## Lightning strikes

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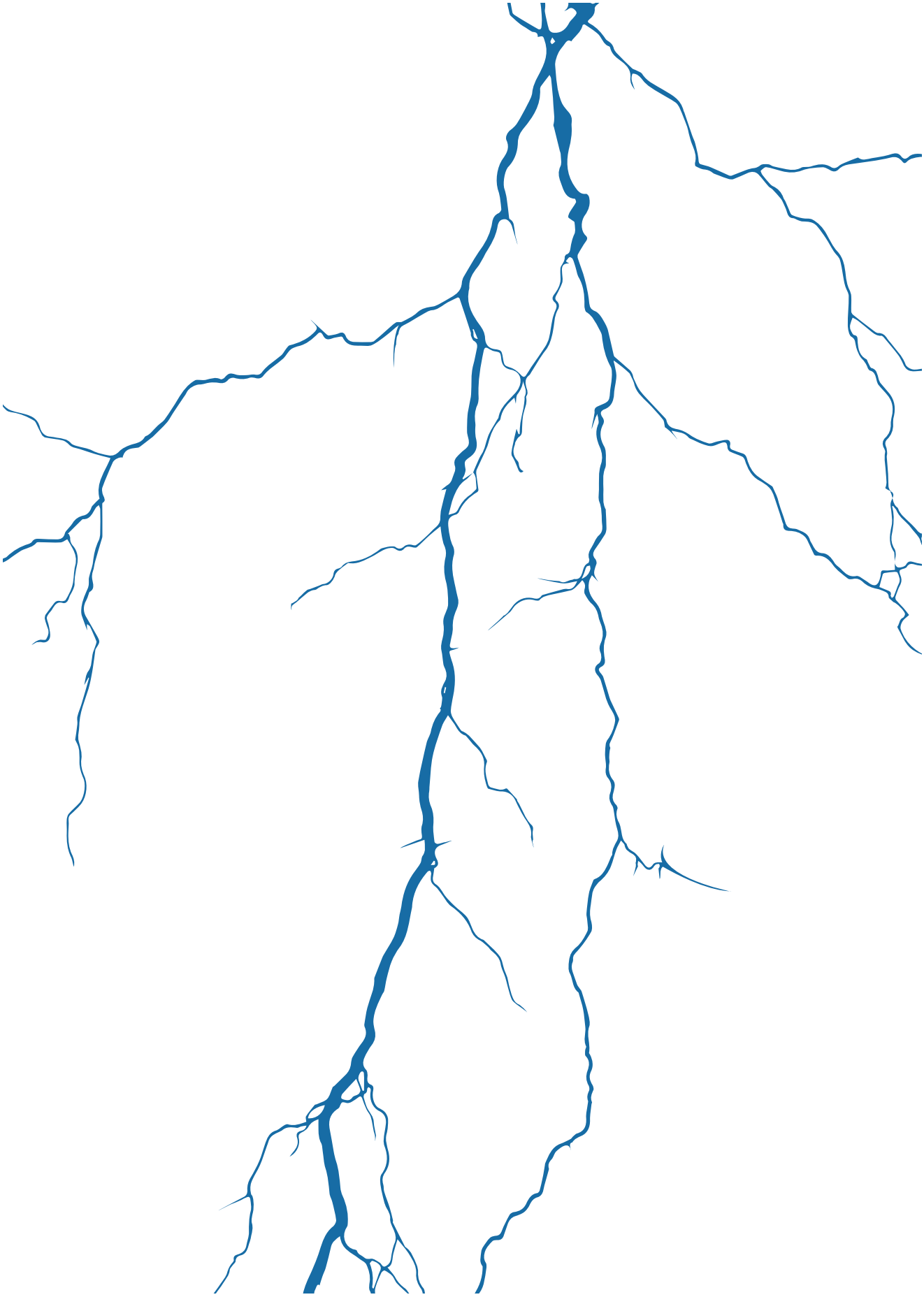

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# Systemic WT-1 specific T cell reactivity in relation to immune status and survival following ablative treatment of locally advanced pancreatic cancer by irreversible electroporation

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## Abstract

### Objective

For patients with locally advanced pancreatic cancer (LAPC), local ablation through irreversible electroporation (IRE) may offer a novel therapeutic option. Irreversible electroporation induces apoptosis of tumor cells by creating nanopores through high-voltage electric pulses. This leaves much of the vasculature intact, allowing for effective immune infiltration. To obtain evidence of the induction of systemic antitumor immunity following local IRE-mediated ablation, we performed an immune monitoring pilot study.

### Materials and Methods

In the first ten patients enrolled in a clinical trial exploring the safety, feasibility, and efficacy of percutaneous image-guided IRE in LAPC (the PANFIRE study), flow cytometric analysis was performed to determine the frequency and activation state of various lymphocytic and myeloid subsets in pre- and post-treatment peripheral blood samples. Systemic T cell responses to the pancreatic cancer associated antigens mesothelin and Wilms Tumor (WT)-1 were determined after in vitro stimulation in an interferon (IFN) γ enzyme-linked immunospot assay (Elispot), at baseline and at 2 weeks and 3 months after IRE.

### Results

Our data show a transient decrease in systemic regulatory T cell (Treg) frequencies and a simultaneous transient increase in activated Ki67+CD8+ T cells, consistent with the temporary lifting of Treg imposed immune suppression after the IRE procedure. In addition we found post-IRE boosting of a pre-existing WT-1 specific T cell response in two out of three patients as well as the de novo induction of these responses in another two patients. There was a trend for these WT-1 T cell responses to be related to longer overall survival (p=0.055).

### Conclusion

These findings are consistent with a systemic immune stimulatory effect of IRE and support the combination of percutaneous IRE with therapeutic immune stimulation.

## Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death in Western Europe and the United States.[1] Currently, surgical resection at an early stage is the only potentially curative treatment. Early diagnosis may increase the chance of curative resection but this is only appropriate for up to 15-20% of patients.[2] Nearly 30% of newly diagnosed pancreatic cancer patients have locally advanced pancreatic carcinoma (LAPC) at the time of diagnosis, infiltrating into the surrounding tissue and crucial blood vessels.[2,3] Surgical resection is no option for these patients, leaving only palliative treatment available. The prognosis of LAPC is poor with a median survival of less than a year.[3]

Local ablative therapies have been developed for the treatment of isolated tumors and metastases. These local therapies are mostly based on thermal ablation, causing necrosis of tumor cells by heat induction. Treatment of LAPC with thermal ablation was however found unsafe due to the high risk of complications caused by collateral damage to the pancreas, surrounding organs, and vascular structures. In addition, the risk of development of pancreatitis was shown to be greatly increased.[4] Irreversible electroporation (IRE) provides a promising alternative to heat-induced tumor ablation in LAPC.[5] Irreverible electroporation is a new, imaging-guided technique which causes formation of small defects in the cell membrane by the application of high-voltage electric pulses. These irreversible nanopores cause loss of homeostatic properties of the cell, leading to cell death through apoptosis.[6,7] Irreversible electroporation (a.k.a. "nanoknife" after the device used clinically) is based on the pulsatile application of electric energy delivered between several electrodes that are placed around the tumor.[8] Irreversible electroporation is believed to destroy all cells within the ablation zone, but – due to its primarily non-thermal mechanism of action – to leave supporting extracellular matrix structures unaffected. Therefore, the structural integrity of inlaying and adjacent vulnerable tissue structures like vessels and bile ducts remains intact.[7] This renders the technique potentially ideal for the selective ablation of diffusely growing malignancies that surround such structures, as is typically the case for LAPC. Early clinical studies have investigated the safety and efficacy of IRE for LAPC, with an overall modest complication rate of 13%.[9,10] One study reported a prolonged overall survival by 9 months as compared to chemotherapy with or without radiation.[11]

We recently conducted the PANFIRE-I phase I study (NCT01939665, clinicaltrials.gov), investigating the safety of percutaneous IRE for LAPC in 25 patients. Overall, the observed complications were acceptable. There were no deaths directly attributable to IRE and twelve minor (grade I/II) and eleven major (9 grade III; 2 grade IV) complications were recorded. Findings further suggest prolonged time to local recurrence, and consequently overall survival, as compared to chemotherapy or no treatment, but require confirmation on follow-up trials (Scheffer et al., PANFIRE manuscript accepted for publication).

Beside the induction of local tumor destruction, IRE may also induce a systemic antitumor response through the priming or boosting of tumor specific immunity. Little is known about immunological responses induced by IRE, but observed abscopal effects are in line with the induction of systemic antitumor immunity.[12] Pancreatic carcinoma appears to be moderately immunogenic, and to barely induce spontaneous antitumor immune responses.[13] Accumulating evidence suggests that this may in part be caused by local and systemic immune suppression caused by tumor-derived factors and stroma.[14,15] Gabitass et al. [16] have





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shown a significant increase in circulating myeloid derived suppressor cell (MDSC) rates in patients with progressive pancreatic carcinoma, which was correlated with similarly elevated frequencies of regulatory T cells (Tregs). The data from Hiraoka et al. [17] confirmed that Tregs indeed play a role in controlling the immune response in pancreatic carcinoma from the premalignant stage to established cancer. The prevalence of Tregs was significantly increased in the stroma of pancreatic invasive carcinomas as compared to the stroma of non-neoplastic inflammation of the pancreas. This may explain the aggressive behavior and the ability of pancreatic tumors to evade the immune system. Nevertheless, pancreatic tumors are amenable to immunotherapy with clinical benefit demonstrated after tumor-specific vaccination approaches.[18-20] Indeed, numerous trials are now exploring the safety and efficacy of (combinations of) immune checkpoint inhibitors, vaccines, adoptive T cell transfer (including T cells transduced to express chimeric antigen receptors [CAR] to mesothelin), and monoclonal antibodies.

The use of IRE in LAPC results in apoptosis and a decrease in tumor mass, which may lead to reduction of tumor-associated immune suppression and the simultaneous release of immunogenic apoptotic tumor cell remnants. This could potentially lead to the generation of antitumor immunity. Moreover, because the larger vessels remain intact, antigen presenting cells like dendritic cells (DC) should be able to infiltrate the lesion and induce an immune response against (neo-)antigens contained within the apoptotic bodies, after transporting them to the draining lymph nodes.[7,21]

In the present immune monitoring pilot study we aimed to obtain evidence of a systemic immune stimulatory effect caused by local IRE-mediated ablation of LAPC.

## Materials and Methods

### Patients and IRE procedure

The first ten patients (out of 25) with LAPC who were enrolled in the PANFIRE study between February 2013 and June 2014 were selected for this immune monitoring side study (**Table 1**). The local institutional review board approved this trial (PANFIRE, registered at clinicaltrials.gov NCT01939665). Study design and conduct were in accordance with the Declaration of Helsinki and were undertaken in accordance with the STROBE statement for observational studies.[22] Written informed consent was obtained prior to treatment. Patients were enrolled when they were shown to suffer from histologically proven LAPC with a maximum axial diameter of the tumor of 5 cm. Locally advanced disease (stage III) was defined as per the 7th edition of the American Joint Committee on Cancer (AJCC) staging system for pancreatic cancer.[23,24] Previous chemotherapy was allowed as long as treatment had been completed six weeks prior to the procedure. The IRE procedure was performed percutaneously using computed tomography (CT)-fluoroscopy guidance, as described earlier.[25] Size and shape of the tumor, including a 5mm tumor-free margin, determined the number and configuration of the electrodes (NanoKnife, AngioDynamics, Latham, NY). Needle electrodes with an exposure length of 15mm were placed parallel to each other within and around the tumor site using CT-fluoroscopy. Correct electrode position and inter-electrode distances were verified with transcatheter CT arteriography using multiplanar image reconstruction. Next, pulses were delivered until complete ablation of the macroscopically visible tumor was achieved. Three cycles of 30 pulses (1500 V/cm, 90 usec duration) were administered sequentially for

each electrode pair, to reach a total of 100 pulses per pair. For larger tumors, electrodes were repositioned or pulled back to ablate the remaining part of the tumor. Upon completion of the ablation procedure, a transcatheter arterial and portal venous phase CT scan was made to confirm technical success (i.e. the absence of any residual tumor enhancement) and to detect early complications. At the time of writing all ten patients included in this side study had died of the disease at a median overall disease specific survival of 11.1 months (range 6.3-17.1 months).

### Collection and processing of peripheral blood

For immune monitoring, peripheral blood samples (50 ml heparinised blood) were collected from the patients just before start of therapy (T=0), at 2 weeks (T=2 wks) and at 3 months (T=3 mnth) after treatment. Immediately after sampling, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation (Nycomed AS, Oslo, Norway) and cryopreserved for later analysis, as described previously.[26]

### Flow Cytometry

Multiparametric flow cytometry was performed to compare circulating frequencies and activation status of both lymphocytic and myeloid subsets in PBMC before and after treatment, as described previously.[26,27] The following antibodies were used for T cell/Treg surface antigens: PerCP-Cy5.5-conjugated CD3, PE-Cy7-conjugated CD27, APC-conjugated CD25, AF700-conjugated CD4, APC-H7 conjugated CD45RA, BV421-conjugated CD127, V500-conjugated CD8 (all from BD Biosciences, San Jose, CA) and BV786-conjugated HLA-DR (Biolegend). After surface staining, PBMC were permeabilized and stained for intracellular FITC-conjugated Ki67 and PE-conjugated FoxP3 following the manufacturer's instructions (anti-FoxP3 antibody clone PCH101 and FoxP3 staining buffer kit; eBioscience, San Diego, CA). Activated (i.e. functionally suppressive) and resting Tregs (aTregs and rTregs, respectively) were discerned according to Myara et al. and as recently described in a consensus white paper.[28,29] Peripheral Blood Dendritic Cell (PBDC) subsets were stained using: FITC-conjugated BDCA-3, PE-conjugated BDCA-2, APC-conjugated M-DC8 (all from Miltenyi Biotec, Bergisch Gladbach, Germany), PE-CF594-conjugated CD19, PerCP-conjugated CD14, PE-Cy7-conjugated CD80, BV421-conjugated CD40, BV786-conjugated PD-L1 (all from BD Bioscience), AF700-conjugated CD1c and APC-Cy7-conjugated CD11c (both from Biolegend). Surface antigens on Myeloid Derived Suppressor Cells (MDSC) were stained with FITC-conjugated CD3, CD19 and CD56, PE-conjugated CD16, PE-CF594-conjugated CD33, PerCP-conjugated CD14, PE-Cy7-conjugated CD11b, APC-conjugated HLA-DR and V500-conjugated CD15 (all from BD Bioscience). Daily performance run was performed using CS&T beads (BD Biosciences) for laser alignment verification and standardization. Stained PBMC were acquired by a FACS LSR FortessaTM X20 (BD Biosciences) and the data were analyzed using Kaluza software (Beckman Coulter Inc., Fullerton, CA).

### T cell in vitro restimulation and IFNγ Elispot assay

Functional T cell responses to mesothelin, WT-1 and recall antigens were determined following a previously described in vitro (re)stimulation and expansion protocol.[30] Cryopreserved PBMC were thawed and incubated at a 1:1 ratio with irradiated autologous PBMC pulsed with one of the following long peptide pools at 1 ug/ml each: 1) a pool of five 20-mer peptides selected to cover the major HLA-A1, A2, A3, and A24 restricted T cell epitopes from mesothelin; MSLN16-35 (PALGSLLFLLFSLGWVQPSR), MSLN411-430

(VATLIDRFVKGRGQLDKDTL), MSLN425-444 (LDKDTLDTAFYPGYLCSL), MSLN520-539 (LATFMKLRTDAVLPLTVAEV) and MSLN531-550 (VLPLTVAEVQKLLGPHVEGL);[31-33] 2) a pool of 110 15-mer overlapping peptides spanning the entire Wilms tumor 1 protein (JPT Peptide Technologies); 3) CEFT positive control pool of 27 peptides selected from defined HLA Class I and II-restricted epitopes from CMV, EBV, Influenza and Tetanus Toxoid (JPT Peptide Technologies). PBMC were cultured for 10 days in the presence of IL-2 (10 IU/ml) and IL-15 (10 ng/ml). The culture medium was changed every 3 to 4 days during in vitro stimulation. The cells were harvested at day 10 and seeded in 2x6 replicate split wells at a density of 2x10e5/well in a Multiscreen 96-well plate (Millipore, the Netherlands) coated with an IFNγ catching antibody (Mabtech, Sweden). Cells were either rechallenged overnight with the peptides to which they were initially stimulated or were cultured with a DMSO vehicle control. Next day the cells were removed and the plates rinsed and developed according to manufacterer's instructions (Mabtech). The spots were counted with a fully automated ELISpot reader system (AID). Specific spots (i.e. antigen-specific T cell frequencies) were calculated by subtracting the mean number of spots of the DMSO control from the mean number of spots in the experimental wells. Antigen-specific T cell frequencies were considered to reflect positive responses when I) antigen-specific frequency was more than 2 times the background, II) the mean spot counts in the experimental wells exceeded those in the control wells by at least 10, and III) the mean number of spots in the experimental wells was significantly higher than the mean number of spots in the control wells as determined by an unpaired T-test.

### Statistical analyses

Statistically significant decreases in the percentage of Tregs cells after treatment were analyzed with one-sided repeated measures ANOVA and a post-hoc Dunnet's multiple comparisons test. The two-sided Student's unpaired T-test was used to test significance of the difference in antigen specific T cell frequencies compared to DMSO control wells and to test significance of differences in survival and WT-1 specific T cell frequencies between patient subgroups. Above listed statistical analyses were performed with Prism GraphPad software (version 6.02). To identify clusters of correlated markers, hierarchical cluster analysis using TIGR software was performed and complete linkage analysis was done by Euclidean correlation analysis.

## Results

### T cell subset differentiation and activation

Flowcytometric analysis of PBMC was performed at baseline (t=0, i.e. at the time of IRE), two weeks after IRE (t=2wks), and 3 months after IRE (t=3mnth), to assess the systemic immune modulatory effects of the IRE procedure. Pre- and post-treatment frequencies of CD4+ and CD8+ T cells remained unchanged (**figure 1A and 1B**). Similarly, the distribution between effector, effector-memory, central-memory, and naive T cells remained stable after IRE treatment (data not shown). No post-treatment CD4+ or CD8+ T cell activation was observed by HLA-DR or CD25 expression (data not shown). There was however evidence of a transient and moderate, non-significant increase in the proliferative fraction of CD8+ T cells which was not observed for CD4+ T cells (**figure 1C and 1D**).

### Transient decrease in activated and resting regulatory T cells following IRE treatment

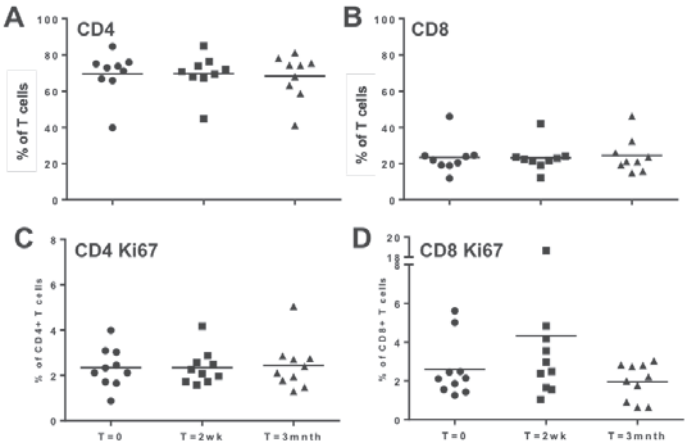


**Figure 1: The effects of IRE on circulating T cel subsets.** Shown are pre- and post-treatment frquencies of (A) CD4+ T cells, (B) CD8+ T cells, (C) CD4+Ki67+ proliferative T cells, and D) CD8+Ki67+ proliferative T cells. N=10.

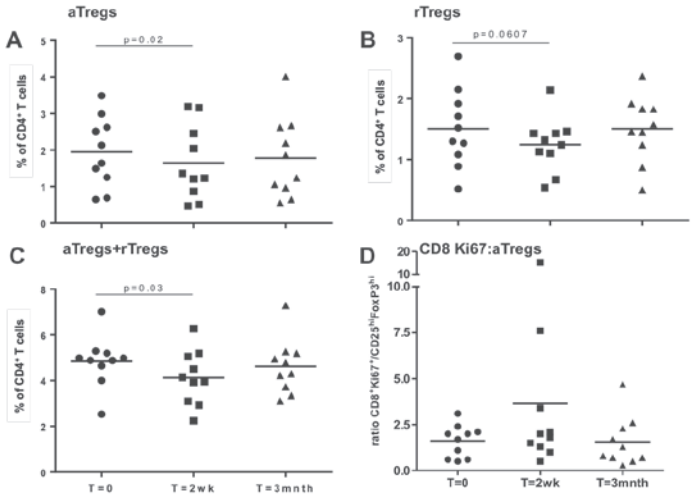


**Figure 2: The effects of IRE on circulating regulatory T cells (Tregs).** Shown are pre- and post-treatment frequencies of (A) activated CD4+CD127-CD25hiFoxP3hi Tregs (aTregs), (B) resting CD4+CD127-CD25+FoxP3+CD45RA+ Tregs (rTregs), (C) aTregs + rTregs, and (D) CD8+Ki67+ proliferative T cell:aTreg ratios. N=10. Indicated statistical significance levels are by one-sided repeated measures ANOVA and post-hoc Dunnet's multiple comparisons test.

A transient but significant decrease in activated (i.e. suppressive) regulatory T cells (aTregs) [28] was observed at 2 weeks following IRE (p=0.02, **figure 2A**). These aTregs were gated as CD4+CD127- T cells with high expression levels of CD25 and FoxP3, further characterized by an absence of CD45RA and expression of Ki67 (data not shown). Resting CD4+CD127-CD25+ Tregs (rTregs) defined by intermediate expression levels of FoxP3, CD45RA positivity, and an absence of Ki67, were similarly transiently decreased (**figure 2B**), resulting in a significant decrease at 2 weeks post-IRE of all Tregs, both rested and activated (p=0.03, Fig.2C). As a measure of Treg suppressive capacity CD8+Ki67+ Tcell/aTreg ratios were calculated. As shown in **figure 2D**, these ratios went up after IRE (although not significantly so), suggesting a decreased Treg-mediated suppression of CD8+ T cell activation.

## Myeloid subset rates and activation state

No effects of IRE were observed on the frequency or activation state (by CD40, CD80,

and PD-L1 expression) of the peripheral blood DC subsets CD1c+ cDC1, CD141+ cDC2, SLAN-cDC, and pDC (**figure 3A-D**). In contrast, a transient and non-significant increase in monocytes (**figure 3E**) and decrease in potentially suppressive Li-CD33+HLA-DR- myeloid derived suppressor cells (MDSC, **figure 3F**) was observed at 2 weeks post-IRE treatment.

### Tumor antigen specific T cell responses

Pre- and post-treatment tumor specific T cell reactivity was assessed by IFNγ Elispot read-out, after in vitro stimulation with peptides covering known CD4 and CD8 T cell epitopes derived from the pancreatic tumor antigen mesothelin (MSLN) or 15-mer overlapping peptides covering the full length of the tumor antigen WT-1. As a measure of immune competence, T cell reactivity against peptides covering CD4 and CD8 T cell epitopes from CMV, EBV, Influenza (Flu), and Tetanus Toxoid (CEFT) was determined. PHA reactivity served as a technical assay control. Whereas CEFT responses were uniformly high and apparently unaffected by IRE treatment, no responses, either pre- or post-treatment, were observed against the tested mesothelin epitopes (**figure 4**). In contrast, T cell responses against WT-1 were detected both pre- and post-treatment (**figure 4**). Remarkably, three patients harbored pre-treatment WT-1 specific T cell responses; in two of these, elevated responses were observed post-treatment. In addition, in two patients WT-1 specific T cell responses were *de novo* induced after IRE treatment.

### WT-1 T cell reactivity in relation to changes in CD8+Ki67+ T cell, aTreg, monocyte, MDSC frequencies and overall survival

We subsequently performed an unsupervised cluster analysis based on changes in CD8+Ki67+ T cell, aTreg, monocyte and MDSC frequencies after IRE. In **figure 5A** this cluster analysis is shown, related to overall survival from the time of IRE and WT-1 T cell reactivity post-IRE. Of note, clustering in roughly two groups appeared to be dominated by

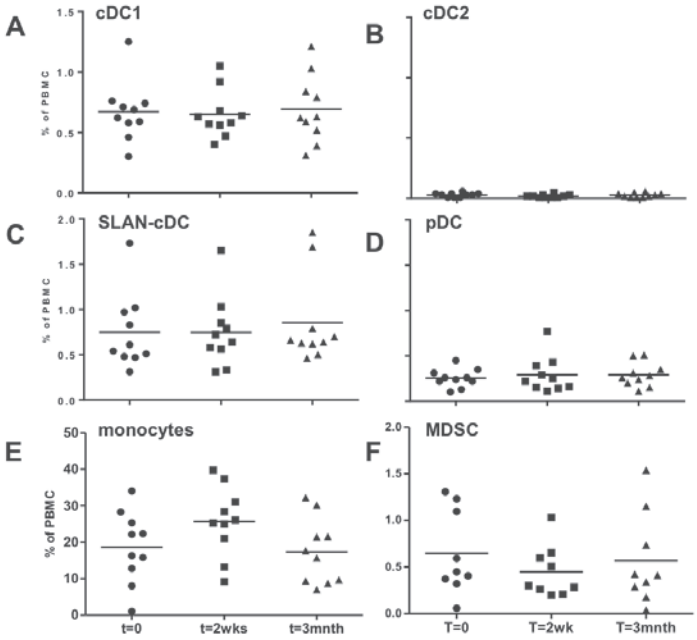


**Figure 3: The effects of IRE on myeloid subsets.** Shown are pre- and post-treatment frequencies of (A) CD1c+ conventional DC (cDC) type-1 (cDC1), (B) CD141+ cDC2, (C) M-DC8+ (Sulph-Lac-Nac) SLAN-cDC, (D) CD202+ plasmacytoid DC (pDC), (E) CD14+ monocytes, and (F) Li-CD33+HLA-DRlo/- myeloid derived suppressor cells (MDSC). N=10, except MDSC, n=9.

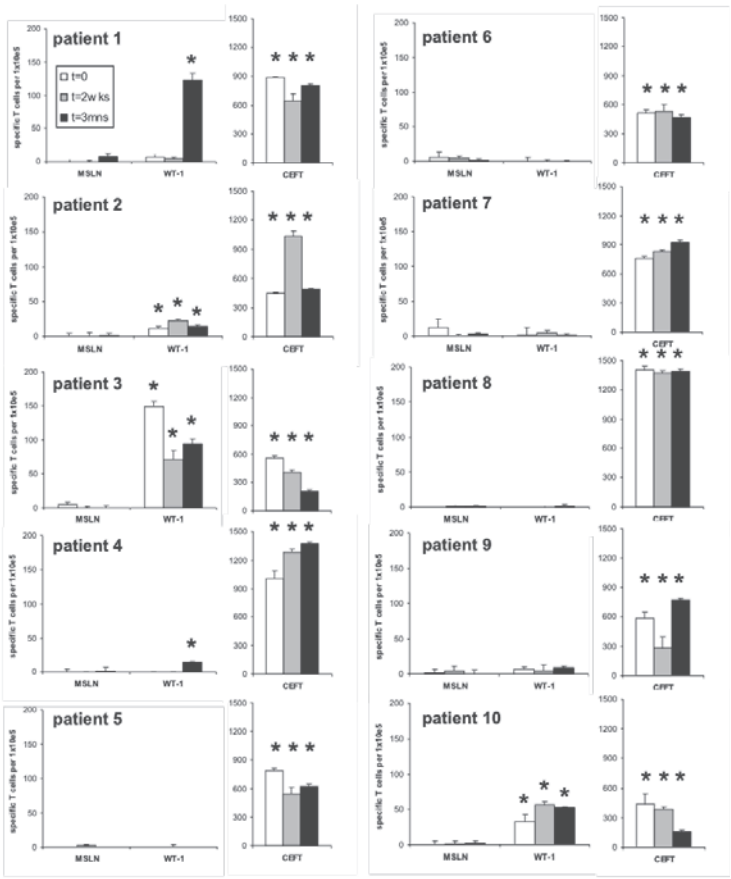


**Figure 4: Pre-existent and IRE-induced WT-1 specific T cell responses.** Shown are pre-and post-IRE specific T cell frequencies against mesothelin (MSLN), WT-1, and CEFT (i.e. CMV, EBV, Flu and Tetanus Toxoid derived recall epitopes) as measured by IFNγ elispot assay (expressed as number of spot-forming T cells per 100,000) after a 10-day in vitro stimulation and expansion culture. Shown are means and standard deviations of six parallel cultures per tested antigen. Asterisks denote positive responses –as defined in the Materials and Methods section.

relatively high versus low increases in CD8+Ki67+ T cell frequencies, with high increases in proliferative CD8+ T cell rates being more often accompanied by decreases in aTregs. Of note, whereas the observed decrease in aTregs reached statistical significance, the increase in the CD8+Ki67+ T cell proliferative fraction did not (**figure 1D** and **figure 2A**). Two groups could clearly be distinguished based on low versus relatively higher CD8+Ki67+ T cell :aTreg ratios. In contrast, decreases in monocyte and MDSC frequencies did not contribute to the clustering of these two groups. Remarkably, 4 out of 5 patients in the high CD8+Ki67+ T cell :aTreg group had a positive WT-1 specific T cell response post-IRE (including the two patients with de novo induced responses) as compared to only 1 out of 5 in the low CD8+Ki67+ T cell :aTreg group. When patients were ordered by overall survival (OS), i.e. for all patients time (in months) from IRE to death, it became clear that higher WT-1 specific T cell frequencies (at any time during follow-up) were observed in patients with above median OS (11.1 months, **figure 5B**). Indeed, when patients were divided by lower than, or higher than, median OS (from the time of IRE), higher WT-1 T cell responses (by frequency) were found in the above median group (p=0.055, **figure 5C**). *Vice versa*, higher mean OS was observed in patients with a positive post-IRE WT-1 T cell response as compared to patients with a negative response (p=0.055, **figure 5D**).

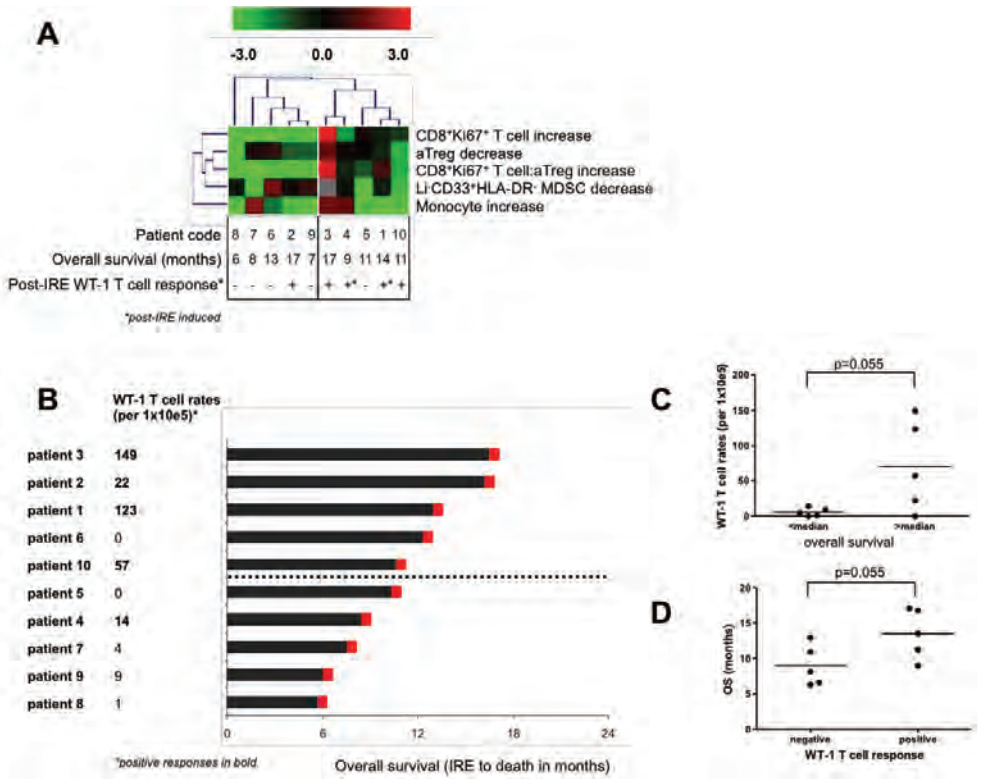


**Figure 5: WT-1 T cell reactivity in relation to changes in CD8+Ki67+ T cell, aTreg, monocyte, MDSC frequencies and overall survival (OS).** A) Unsupervised cluster analysis shows two groups of patients (each of n=5): clustering is dominated by relatively higher or low increases in CD8+Ki67+ proliferative T cell responses. OS and post-IRE WT-1 responsiveness are shown per patient in relation to the relative parameter expression levels. B) Bar graphs of OS, ordered by OS in months counted from IRE, i.e. time from IRE to death (red caps on the bars represent the patient's time of death). Highest measured WT-1 specific T cell rates at any time during follow-up are listed in relation to OS per patient and reveal highest frequencies in patients with above median OS (dotted line indicates median OS). Frequencies in bold face denote positive responses (n=5) as defined in the Materials and Methods section. C) WT-1 specific T cell rates (highest rates are shown at any time during follow-up) in patients with below and above median OS and D) OS in patients with positive versus negative WT-1 specific T cell responses. Significance levels indicated were based on 2-sided unpaired T-tests.

## Discussion

Little is known about the immunological responses induced by IRE and the ability of these responses in pancreatic cancer to provide both local and systemic protection against recurrence. Our findings are encouraging in that they provide evidence for a transient lifting of systemic suppression by Tregs, accompanied by a transient increase in the CD8+Ki67+ T cell proliferative fraction. Hierarchical clustering analysis showed this phenomenon to coincide with detectable (and durable) T cell responses to WT-1, which in turn were more prominent in patients with above median OS.

Minimally invasive interventional techniques for in situ tumor destruction are gaining

ground clinically. Unlike surgery, the treated malignancy is not removed from the body, but apoptotic or necrotic cell remnants induced by the ablative technique remain available to be taken up by phagocytic leukocytes. If apoptosis induction is accompanied by the release of damage-associated molecular patterns (like e.g. ATP and HMGB1), which serve as so-called "find me" and "eat me" signals, infiltrating antigen-presenting cells (like DC) will become activated and transport tumor fragments to draining lymph nodes where adaptive immune activation can take place. In effect this local ablation, through e.g. thermal techniques such as radio frequency ablation (RFA), thus serves to achieve in situ tumor vaccination -reviewed by Bastianpillai et al. [34] and O'Brien et al.[35] As a result, such local therapies can induce a durable and systemic antitumor T cell response that in turn can induce regression in distant, non-treated metastases, a phenomenon known as the abscopal effect. In keeping with this notion, case reports of spontaneous regression of metastases following RFA of a primary tumor and enhancement of tumor-specific T-cell responses have been reported.[36] These observations hold true for intrinsically immunogenic tumors (e.g. with high mutation rates and high neo-antigen load), but are much less pronounced in weakly immunogenic tumors. The latter may benefit from therapies combining local ablation with immune stimulation, e.g. by intratumoral delivery of Toll-like receptor ligands (TLR-L) and/or perioperative immune checkpoint inhibition.[34,35]

Immune stimulation could be particularly useful in pancreatic cancer, where a lack of infiltrating DC and prevailing immune suppression is well documented and where cellular apoptosis is a rare event.[13,14,37] These circumstances all conspire to restrict the immunogenic potential of pancreatic cancer and could well be overcome by immunogenic tumor cell death induced by local ablation combined with further immune stimulation. IRE may offer an attractive therapeutic platform in this regard.

Thus far evidence of post-IRE induced antitumor immunity in man has been lacking. A major theoretic benefit of IRE is the sparing of larger vessels, which remain intact due to the largely non-thermal mechanism of action. Consequently, immune effector cells should be able to infiltrate the lesion and DC should be able to migrate to draining lymph nodes to induce a systemic immune response subsequent to IRE. An early report by Al-Sakere et al. [38] on the effects of IRE in subcutaneously injected tumors in a mouse model were not encouraging as IRE did not appear to induce substantial infiltration of immune cells into the treated tissue after 0, 2 and 6 hours post-ablation. However, this might well have been due to the subcutaneous localization of these transplanted tumors which are relatively poorly vascularised, restricting immune effector cell access and in some instances resulting in 'immunological ignorance'. Moreover, since their results only comprised the first 6 hours after ablation, there is the possibility of immune infiltration and priming of an immune response at later time points post-IRE. More promising observations were reported in subsequent publications. Rubinsky et al. studied the effects of IRE in a canine prostate cancer model and reported rapid resolution of the lesional debris after IRE, which is consistent with intact (lymph) vessels. They also noticed lymph node reactivity in the ablation drainage area, which they interpreted as IRE-induced immune activation.[7] In a rat osteosarcoma model increased T cell infiltration and reduced levels of the immunosuppressive cytokine IL-10 were reported post-IRE.[39] Similarly, José et al. [40] observed extensive areas of necrotized tissue in a xenograft model of intra-pancreatically injected tumors, with the presence of lymphocytic infiltrates and histiocytes at day 7 post-IRE and massive infiltration by day 14. In a particularly interesting

experimental set-up Neal and colleagues studied the effects of IRE of subcutaneously injected renal carcinoma tumors in an immune competent versus immune compromised mouse model.[41] Based on tumor burden and the progression-free disease period, antitumor responses were substantially more durable in the IRE-treated immune competent mice relative to the IRE-treated immune deficient mice and sham controls. This was accompanied by robust T cell infiltration rates at the ablation border. Tumor rechallenge after IRE-mediated tumor ablation in the immune competent mice resulted in an increased delay in tumor outgrowth or even complete prevention of tumor growth. These findings clearly point to a protective antitumor immune response induced by IRE.[41] In a more recent report Bulvik et al. have compared the effects of IRE with those of RFA and found higher levels of systemic IL-6 post-IRE, which may indicate Damage-Associated Molecular Pattern (DAMP) mediated immune activation.[12] In a subcutaneous hepatocellular carcinoma model superior delayed tumor outgrowth was observed after IRE. Moreover, in the border zone surrounding the treated lesions leukocyte infiltration into the ablation zone was demonstrated in IRE-treated, but not in RFA-treated lesions. This led the authors to conclude that not only larger vessels, but also the microvasculature was preserved after IRE, which should greatly facilitate leukocyte trafficking.[12]

The observed immune stimulatory effects of IRE, as enumerated by the above listed reports are consistent with our own post-IRE observations of decreased Treg levels and expansion of WT-1 specific T cells in a number of patients. Similar decreases in systemic frequencies of Tregs were previously reported after RFA and may be due to a decrease in tumor-derived immunosuppressive soluble factors.[42] Although MDSC were also previously reported to inversely correlate with clinical benefit in patients treated by RFA,[43] clustering analysis rather showed a relationship between decreased Treg (and not MDSC) rates, increased rates of proliferative CD8+ T cells and post-IRE WT-1 specific T cell responses in the monitored patients with LAPC.

We found pre-treatment T cell reactivity to WT-1 in 3/10 patients. This is surprisingly high for a supposedly weakly immunogenic tumor type like pancreatic cancer [13] and is promising as such natural immunity may be boosted to enhance antitumor efficacy. Indeed, in two of these patients we found increased WT-1 specific T cell frequencies after IRE. Even more promising was the observation that in two additional patients *de novo* WT-1 specific T cell responses were induced after IRE. WT-1 has been reported to be expressed in 75% of pancreatic tumors and not at all in healthy pancreatic tissues,[44] confirming it as a *bona fide* immune target antigen. Although caution is warranted not to over-interpret our findings in this small group of patients, the seeming relationship between WT-1 responsiveness and longer OS is particularly exciting. In line with this, WT-1 specific vaccination in patients with pancreatic cancer was shown to provide clinical benefit in a subgroup.[18] In contrast to WT-1, no mesothelin specific T cell responses were observed in this study, either prior to or after IRE. Others have reported naturally occurring mesothelin-specific T cell responses [32] and that induction or boosting of such responses after vaccination was associated with clinical benefit.[19,20] This apparent discrepancy may be due to the fact that unlike for WT-1 we didn't use overlapping peptides to cover the entire length of mesothelin but rather selected 20-mer peptides with reported epitopes. As a result, responses to alternative epitopes may have been missed.

## Conclusion

Our observations show, to our knowledge for the first time, that IRE has immune modulating effects that can result in the boosting or even priming of systemic antitumor T cell responses, as exemplified by the monitoring of WT-1 specific T cell responses. Indeed, in 5 out of 10 tested patients with LAPC, responses to WT-1 were detectable 3 months after IRE. This suggests a durable immune response that could conceivably offer long-term protection against distant tumor recurrence. Encouragingly, these responses were most prevalent in patients with above median OS. These observations argue in favor of combining IRE with immune stimulatory therapies to enhance antitumor efficacy. An attractive approach would be the local delivery of TLR-L or immune checkpoint blocking antibodies at the tumor site, e.g. through imaging-guided injections immediately following the IRE procedure. Such local immune potentiation strategies are actively pursued and are shown to boost systemic antitumor immunity.[45,46] Although it is important to realize that this pilot study was not sufficiently powered to draw definitive conclusions, our findings suggest that IRE offers an attractive and effective *in situ* vaccination platform to combine with such immunotherapeutic approaches.

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