coulter, Miami, Florida, USA). The Treg was defined as CD3+/CD4+/CD25+/CD127- lymphocyte subpopulation.

Testing was repeated on days 1, 4, 7, 18 and at every other intravenous (I.V.) IL-2 therapy cycle, and after therapy cessation, and on day 1, every other cycle of subcutaneous (SC) administration of IL-2, and after therapy cessation.

2.3. Statistical considerations

Descriptive statistics included median and range as a measure of variability. The PFS and OS were computed by the Kaplan–Meier method, and Log-rank test was used to test the differences between subgroups. Differences were considered significant when P values were < 0.05.

2.4. Treatment

From February 2014, the therapy included Cyt 300 mg/sqm IV on day 1, followed by intravenous (I.V.) chronomodulated or continuous infusion (C.I.) of IL-2 18 MUI/sqm on days 4–6 and 18–20. The cycle was repeated on day 29.

Subsequently, the treatment was modified by adding FU 500 mg/sqm I.V. bolus at day 1, followed by IL-2 I.V.C.I. regimen or IL-2 4.5 MUI S.C on days 3–6 and 17–20. The cycle was repeated on day 29. The treatment with Cyt-FU was performed as out-patient in both regimens while IL-2 I.V. and S.C was administered as in-patient and out-patient settings, respectively.

Responses were evaluated according to RECIST version 1.1 [65], every 2 cycles, and therapy was continued in stable (SD) and responding (CR, PR) patients for other 4 cycles.

A dose reduction of IL-2 of 14 MUI/sqm was adopted in the case of renal, gastrointestinal or cardiovascular G2 toxicity and of 10 MUI/sqm for G3 cardiovascular toxicity. For G3 renal, pulmonary or greater than G1 sensory or motor neurological toxicity, the treatment was interrupted. No reduction in S.C. IL-2 for G2-3 toxicity was required, but in case of subjective intolerance or severe toxicity, the therapy was suspended.

3. Results

From February 2014 to January 2017 13/14 patients were treated (Table 2).

3.1. Toxicity

Table 3 shows the toxicity, according to CTCAE v3.0 criteria [66], reported during treatment. For six patients treated with 9 cycles (range 1–2) I.V. IL-2 administration, toxicity was manageable in the standard care unit. Grade 3 were represented by diarrhea in 2, proteinuria and erhytroderma G3 with hypereosinophilia in 1 patient, respectively. Vascular, gastrointestinal and general disorders treatment-related were reversible and side effects regressed upon discontinuation of therapy. Regarding other toxicities, proteinuria improved after 6 days from discontinuation therapy whereas hypothyroidism required continuous replacement therapy. Seventeen cycles (range 1–4) of S.C IL-2 were administered. Toxicity was mild although G3 toxicities as bleeding anemia was reported in 2 patients. The general disorders were mild with rapid improvement after treatment discontinuation. The hemorrhagic anemia resolved after transfusion of red blood cells, and it did not result in evident signs of bleeding at endoscopy.

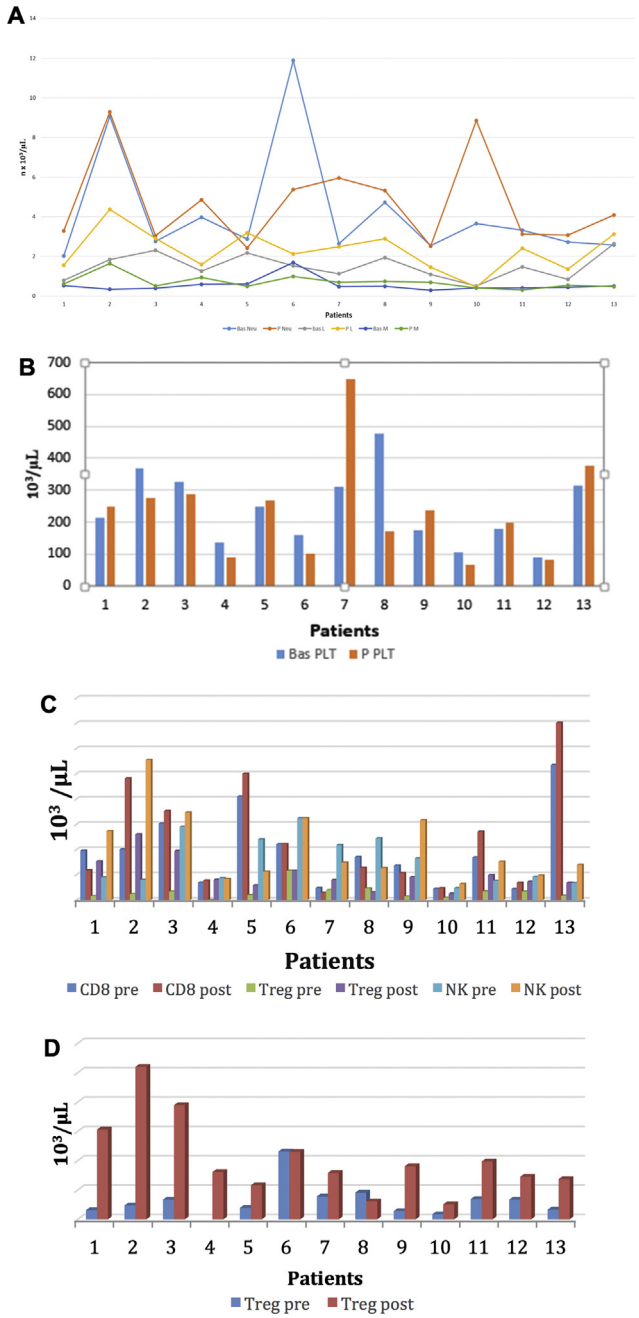

Fig. 5. Hematological parameters outcome during therapy: (A) relative stability in the neutrophils granulocytes, lymphocytes, monocytes was observed almost on all cases by comparing baseline counts with those after treatment; Bas: basal; p: post; neu: neutrophil; L: lymphocytes; M: monocytes; (B) Platelets outcome during therapy: decrease of platelets count on half of cases with a more reduction after IL-2 I.V than S.C. (Bas PLT: basal platelets; P PLT: post-therapy platelets); (C): immunological parameters outcome during therapy: relative increase of CD8, Treg and NK cell count on almost half and a stability in the remaining cases was observed with a more marked increase in Tregs with S.C. IL-2 administration (CD8: Lymphocyte CD8; Treg: Lymphocyte T regulator; NK: natural Killer); (D): Treg outcome during therapy: a relative increase of Treg cell count on almost half and a stability in the remaining cases was observed with a more marked increase in Tregs with S.C. IL-2 administration (Treg pre: Lymphocyte T regulator pre-Therapy;Treg post: Lymphocyte T regulator post-Therapy).

3.2. Response

Objective responses were observed in 2 PR, 1 HCC (Fig. 1A, B) and 1 pancreatic carcinoma (PC) (Fig. 2A, B) patients treated with I.V. IL-2. They were previously treated with *trans*-arterial chemoembolization (TACE) and Sorafenib and with nab-Paclitaxel-Gemcitabine, radiotherapy and Oxaliplatin-Irinotecan-Capecitabine containing regimens respectively.

Moreover, a PR in a HCC patient, not subjected to TACE for portal vein thrombosis, was treated with IL-2 S.C (Fig. 3A, B), after a progression to previous treatment with Sorafenib.

The pathological specimen of HCC patient treated with I.V. IL-2 showed moderately differentiated HCC with trabecular pattern, micronodular cirrhosis with severe lymphoyd infiltrate only in the portal space (Fig. 1C, D) with LCA positivity on micronodular cirrhosis at immunohistochemistry (Fig. 1E). The post-treatment biopsy showed the presence of micronodular cirrhosis only (Fig. 1F). The pathological specimen of HCC patient treated with S.C. IL-2 (Fig. 3C) showed well differentiated HCC with scattered lymphocyte T (CD3) infiltration (Fig. 3D). Furthermore 3 SD (23%) and 7 PD (54%) were observed. Therefore, the overall response rate and disease control rate (PR + SD) were 23% and 46%, respectively (Table 4). Noteworthy, these responder patient typologies are characterized by short-term, poor survival [67,68] and are not susceptible for current effective treatment.

Furthermore, we observed a response on Ca-15-3, CEA, paraneoplastic hypercalcemia with renal insufficiency in breast cancer obtaining SD and on Ca-125 in non-responding ovarian cancer, as well as relevant subjective improvement in a gastro-intestinal stromal tumour (GIST) patient with mediastinal lymph node response and the stabilization of massive hepatomegaly.

The duration of response was 2+ and 3 months in the 2 HCC and 18+ months in PC patient. Pain control was obtained on 7/8 patients. The median progression-free survival and overall survival were 1 (range 1–8+) and 7 (range 1–20) months (Fig. 4A). With the limits of the number of patients' cohort, differentiating by route of administration of IL-2, the median survival for S.C. and I.V. was 6 and 9 months, respectively (Fig. 4B).

Regarding the hematological (Fig. 5A, B) and immunological parameters outcome (Fig. 5C, D) (Table 5), although statistical considerations cannot be made for the small cohort of patients treated and the non-homogeneity of therapy, a relative stability in the neutrophils granulocytes, lymphocytes, monocytes, platelets and CD8 counts and an inverse relationship from the frequency of Plt and Tregs with a more marked Plt reduction with IL-2 I.V. and a more marked increase in Tregs with S.C. administration was observed, but without differences on survival (not shown).

The median variation from post-therapy to pre-therapy of CD8/Treg (Fig. 6A), N/Ly (not shown) and Plt/Ly (Fig. 6B) ratio in the S.C. and I.V IL-2 administration was −0.29, −0.23, 1 and 1, 0.59 and 0.70 respectively without a significant difference on survival as for pre-therapy Plt/Ly ratio (Fig. 6C) as for other parameters (not shown) in the overall group of patients treated Furthermore, in an early evaluation, during the first three weeks of treatment with IL-2 I.V. the lymphocyte subpopulations showed a bimodal pattern with a reduction at day 7 and an increase at days 4 and 18, except for CD16 and HLA-DR/CD3 cells (Table 6) (Fig. 7A, B).

Therefore, given the even modest elevation of the total lymphocyte count after therapy, the variation of lymphocyte subpopulations and ratio can be considered real and Treg elevation after therapy is confirmed also by cD8/Treg reduction.

Finally, a reasonable reduction in CRP was observed after therapy in 5 patients (not shown)

4. Discussion

Immunotherapy is employed for a small group of tumors, with long-

Table 5
Quantitative variation of white blood cells, lymphocyte subpopulations and platelets after therapy compared to baseline values.

Variables	CTX-FU-IL-2 S.C.		CTX-FU-IL-2 I.V	
	Median differences values	Range	Median differences values	Range
Neutrophil (10^3/μL)	+1.02	−0.45 to +1.63	+1.12	−0.93 to +2.41
Lymphocytes (10^3/μL)	+1.28	+1.25 to +2.37	+1.40	−0.90 to +1.63
Monocytes (10^3/μL)	+1.16	−0.58 to +4.8	+1.13	−0.78 to +2.37
Platelets (10^3/μL)	−0.88	−0.62 to +2.09	−0.38	−0.35 to +1.37
CD8 + (10^3/uL)	1	−0.6 to +2.4	1.18	−0.75 to +1.7
Tregs (10^3/uL)	+5.8	−0.99 to +11	+2.93	−0.67 to +6.46
NK (10^3/uL)	−0.96	−0.46 to +3.03	1.6	−0.51 to +2.05

CD8: Lymphocyte CD8; Treg: Lymphocytes T regulator; NK: natural killer; CTX-FU-IL-2 S.C.: cyclophosphamide-fluorouracil-interleukin-2 subcutaneous; CTX-FU-IL-2 I.V: cyclophosphamide-fluorouracil-interleukin-2 intravenous.

term results reported. However, immune surveillance plays an important role in the prognosis of all tumor types, even if it has no direct impact on conventional anticancer therapeutic agents.

Several parameters have been investigated to measure immune status, such as N/Ly [69] and Plt/Ly [70]. These parameters showed an impact on prognosis and survival and a predictive role in the response to chemotherapy in various neoplasms.

Recently, we have witnessed an explosion of immunotherapeutic research as anti-PD-1 and anti-PDL-1 inhibitors, which has led to confirmation of superiority over simple benchmark or standard treatments and has allowed an existing vacuum in some pathologies to be filled [32–36].

There are handy, easily administered outpatient drugs, which provide long-term therapies (until progression or appearance of immune-related side effects requiring expertise). While waiting to evaluate the impact of treatment with these new agents on survival in long-term follow-up, research on older immunotherapeutic agents with confirmed roles in RCC and melanoma related to long-term survival may be justified. The combined use of immunotherapeutic agents and other drugs could improve the therapeutic index and expand their use to a greater number of tumours.

IL-2, the main immunotherapeutic agent known in oncology, has these requirements and the direct induction of immunosuppression through Tregs and indirectly through MDSC expression can be modulated by Cyt (acting on Tregs) and by FU (the main suppressor of MDSCs). These antiblastic drugs could also play a role in antigenic priming, which may have synergistic action with immunotherapy.

This preliminary off-label experience in chemo-immunotherapy was undertaken by combining IL-2 with Cyt and FU in light of its theoretical, experimental and immunomodulating role. Both regimens can be considered safe and slightly and moderately toxic for S.C. and I.V. IL-2 administrations, respectively, in patients heavily pre-treated and chemo-resistant, such as PC, HCC [67,68] and GIST, for which no therapeutic alternatives exist.

However, beyond the constant and transient capillary leak syndrome and other common toxicities, issues such as proteinuria, haemorrhagic anaemia, and skin rash with hypereosinophilia were reported. Proteinuria is an uncommon side effect during treatment with IL-2 [39], which may in contrast exert a protective effect by increasing Tregs [71]. Hemorrhagic anemia is a side effect reported for a high-dose bolus of IL-2 [72].

An extended skin rash, with intense itching and an increase of eosinophils was reported in a responsive PC patient. However, hyper eosinophilia correlates with an unfavorable prognosis and outcome in relation to immunotherapy [73]. Therefore, the relationship between nephropathic, cutaneous and haemorrhagic toxicity and a favorable response could be explained probably by the effect exerted by Cyt on Tregs, increasing the potential toxicity and activity of IL-2. No relationship between toxicity observed at previous treatment can be hypothesized, except bleeding anemia reported in two patients previously treated with Bevacizumab and Sorafenib, respectively.

Another issue to be considered is the possible interference of opioid-based antalgic therapy, in these heavily pre-treated patients, with the immune system [74]. The data are controversial with regard to the reduction of levels of IL-2 and other cytokines such as IL-6 and IL-10 [75], while a negative influence on lymphocyte subpopulations has been reported without any significant influence on disease-free survival in patients submitted to radical surgery [76].

Due to limitations of the type of experience and the small studied cohort of patients, we can only limit ourselves to a descriptive report of the potential reverting role of our regimens on immunosuppressive cells. A certain stability on the cellular blood series globally and an initial reduction of Treg followed by an increase at the end of treatment in the global population was observed. In addition, NK cells tended to increase and CD8/Treg and Plt/Ly ratio tended to decrease.

In the planned study of lymphocyte subpopulations during IL-2 I.V. a Treg increase on day 4, its decrease on day 7 followed by a rebound thereafter were observed.

From this preliminary experience definitive conclusions cannot be drawn due to the limits related to the non-homogeneity of the treatment and the scarce number of cases. However, we found a transient decrease of Treg after therapy, a subjective improvement, objective response on 23% of patients, and a reasonable lengthening in survival that represent a relevant aspect in an unfavorable patients population. Moreover, absence of intra-tumor lymphocyte infiltration pre-treatment in the two cases of HCC and the disappearance of tumor cells in the bioptic control after treatment with IL-2 I.V. were reported.

All these characteristics, in addition to the positive impact of IL-2, regardless of response, on survival corroborates our experiment and justifies further evaluation in the context of a prospective study.

5. Conclusion

This therapeutic combination is an attempt to engineer first-generation cytokine agent that will still play a role in cancer therapy even in the new era of check-point inhibitors.

However these preliminary data require further perspective study in a more selected series with the aim of verifying in a more timely manner the possible temporal variations of lymphocyte subpopulations by comparing chemo-immunotherapy with a control group with immunotherapy alone and its correlation with response to treatment and survival.

6. CRediT authorship contribution statement

Giovanni Lo Re: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Francesco Lo Re:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration,

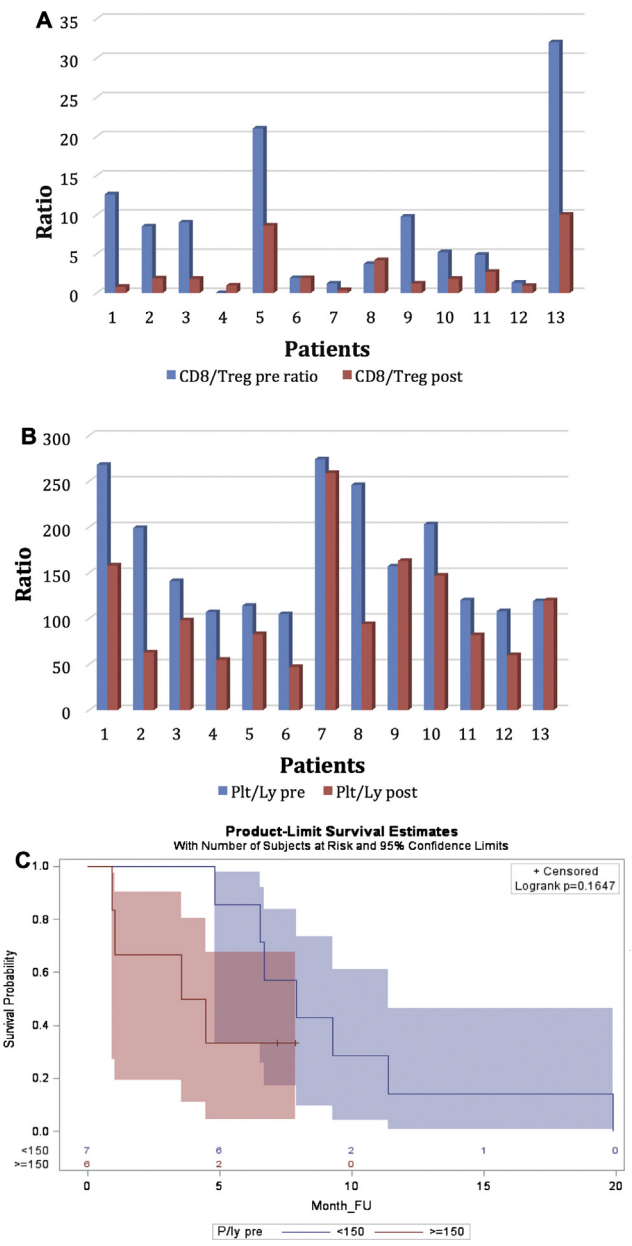

Fig. 6. Relation between CD8/Treg Plt/Ly ratio and therapy, and Plt/Ly and survival. (A) Correlation between CD8 and Treg during therapy: after therapy a reduction of CD8/Treg ratio was reported (CD8/Treg: lymphocyte CD8/T regulator ratio before and after therapy). (B) Correlation between platelets/ lymphocyte ratio during therapy: a reasonable reduction of the platelet/lymphocyte ratio was observed in almost all cases after therapy (Plt/Ly: platelet/ lymphocyte n × 10^3/μL before and after therapy). (C): Overall survival according to basal P/Ly ratio Kaplan-Meier estimates of overall survival (OS) for basal PLT/Ly ratio < 150 (blue line) or > 150 (red line). Log-rank test: p = 0.1647, blue line: basal P/Ly : Platelets/Lymphocytes pre-therapy < 150; red line: P/Ly post: Platelets/Lymphocytes pre-therapy > 150.

Table 6
Quantitative variation of lymphocyte subpopulations during the first cycle of therapy with IL-2 i.v. compared to baseline values.

Variables	MCN (10^3/μL)	Range (10^3/μL)	MCN (10^3/μL)	Range (10^3/μL)	MCN (10^3/μL)	Range (10^3/μL)	MCN (10^3/μL)	Range (10^3/μL)
	Day1		Day4		Day7		Day 18	
Treg	48	17–69	61	42–66	33	5–167	188.5	61–267
CD3	774	305–2313	919	714–1092	737	60–1494	1380	448–1566
CD4	533	207–1512	650	481–618	489.5	41–1146	917	347–1139
CD8	240	88–1069	255	247–268	182.5	17–539	418	91–594
CD16	167.5	94–330	204	119–253	250.5	98–1351	234	129–573
HLA-DR/CD3	121.5	39–697	124	36–246	146.5	25–389	297	103–741

MCN: median count number; Treg: Lymphocytes T regulator; CD3: Lymphocyte CD3; CD4: Lymphocyte CD4; CD8: Lymphocyte CD8; CD16: Lymphocyte CD16; HLA/CD3: Lymphocyte HLA/CD3.

Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Paolo Doretto:** Conceptualization, Formal analysis, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Alessandro Del Conte:** Formal analysis, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Maria Amadio:** Methodology, Project administration, Validation, Visualization, Writing – review & editing. **Cinzia Cozzi:** Formal analysis, Methodology, Project administration,

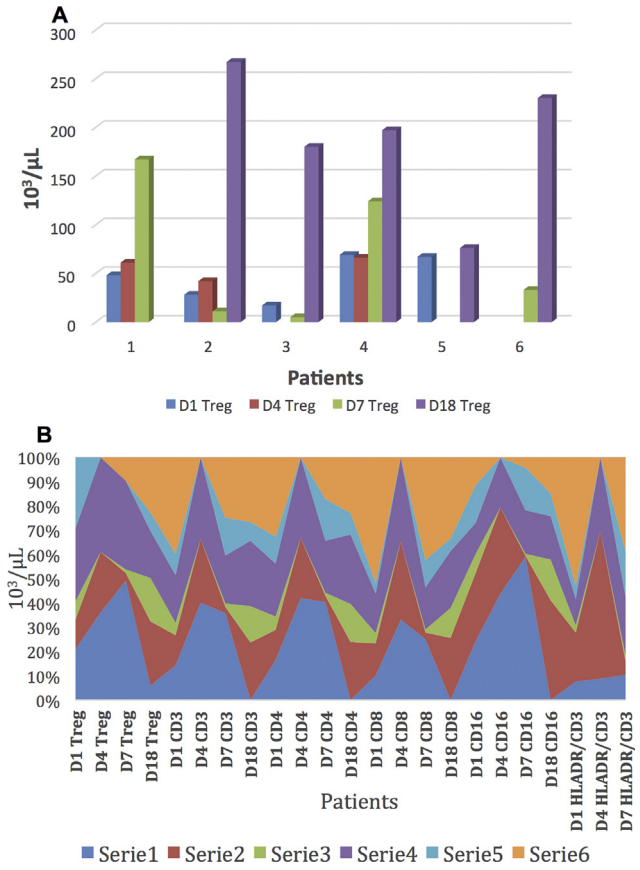

Fig. 7. Outcome of lymphocyte subpopulation and therapy. (A): Outcome of Treg during 1° cycle of I.V IL-2 therapy: Treg count reduction at day 7 and increase on day 4 and 18 during the first cycle of I.V IL-2 therapy was detected (Treg D1, D4, D7, D18: Lymphocyte Treg day 1, 4,7,18). (B): Overall outcome of lymphocyte subpopulation during 1° cycle of I.V IL-2 therapy: All lymphocyte subpopulation except CD16 and HLA-DR/CD3 cells have suffered reduction at day 7 and increase on day 4 and 18 during the first cycle of I.V IL-2 therapy. CD16 and HLA-DR/CD3 were stable on day 4 and progressively increased on day 7 and 18. (Treg, CD3,CD4,CD8, CD16, HLA-DR/CD3 D1, D4, D7,D18: Lymphocyte Treg, CD3,CD4,CD8, CD16, HLA-DR/CD3 day 1, 4,7,18).

Software, Validation, Visualization, Writing – review & editing. **Maria Maddalena Casarotto:** Formal analysis, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Daniele Maruzzi:** Formal analysis, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Wally Marus:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Paolo Ubiali:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Paolo Sandri:** Conceptualization, Formal analysis, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing.

Acknowledgements

We thank the patients, doctors and nurses of oncology who have allowed this experience aimed at improving the therapeutic index and quality of life of patients. We thank L. Dal Maso for assistance with statistical analysis.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors declare that they do not have any financial or non-financial conflict of interests.

References

[1] Y. Shetzer, H. Solomon, G. Koifman, A. Molchadsky, S. Horesh, V. Rotter, The paradigm of mutant p53-expressing cancer stem cells and drug resistance, Carcinogenesis 35 (2014) 1196–1208.
[2] V. Lopes-Rodrigues, H. Seca, D. Sousa, E. Sousa, R.T. Lima, M.H. Vasconcelos, The network of P-glycoprotein and microRNAs interactions, Int. J. Cancer 135 (2014) 253–263.
[3] G.P. Dunn, A.T. Bruce, H. Ikeda, L.J. Old, R.D. Schreiber, Cancer immunoediting: from immunosurveillance to tumor escape, Nat. Immunol. 3 (2002) 991–998.
[4] G.P. Dunn, A.T. Bruce, K.C. Sheehan, et al., A critical function for type I interferons in cancer immunoediting, Nat. Immunol. 6 (2005) 722–729.
[5] R. Kim, M. Emi, K. Tanabe, K. Arihiro, Tumor-driven evolution of immunosuppressive networks during malignant progression, Cancer Res. 66 (2006) 5527–5536.
[6] G. Klein, Immune surveillance – a powerful mechanism with a limited range, Natl. Cancer Inst. Monogr. 44 (1976) 109–113.
[7] R.T. Prehn, J.M. Main, Immunity to methylcholanthrene-induced sarcomas, J. Natl. Cancer Inst. 18 (1957) 769–778.
[8] L.J. Old, E.A. Boyse, Immunology of experimental tumors, Annu. Rev. Med. 15 (1964) 167–186.
[9] F.M. Burnet, Immunological surveillance in neoplasia, Transplant Rev. 7 (1971) 3–25.
[10] L. Thomas, On immunosurveillance in human cancer, Yale J. Biol. Med. 55 (1982) 329–333.
[11] J. Rygaard, C.O. Povlsen, The mouse mutant nude does not develop spontaneous tumours. An argument against immunological surveillance, Acta Pathol. Microbiol. Scand. Microbiol. Immunol. 82 (1974) 99–106.
[12] J.M. Zaretsky, A. Garcia-Diaz, D.S. Shin, et al., Mutations associated with acquired resistance to PD-1 blockade in melanoma, N. Engl. J. Med. 375 (2016) 819–829.
[13] H. Dong, S.E. Strome, D.R. Salomao, et al., Tumor-associated B7–H1 promotes T-cell apoptosis: a potential mechanism of immune evasion, Nat. Med. 8 (2002) 793–800.
[14] Y. Iwai, M. Ishida, Y. Tanaka, et al., Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade, Proc. Natl. Acad. Sci. USA 99 (2002) 12293–12297.
[15] W. Zou, L. Chen, Inhibitory B7-family molecules in the tumour microenvironment, Nat. Rev. Immunol. 8 (2008) 467–477.
[16] M.W. Bennett, J. O'Connell, G.C. O'Sullivan, et al., The Fas counterattack in vivo: apoptotic depletion of tumor-infiltrating lymphocytes associated with Fas ligand expression by human esophageal carcinoma, J. Immunol. 160 (1998) 5669–5675.
[17] C. Leng, Y. Li, J. Qin, et al., Relationship between expression of PD-L1 and PD-L2 on esophageal squamous cell carcinoma and the antitumor effects of $CD8^+$ T cells, Oncol. Rep. 35 (2016) 699–708.
[18] D.I. Gabrilovich, S. Nagaraj, Myeloid-derived suppressor cells as regulators of the immune system, Nat. Rev. Immunol. 9 (2009) 162–174.
[19] L. Strauss, C. Bergmann, M. Szczepanski, W. Gooding, J.T. Johnson, T.L. Whiteside, A unique subset of CD4 + CD25highFoxp3 + T cells secreting IL-10 and TGF-β1 mediates suppression in the tumor microenvironment, Clin. Cancer Res. 13 (2007) 4345–4354.
[20] L. Strauss, C. Bergmann, W. Gooding, J.T. Johnson, T.L. Whiteside, The frequency and suppressor function of CD4 + CD25highFoxp3 + T cells in the circulation of patients with squamous cell carcinoma of the head and neck, Clin. Cancer Res. 13 (2007) 6301–6311.
[21] H. Nishikawa, S. Sakaguchi, Regulatory T cells in tumor immunity, Int. J. Cancer 127 (2010) 759–767.
[22] D. Mougiakakos, A. Choudhury, A. Lladser, R. Kiessling, C.C. Johansson, Regulatory T cells in cancer, Adv. Cancer Res. 107 (2010) 57–117.
[23] C. Bergmann, L. Strauss, Y. Wang, et al., T regulatory type 1 cells (Tr1) in squamous cell carcinoma of the head and neck: mechanisms of suppression and expansion in advanced disease, Clin. Cancer Res. 14 (2008) 3706–3715.
[24] R.P. Petersen, M.J. Campa, J. Sperlazza, et al., Tumor infiltrating Foxp3 + regulatory T-cells are associated with recurrence in pathologic stage I NSCLC patients, Cancer 107 (2006) 2866–2872.
[25] T.J. Curiel, G. Coukos, L. Zou, et al., Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival, Nat. Med. 10 (2004) 942–949.
[26] G. Asma, G. Amal, M. Raja, D. Amine, C. Mohammed, B.A. Amel, Comparison of circulating and intratumoral regulatory T cells in patients with renal cell carcinoma, Tumour Biol. 36 (2015) 3727–3734.
[27] X. Wang, H. Dong, Q. Li, Y. Li, A. Hong, Thioredoxin induces Tregs to generate an immunotolerant tumor microenvironment in metastatic melanoma,

Oncoimmunology 4 (2015) e1027471.
[28] T. Horn, J. Grab, J. Schusdziarra, et al., Antitumor T cell responses in bladder cancer are directed against a limited set of antigens and are modulated by regulatory T cells and routine treatment approaches, Int. J. Cancer 133 (2013) 2145–2156.
[29] O. Draghiciu, J. Lubbers, H.W. Nijman, T. Daemen, Myeloid derived suppressor cells-An overview of combat strategies to increase immunotherapy efficacy, Oncoimmunology 4 (2015) e954829.
[30] B. Hoechst, J. Gamrekelashvili, M.P. Manns, T.F. Greten, F. Korangy, Plasticity of human Th17 cells and iTregs is orchestrated by different subsets of myeloid cells, Blood 117 (2011) 6532–6541.
[31] C.M. Diaz-Montero, M.L. Salem, M.I. Nishimura, E. Garrett-Mayer, D.J. Cole, A.J. Montero, Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy, Cancer Immunol. Immunother. 58 (2009) 49–59.
[32] R.J. Motzer, B. Escudier, D.F. McDermott, S. George, CheckMate 025 Investigators. Nivolumab versus Everolimus in Advanced Renal-Cell Carcinoma, N. Engl. J. Med. 373 (2015) 1803–1813.
[33] J. Larkin, V. Chiarion-Sileni, R. Gonzalez, et al., Combined Nivolumab and ipilimumab or monotherapy in untreated melanoma, N. Engl. J. Med. 373 (2015) 23–34.
[34] J.K.L. Brahmer, P. ReckampBaas, et al., Nivolumab versus Docetaxel in Advanced Squamous-Cell Non-Small-Cell Lung Cancer, N. Engl. J. Med. 373 (2015) 123–135.
[35] J. Bellmunt, R. de Wit, D.J. Vaughn, et al., KEYNOTE-045 Investigators. Pembrolizumab as Second-Line Therapy for Advanced Urothelial Carcinoma, N. Engl. J. Med. 376 (2017) 1015–1026.
[36] L.Q. Chow, R. Haddad, S. Gupta, et al., Antitumor activity of pembrolizumab in biomarker-unselected patients with recurrent and/or metastatic head and neck squamous cell carcinoma: results from the phase Ib KEYNOTE-012 expansion cohort, J. Clin. Oncol. (2016) pii: 681478.
[37] R. Nanda, L.Q. Chow, E.C. Dees, et al., Pembrolizumab in patients with advanced triple-negative breast cancer: phase Ib KEYNOTE-012 study, J. Clin. Oncol. 34 (2016) 2460–2467.
[38] P.T. Nghiem, S. Bhatia, E.J. Lipson, et al., PD-1 blockade with pembrolizumab in advanced merkel-cell carcinoma, N. Engl. J. Med. 374 (2016) 2542–2552.
[39] S.A. Rosenberg, Interleukin-2 and the development of immunotherapy for the treatment of patients with cancer, Cancer J. Sci. Am. 6 (Suppl 1) (2000) S2–S7.
[40] J.D. Fontenot, J.P. Rasmussen, M.A. Gavin, A.Y. Rudensky, A function for interleukin 2 in Foxp3-expressing regulatory T cells, Nat. Immunol. 6 (2005) 1142–1145.
[41] J.C. Yang, R.M. Sherry, S.M. Steinberg, et al., Randomized study of high-dose and low-dose interleukin-2 in patients with metastatic renal cancer, J. Clin. Oncol. 21 (2003) 3127–3132.
[42] F.M. Cappa, M.C. Cantarini, G. Magini, et al., Effects of the combined treatment with thalidomide, megestrol and interleukine-2 in cirrhotic patients with advanced hepatocellular carcinoma. A pilot study, Dig Liver Dis. 37 (2005) 254–259.
[43] K. Hayakawa, T. Fukushima, D. Seito, et al., Continuous arterial infusion therapy of recombinant interleukin-2 and cyclophosphamide in hepatoma and liver metastasis, Gan To Kagaku Ryoho 16 (1989) 2842–2844.
[44] S. Onishi, T. Saibara, M. Fujikawa, H. Sakaeda, Y. Matsuura, Y. Matsunaga, Y. Yamamoto, Adoptive immunotherapy with lymphokine-activated killer cells plus recombinant interleukin 2 in patients with unresectable hepatocellular carcinoma, Hepatology 10 (1989) 349–353.
[45] H.J. Zhang, J.J. Yang, W.X. Wang, et al., Effects of Jinlong Capsule on expressions of interleukin-2 and soluble interleukin-2 receptor in patients with primary liver cancer after transarterial chemoembolization therapy, Zhong Xi Yi Jie He Xue Bao 6 (2008) 907–910.
[46] R. Aldeghi, P. Lissoni, S. Barni, et al., Low-dose interleukin-2 subcutaneous immunotherapy in association with the pineal hormone melatonin as a first-line therapy inlocally advanced or metastatic hepatocellular carcinoma, Eur. J. Cancer 30A (1994) 167–170.
[47] F. Uggeri, R. Caprotti, L. De Grate, et al., Short-term preoperative IL-2 immunotherapy in operable pancreatic cancer: a randomized study, Hepatogastroenterology 56 (2009) 861–865.
[48] R. Caprotti, F. Brivio, L. Fumagalli, et al., Free-from-progression period and overall short preoperative immunotherapy with IL-2 increases the survival of pancreatic cancer patients treated with macroscopically radical surgery, Anticancer Res. 28 (2008) 1951–1954.
[49] Mayer N. Fishman, Daniel A. Vaena, et al., Phase Ib/II study of an IL-2/T-cell receptor fusion protein in combination with gemcitabine and cisplatin in advanced or metastatic chemo-refractory urothelial cancer (UC), J Clin Oncol 33 (2015) (suppl; abstr 4515).
[50] S. Wei, I. Kryczek, R.P. Edwards, et al., Interleukin-2 administration alters the CD4+FOXP3+ T-cell pool and tumor trafficking in patients with ovarian carcinoma, Cancer Res. 67 (2007) 7487–7494.
[51] A. Nicolini, A. Carpi, Beta-interferon and interleukin-2 prolong more than three times the survival of 26 consecutive endocrine dependent breast cancer patients with distant metastases: an exploratory trial, Biomed. Pharmacother. 59 (2005) 253–263.
[52] G. Kimmick, M.J. Ratain, D. Berry, S. Woolf, L. Norton, H.B. Muss, Cancer and Leukemia Group B (CALGB 9041), subcutaneously administered recombinant humaninterleukin-2 and interferon alfa-2a for advanced breast cancer: a phase II study of the Cancer and Leukemia Group B (CALGB 9041), Invest. New Drugs 22 (2004) 83–89.
[53] C. Catania, M. Maur, R. Berardi, et al., The tumor-targeting immunocytokine F16-IL2 in combination with doxorubicin: dose escalation in patients with advanced solid tumors and expansion into patients with metastatic breast cancer, Cell Adh. Migr. 9 (2015) 14–21.
[54] W. Maes, T. Verschuere, A. Van Hoylandt, L. Boon, S. Van Gool, Depletion of regulatory T cells in a mouse experimental glioma model through anti-CD25 treatment results in the infiltration of non-immunosuppressive myeloid cells inthe brain, Clin. Dev. Immunol. 2013 (2013) 952469.
[55] T. Pentcheva-Hoang, T.R. Simpson, W. Montalvo-Ortiz, J.P. Allison, Cytotoxic T lymphocyte antigen-4 blockade enhances antitumor immunity by stimulating melanoma-specific T-cell motility, Cancer Immunol. Res. 2 (2014) 970–980.
[56] D. Coe, S. Begom, C. Addey, M. White, J. Dyson, J.G. Chai, Depletion of regulatory T cells by anti-GITR mAb as a novel mechanism for cancer immunotherapy, Cancer Immunol. Immunother. 59 (2010) 1367–1377.
[57] I. Shevchenko, S. Karakhanova, S. Soltek, et al., Low-dose gemcitabine depletes regulatory T cells and improves survival in the orthotopic Panc02 model of pancreatic cancer, Int. J. Cancer 133 (2013) 98–107.
[58] J. Zhao, Y. Cao, Z. Lei, Z. Yang, B. Zhang, B. Huang, Selective depletion of CD4+CD25+Foxp3+ regulatory T cells by low-dose cyclophosphamide is explained by reduced intracellular ATP levels, Cancer Res. 70 (2010) 4850–4858.
[59] Y. Zhu, N. Liu, S.D. Xiong, Y.J. Zheng, Y.W. Chu, CD4+Foxp3+ regulatory T-cell impairment by paclitaxel is independent of toll-like receptor 4, Scand. J. Immunol. 73 (2011) 301–308.
[60] F. Ghiringhelli, L. Apetoh, Enhancing the anticancer effects of 5-fluorouracil: current challenges and future perspectives, Biomed. J. 38 (2015) 111–116.
[61] J.S. Ko, A.H. Zea, B.I. Rini, et al., Sunitinib mediates reversal of myeloid-derived suppressor cell accumulation in renal cell carcinoma patients, Clin. Cancer Res. 15 (2009) 2148–2157.
[62] S. Kusmartsev, E. Eruslanov, H. Kübler, et al., Oxidative stress regulates expression of VEGFR1 in myeloid cells: link to tumor-induced immune suppression in renal cell carcinoma, J. Immunol. 181 (2008) 346–353.
[63] A. Sevko, M. Sade-Feldman, J. Kanterman, et al., Cyclophosphamide promotes chronic inflammation-dependent immunosuppression and prevents antitumor response in melanoma, J. Invest. Dermatol. 133 (2013) 1610–1619.
[64] M. Tongu, N. Harashima, H. Monma, et al., Metronomic chemotherapy with low-dose cyclophosphamide plus gemcitabine can induce anti-tumor T cell immunity in vivo, Cancer Immunol. Immunother. 62 (2013) 383–391.
[65] E.A. Eisenhauer, P. Therasse, J. Bogaerts, et al., New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1), Eur J Cancer (2009).
[66] Common Terminology Criteria for Adverse Events v3.0 (CTCAE) Publish Date: August 9, 2006.
[67] A.L. Cheng, Y.K. Kang, Z. Chen, et al., Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial, Lancet Oncol. 10 (2009) 25–34.
[68] D.D. Von Hoff, T. Ervin, F.P. Arena, et al., Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine, N. Engl. J. Med. 369 (2013) 1691–1703.
[69] A.G. Sahin, C. Aydin, M. Unver, K. Pehlivanoglu, Predictive value of preoperative neutrophil lymphocyte ratio in determining the stage of gastric tumor, Med. Sci. Monit. 23 (2017) 1973–1979.
[70] C. Pedrazzani, G. Mantovani, E. Fernandes, et al., Assessment of neutrophil-to-lymphocyte ratio, platelet-to-lymphocyte ratio and platelet countas predictors of long-term outcome after R0 resection for colorectal cancer, Sci. Rep. 7 (2017) 1494.
[71] T. Polhill, G.Y. Zhang, M. Hu, A. Sawyer, et al., IL-2/IL-2Ab complexes induce regulatory T cell expansion and protect against proteinuric CKD, J. Am. Soc. Nephrol. 23 (2012) 1303–1308.
[72] M.P. MacFarlane, J.C. Yang, A.S. Guleria, et al., The hematologic toxicity of interleukin-2 in patients with metastatic melanoma and renal cell carcinoma, Cancer 75 (1995) 1030–1037.
[73] M. Moroni, C. Porta, M. De Amici, S. Quaglini, M.A. Cattabiani, C. Buzio, Eosinophils and C4 predict clinical failure of combination immunotherapy with very low dose subcutaneous interleukin-2 and interferon in renal cell carcinoma patients, Haematologica 85 (2000) 298–303.
[74] C. Börner, B. Warnick, M. Smida, et al., Mechanisms of opioid-mediated inhibition of human T cell receptor signaling, J. Immunol. 183 (2009) 882–889.
[75] J.W. Boland, K. McWilliams, S.H. Ahmedzai, A.G. Pockley, Effects of opioids on immunologic parameters that are relevant to anti-tumour immune potential in patients with cancer: a systematic literature review, Br. J. Cancer 111 (2014) 866–873.
[76] S.Y. Kim, N.K. Kim, S.H. Baik, et al., Effects of postoperative pain management on immune function after laparoscopic resection of colorectal cancer: a randomized study, Medicine (Baltimore) 95 (2016) e3602.