Highlights

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- Immunotherapy is emerging as the most promising approaches in cancer treatment
- There is no personalized assay that can predict patient response to immunotherapy
- We introduced fully humanized microfluidic chip to test immunotherapeutic drugs
- The chip is loaded with isolated cancer cells, patients´ serum and immune cells
- Several immunotherapeutic drugs can be tested for each patient simultaneously

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1 # *In vitro* Humanized 3D Microfluidic Chip for Testing Personalized
2 # Immunotherapeutics for Head and Neck Cancer Patients

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Abstract

Objectives. Immunotherapy and personalized medicine therapeutics are emerging as promising approaches in the management of head and neck squamous cell carcinoma (HNSCC). In spite of that, there is yet no assay that could predict individual response to immunotherapy.

Methods. We manufactured an *in vitro* 3D microfluidic chip to test the efficacy of immunotherapy. The assay was first tested using a tongue cancer cell line (HSC-3) embedded in a human tumour-derived matrix "Myogel/fibrin" and immune cells from three healthy donors. Next, the chips were used with freshly isolated cancer cells, patients´ serum and immune cells. Chips were loaded with different immune checkpoint inhibitors, PD-L1 antibody and IDO 1 inhibitor. Migration of immune cells towards cancer cells and the cancer cell proliferation rate were evaluated.

Results. Immune cell migration towards HSC-3 cells was cancer cell density dependent. IDO 1 inhibitor induced immune cells to migrate towards cancer cells both in HSC-3 and in two HNSCC patient samples. Efficacy of PD-L1 antibody and IDO 1 inhibitor was patient dependent.

Conclusion. We introduced the first humanized *in vitro* microfluidic chip assay to test immunotherapeutic drugs against HNSCC patient samples. This assay could be used to predict the efficacy of immunotherapeutic drugs for individual patients.

Keywords. Head and neck cancer; in vitro; microfuidic chip; personalized medicine; PD-L1; IDO1; immunotherapy

59 Introduction

60 Immunotherapy is considered as the newest treatment modality for head and neck squamous cell carcinoma
61 (HNSCC) patients. Surgical removal of the tumour or definitive radiotherapy are the main approaches with
62 curative intent for selected groups of patients with an early stage tumour. Patients with advanced stage
63 disease at the time of diagnosis typically receive an individually tailored combination of surgery and
64 (chemo)radio-, targeted-, and recently immunotherapy. With all these available treatments the five-year
65 survival amongst HNSCC patients still is around 50% [1].

66 After introducing immunotherapy, there was a hope that this new treatment approach could significantly
67 enhance HNSCC patients' survival. Pembrolizumab, a PD-1 inhibitor, is the first immunotherapy, which has
68 received FDA approval for treating HNSCC patients and was followed by another anti-PD-1 drug, nivolumab [2-
69 59]. These agents are used as second line treatment after failure of platinum-based chemotherapy. In a phase 3
70 clinical trial (NCT02252042) pembrolizumab gave a better objective response rate (14.6%, 95%CI 10.4 to 19.6)
71 compared with the standard therapy (10.1%, 95%CI 6.6 to 14.5) for HNSCC patients [6]. Other
72 immunotherapies are still investigated in ongoing clinical trials, such as durvalumab, avelumab (both are anti
73 PD-1) and tremelimumab (anti CTLA-4) [7]. Similar to the other types of HNSCC treatments, there are currently
74 no methods to predict the patients' response to immunotherapy. This leads to a significant problem in terms of
75 subjecting patients to unnecessary side effects and ineffective treatments, especially when the percentage of
76 the responsive patients remains low (14.6% for pembrolizumab). Expression of PD-L1 with a cut-off of 1% was
77 used as a requirement for giving the PD-1 antibody in a study by Seiwert et al. [5] in a series of 60 HNSCC
78 patients, but unfortunately it did not guarantee treatment response.

79 Although, personalized drug testing has been recently undergoing active investigation, the results have still not
80 been translated into clinical practice despite that several models have been suggested for testing chemo-,
81 radio- and targeted therapies. On the other hand, testing immunotherapy in *in vitro* setting seems to be more
82 complicated, especially as a co-culture of two different cells i.e. cancer cells and immune cells with different
83 adherent properties is needed. *In vitro* 2D culture has been considered as the gold standard for cancer
84 research. However, with our recent knowledge, researchers have started to regard the 2D cell culture as being
85 too far from the real *in vivo* condition. Thus, there is a clear shift in research setting towards using 3D cell
86 cultures and many extracellular matrices (ECM) have been proposed to be used for this purpose. The majority
87 of these matrices have either been extracted from animals (sarcoma mouse-derived Matrigel, rat tail type one
88 collagen) or derived from synthetic material. Even though these matrices can provide a 3D structure for the
89 cancer cells, they still have major limitations including missing of important elements present in the human
90 tumour microenvironment. To overcome this problem, our group developed human tumour-based matrix
91 "Myogel" from leiomyoma tissue [8]. Due to its neoplastic origin, Myogel seems to be suitable for human
92 cancer 3D *in vitro* assays providing an ideal tumour environment for the cultured cancer cells [8,9].

93 Microfluidic chip has been applied as one solution for studying the efficacy of immunotherapy by evaluating
94 immune cell migration towards cancer cells [10-12]. The chip was developed by Businaro et al. [10] and was
95 used for testing the effects of type I interferons on melanoma cells [11]. Matrigel was used in these assays to
96 provide 3D environment for the cancer cells. Here, we aim to provide a fully human *in vitro* microfluidic chip
97 assay to test immunotherapy for personalized medicine purposes for HNSCC patients. The cancer cells
98 (including carcinoma and stromal cells) are isolated from patient-derived tumour tissue pieces and embedded
99 in human tumour-based ECM "Myogel/fibrin". Additionally, the immune cells and serum are collected from the

100 patient's blood. All these elements are loaded into the microfluidic chip with or without immunomodulators to
101 test both the immune cell migration towards cancer cells and their cytotoxic activity.

102

103 Materials and methods:

104 **Cell line**

105 Human tongue SCC (HSC-3; Japan Health Sciences Foundation, Japan) cells were cultured in 75 cm2 flasks
106 containing Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco, Paisley, UK) supplied with 10% heat-
107 inactivated foetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml fungizone and
108 50 µg/ml ascorbic acid (all from Sigma-Aldrich, St. Louis, Mo, USA). To prepare the cell suspension for injection
109 into the chip, cells were detached from the flask using trypsin/EDTA and suspended in complete media.

110 **Patient samples**

111 Samples were collected according to our institutional Research Ethics Board (14.03.2016 Eettmk 84) approval.
112 Patient participation was voluntary and required informed consent. Clinical and pathological characteristics of
113 the patients are presented in Table 1.

114 The fresh tissue samples were obtained perioperatively and immersed in ice-cold Hanks' Balanced Salt solution
115 (HBSS; supplied with 100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml fungizone). The samples were
116 taken from the area adjacent to the centre of the tumour to assure the the presence of the carcinoma tissue
117 cells, including mostly carcinoma cells and some cancer associated fibroblasts. Each sample was placed in a
118 petri dish containing ice-cold HBSS and necrotic tissues were removed using a scalpel. Vital tissue pieces were
119 placed into a new petri dish containing HBSS and minced into small (1–2 mm) pieces with a scalpel. The tissue
120 pieces were transferred to 15-ml falcon tube and centrifuged for 5 min at 1000 rpm (200 × g) at 4°C. The
121 supernatant was discarded and a fresh HBSS buffer was added before another round of centrifugation. The
122 tissue piece pellet was suspended in a 5 ml HBSS buffer containing 1 mg/ml collagenase type I from Clostridium
123 histolyticum (Sigma-Aldrich, St. Louis, Mo, USA) and placed on a rocker platform at 37°C for two hours. The
124 tube was centrifuged and the supernatant was discarded and replaced with a fresh HBSS buffer before another
125 round of centrifugation. The digested sample was suspended in an HBSS buffer, filtered using a 100-µm cell
126 strainer (Falcon™ Cell Strainer, Fisher Scientific, NH, USA) and the flow-through (single cells) was collected and
127 centrifuged. The supernatant was discarded and the cell pellet was suspended in DMEM/F-12.

128 **Isolation of the human peripheral blood mononuclear cells (MNCs) and serum from the buffy coat of healthy**
129 **donors and blood of cancer patients**

130 Human peripheral blood MNCs were isolated from a buffy coat of three healthy donors provided of by the
131 Finnish Red Cross and blood of two HNSCC patients. A density gradient technique was followed to isolate MNCs
132 using Ficoll–Paque PLUS (GE Healthcare, Piscataway, NJ, USA). As peripheral blood MNCs consists of both
133 adaptive and innate immune cells (T cells, B cells, NK cells, monocytes, and dendritic cells), we will refer to
134 them as immune cells.

135 For serum collection, blood was allowed to clot at room temperature for 30 minutes and clot was removed by
136 centrifugation at 2000 rpm for 10 minutes in a refrigerated centrifuge at 4C.

137 **Proliferation luminescent cell viability assay**

Wells of 96-well plates with black well walls and clear bottoms (PerkinElmer, Waltham, MA, USA) were coated with 50 µl/well of 0.5 mg/ml Myogel (lab made) and Matrigel (Corning, Corning, NY, USA); control wells were left uncoated. The plate was incubated at the cell culture incubator overnight. Isolated cancer cells were seeded at the density of 1000 cells/well in 100 µl of complete medium. After 3 days, the plate was taken out from the incubator to room temperature for 15 min before starting the assay. One hundred µl of CellTiter-Glo was dispensed in each well. The plate was put on a plate shaker (Heidolph, Schwabach, Germany) for 5 min at 450 rpm and then in plate spinner (Thermo Scientific, Massachusetts, USA) for 5 min at 1000 rpm. Finally, the plate was placed in the BMG Pheraster FS (BMG Labtech, Offenburg, Germany) plate reader to detect cell viability.

Microfluidic chip design and fabrication process

Standard soft lithography process was followed to fabricate the PDMS poly(dimethylsiloxane) microfluidic devices. The chip was designed with slight modification from Businaro et al. [10]. CAD software was used to design the microchannel patterns, which were then plotted on high resolution polymer films, consequently applied as photomasks for the photolithography steps. Figure 1 shows the microchannel layout, which was translated onto a master mould, containing two layered SU8 negative photoresist structures on silicon wafer following MicroChem Nano™ protocols.

Each microfluidic structure contains a central 16 mm long 1.2 mm wide and 150 µm deep chamber for the immune cells, which is connected to the neighbouring cancer cell containing channels via 200 µm long, 10 µm deep and 12 um wide microchannels. The channels incorporating the cancer cells are 150 µm deep, 600 µm wide and linked to the immune cell containing channel along a 10 mm section from both sides.

For preparing the PDMS replicas, Sylgard 184 (Dow Corning) was mixed with crosslinking agent in 10:1 ratio and casted over the master mould template. After keeping in vacuum for 45 min to eliminate air bubbles it was placed in a preheated oven and cured at 70 C for 2 hours. Upon cooling back to room temperature the PDMS replica was peeled off the template and the chips were cut out, the fluidic inlets and outlets punched through, including the larger circular vias defining the open liquid reservoirs. Then the PDMS surfaces were cleaned from dust with nitrogen gun and scotch tape. Glass microscope slides were thoroughly wiped and cleaned with ethanol and dried with nitrogen gun. After exposing the slides and the PDMS replicas to oxygen plasma in a PVA TePla 400 plasma system for 1 minute (power: 60 W, O_2 flow rate: 500 ml/min) the PDMS chips were immediately placed on and bonded to the glass slide, providing a lid over the microchannels. Prior to loading the samples the chips were autoclaved at 120 C for 30 min.

Microfluidic chip assay

Cancer cells (cell line or freshly isolated tumour cells) were stained with Celltrace Far Red (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and suspended in Myogel/fibrin. Myogel/fibrin was prepared using the following concentrations: 2.4 mg/ml Myogel, 0.5 mg/ml fibrinogen (Merck, Darmstadt, Germany), 0.3 U/ml thrombin (Sigma-Aldrich), and 33.3 ug/ml aprotinin (Sigma-Aldrich); these reagents were diluted in DMEM/F12 media with 10% fetal bovine serum for the cell line experiment and patient serum for the patient sample experiment. Three microliters of cell suspension (containing 20 000 cells) in gel was loaded in each small channel (B).

Immune cells were stained with CellTrace Violet (Invitrogen) and suspended in DMEM/F12 media supplied with 10% serum, foetal bovine serum for cell line experiment and patient serum for the patient samples experiment. Immune cells were divided into 3 groups: control (with no drug), 9.6 µg/ml PDL-1 antibody (Bio X Cell, Hanover,

179 NH, USA) and 5 µg/ml IDO 1 inhibitor (NLG-919, Cayman Chemical, Ann Arbor, MI, USA). 150 µl of cells
180 suspension (containing 1 million immune cells) was injected into the large channel (A). PD-L1 antibody and IDO
181 1 inhibitor were added with the immune cells due to large volume of cells suspension added to the channel
182 (150 µl) compared with only 3 µl for the cancer cells channel which allow adding higher volume of drug.
183 Additionally, this setup is closer to the *in vivo* situation where the patients receive immunomodulator drugs
184 through intravenous injection.

185 Chips were incubated in the cell culture incubator for three days and imaged daily under fluorescence
186 microscope using Leica DM6000 B/M light microscope connected to a digital camera (DFC420 and DFC365FX;
187 Leica Microsystems, Wetzlar, Germany).

188 **Cells counting**

189 The number of immune and cancer cells were analysed using Matlab's (Mathworks, Natick, MA, USA) built-in
190 algorithm. The algorithm is semi-automated with manual thresholding. The algorithm detects positive cells
191 when the intensity of their staining is substantially higher than the background [13]. Matlab's built-in functions
192 trace boundaries cells/objects.
193

194 Tumour-associated immune cells were identified based on their size and morphology. For cancer cell detection,
195 objects with smaller size than the average cell size were excluded from the quantification using object size
196 threshold set manually. Cancer cell proliferation rate was measured by dividing the number of cancer cells each
197 day by the number of cancer cells of day 1.

198

199 Results

200 **Isolated cancer cells proliferate more on top of Myogel compared with Matrigel and uncoated wells**

201 Cancer cells were isolated from three HNSCC patients and seeded on top of plastic, Myogel and Matrigel to
202 study their proliferation. For all the three patient samples, cancer cells had higher proliferation rate on top of
203 Myogel compared with plastic or Matrigel (Figure 2).

204 **Immune cells migration towards cancer cells depends on cancer cells density**

205 Two chips were used to study the immune cells migration towards cancer cells. The first chip (Figure 3a) was
206 loaded with immune and cancer cells (channels A and B, respectively). The second one (Figure 3b) was loaded
207 only with immune cells, where instead of the cancer cells, a Myogel/fibrin gel was applied. In the first chip,
208 during three days there was a continuous migration of the immune cells towards cancer cells. On the other
209 hand, immune cells did not pass to channel B in the second chip (Figure 3a & b).

210 To study if the migration of the immune cells towards the cancer cells is cancer cell density dependent, chips
211 were loaded with two different cancer cells densities (20 000 and 10 000 cells). Immune cells from three
212 healthy donors were used in this experiment. In all the 3 chips with high cancer cells density, more immune
213 cells migrated towards the cancer cells compared with the chips, which were loaded with lower number of
214 cancer cells (Figure 3c).

215 **IDO 1 inhibitor induces immune cells migration towards cancer cells**

216 To study the effect of PD-L1 antibody and IDO 1 inhibitor on the immune cells migration, we first used HSC-3
217 cell line and immune cells from three healthy donors. For all the three donors, IDO 1 inhibitor induced the
218 immune cell migration (Figure 4). On the other hand, PD-L1 antibody did not increase immune cell migration,
219 but instead it reduced it in two cases (Donor 1 and 3; Figure 4).

220 Next, using patients´ cancer cells and their immune cells in the presence of the patients´ serum, we tested the
221 effect of PD-L1 antibody and IDO 1 inhibitor on the immune cell migration. Similar to the cell line results, IDO 1
222 inhibitor induced the immune cell migration towards cancer cells (Figure 5a & b).

223 **Patient dependent response to immunotherapy**

224 Two patient samples were used to test the effect of anti PD-L1 and IDO 1 inhibitor on tumour cell proliferation.
225 While PD-L1 antibody was the most effective drug for the first patient, IDO 1 inhibitor gave the best, still
226 modest, result for the second patient (Figure 5c).

227

228 Discussion

229 Immunotherapy is emerging as a novel treatment approach for several cancer types including HNSCC. In spite
230 of its promising results, the percentage of responder patients remains very low [3, 4]. Due to the low number
231 of responders, the majority of patients are subjected to ineffective drugs and unnecessary side effects. Until
232 now, there is no marker, which could predict the patients' response to these treatments. Here, we introduced
233 a 3D *in vitro* microfluidic chip supplied with the patients' cancer cells, their immune cells and serum to test the
234 efficacy of immunotherapeutic drugs. The system is superior to the other existing methods since it provides a
235 3D fully human tumour microenvironment to the cancer cells as they are embedded in human Myogel/fibrin
236 matrix. We tested this system using a HSC-3 cell line and two HNSCC patient samples. Immune checkpoint
237 inhibitors (PD-L1 antibody and IDO 1 inhibitor) gave variable cytotoxic activity between the patients.
238 Additionally, we reported for the first time that IDO1 inhibitor induced immune cells migration towards cancer
239 cells.

240 Several immune checkpoints have been identified, such as PD-L1, B7-H3, IDO1 and CTL-4. While all these
241 checkpoints contribute to cancer progression through immune system suppression, each one has a different
242 mechanism. In this work we targeted two immune checkpoints, PD-L1 and IDO 1. PD-L1 is reported to be highly
243 expressed on the HNSCC tumour cells [14]. This upregulation allows tumour cells to escape from the host
244 immune system through induction of T-cell apoptosis, anergy, exhaustion and secretion of the anti-
245 inflammatory cytokines IL-10 [15]. IDO 1 is also upregulated in HNSCC and it induces T-cell apoptosis, and
246 promotion of Treg differentiation through depletion of tryptophan and production of kynurenine [16, 17].

247 Testing the immunotherapeutic efficacy is more complicated compared with that of chemo-, targeted- and
248 radiotherapy due to the need of co-culturing the cancer and immune cells. Although many co-culture models
249 are available, still co-culturing cancer and immune cells warrants improvement due to different adherent
250 properties of these cells. The microfluidic chip which was designed first by Businaro et al. [10] provides a
251 solution for this problem by using two chambers connected by micro-channels. The first chamber is filled with
252 suspended immune cells and the other one is injected with cancer cells embedded in gel. In this study, we
253 replaced the commonly used mouse sarcoma derived Matrigel with the human leiomyoma originated Myogel
254 to provide better microenvironment to the cancer cells mimicking the *in vivo* tumour matrix. We have shown in
255 previous studies that cancer cells migrate and invade faster on Myogel than on Matrigel [8, 9]. Here we showed

that the freshly isolated cancer cells from three HNSCC patients proliferated more on top of Myogel compared with plastic or Matrigel. This result indicates the importance of providing a suitable ECM for the 3D cell culture, which has both human and tumour properties. The chip is also supplied with the patient's own serum instead of the commonly used FBS. This provides the cancer and immune cells with the similar mixture of proteins, which are in contact with them *in vivo*.

Studying immune cell migration is a common method to investigate the cross-talk between cancer and immune cells [18]. It is also used as a parameter for studying the efficacy of immunotherapy [11, 12]. Interestingly, our results indicate that IDO 1 inhibitor, but not PD-L1 antibody, induced immune cells migration towards cancer cells. To the best of our knowledge, this is the first report showing the induction of immune cells migration by inhibiting IDO1. Such observation of IDO 1 inhibitor being able to induce immune cell infiltration towards cancer could change the tumour from cold to hot, if validated *in vivo*. This could, in theory, enhance the efficacy of the other immunotherapeutic drugs, such as PD-1 and PD-L1 antibodies, if given in combination with IDO 1 inhibitor. Therefore, we aim as the next step to test various combination therapies using our microfluidic chip assay.

Our results demonstrated the variability of the drugs` efficacy between the two HNSCC patients. As PD-L1 antibody was the most effective one in the patient No. 4, IDO 1 inhibitor was the most effective in the patient No. 5. To be noticed, since immune cell migration did not parallel the proliferation results, we consider the immune cell migration is not sufficient parameter to evaluate the response of the immunotherapeutic drugs, as suggested before [11]. Unfortunately, we do not have *in vivo* evidence of the effects of these immunotherapy drugs on our patients, since these drugs are not in clinical use yet.

In conclusion, we describe here a novel humanized *in vitro* microfluidic chip assay for testing the efficacy of immunotherapeutic drugs against patient samples. This assay could give preliminary knowledge to the clinician on the efficacy of these drugs for individual patients.

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328 Figure legends:

329 **Figure 1. Microfluidic chip design.** Immune cells are loaded in Channel A and cancer cells in Channels B.
330 Channels C are used for hydration.

331 **Figure 2. Cancer cells have higher proliferation on top of Myogel compared with plastic and Matrigel.** Freshly
332 isolated cancer cells from three HNSCC patients were cultured on top of plastic, Matrigel and Myogel for three
333 days. Cancer cells´ proliferation was measured using luminescent cell viability assay. Results are presented as
334 mean ± standard deviation.

335 **Figure 3. Immune cells migration towards cancer cells depends on cancer cells density.** Cancer cells were
336 labelled with cell trace far red, embedded in Myogel/fibrin and injected in channel B of the first chip (a).
337 Immune cells were labelled with cell trace violet and loaded in channel B in the first and second chips (a and b).

338 Migration of the immune cells was only detected in the first chip (a). Immune cells migration was dependent
339 on the number of the injected cancer cells (c).

340 **Figure 4. IDO 1 inhibitor induces immune cells migration towards cancer cells.** Cancer (red) and immune cells
341 (blue, from three healthy donors) were loaded in the microfluidic chips. The chips were supplied with PD-L1
342 antibody and IDO1 inhibitor, no drugs were added to the control chips. Number of infiltrated immune cells
343 were counted over three days.

344 **Figure 5. Migration of the patients´ immune cells and their effects on the cancer cells proliferation.** Isolated
345 cancer and immune cells (both from HNSCC patients) were labelled with cell trace far red and violet,
346 respectively. Cells were injected in the microfluidic chips in the presence or absence of PD-L1 antibody and
347 IDO1 inhibitor (a). Number of the infiltrated immune cells (b) and the cancer cells proliferation rate (c) were
348 measured over three days.

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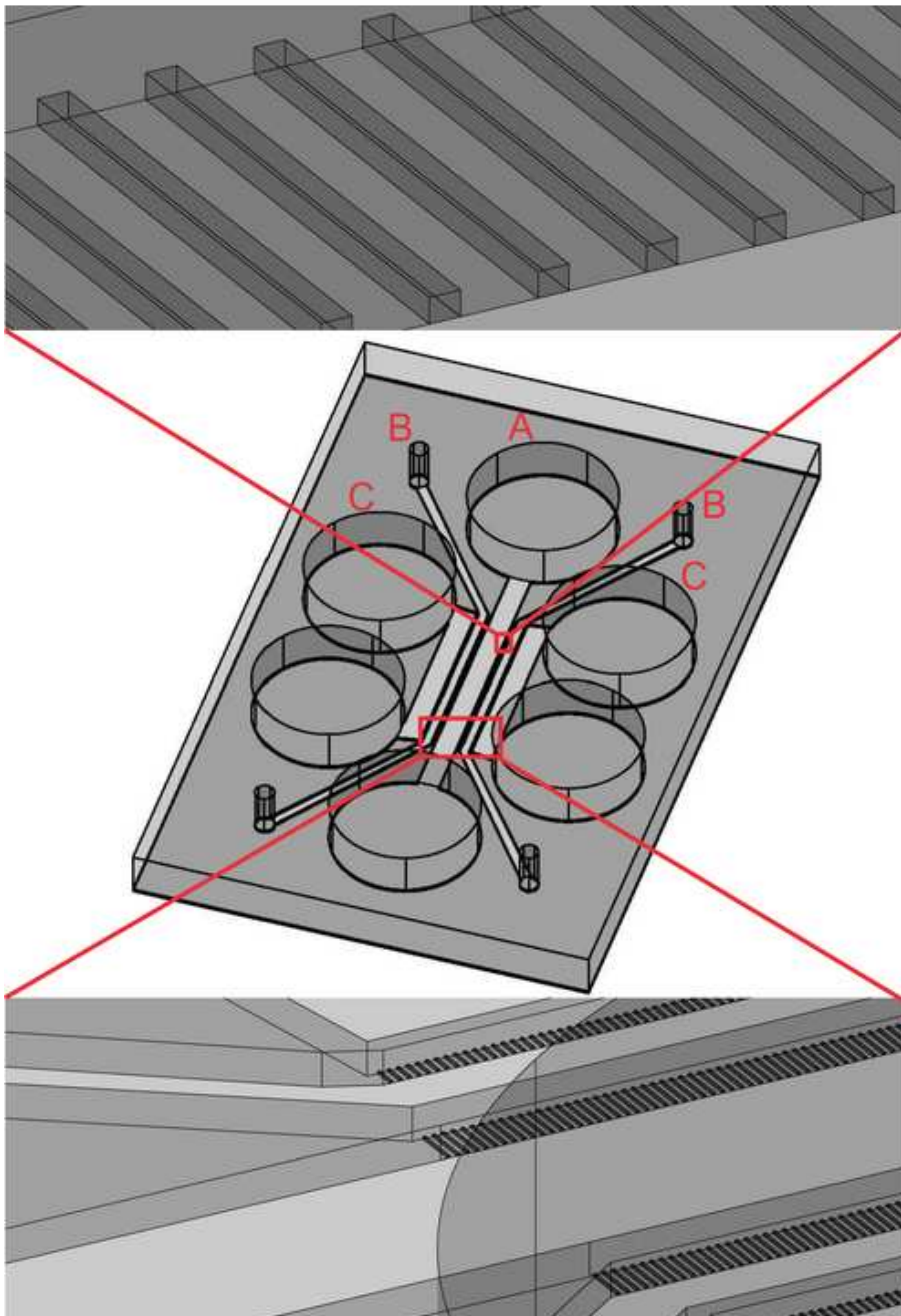

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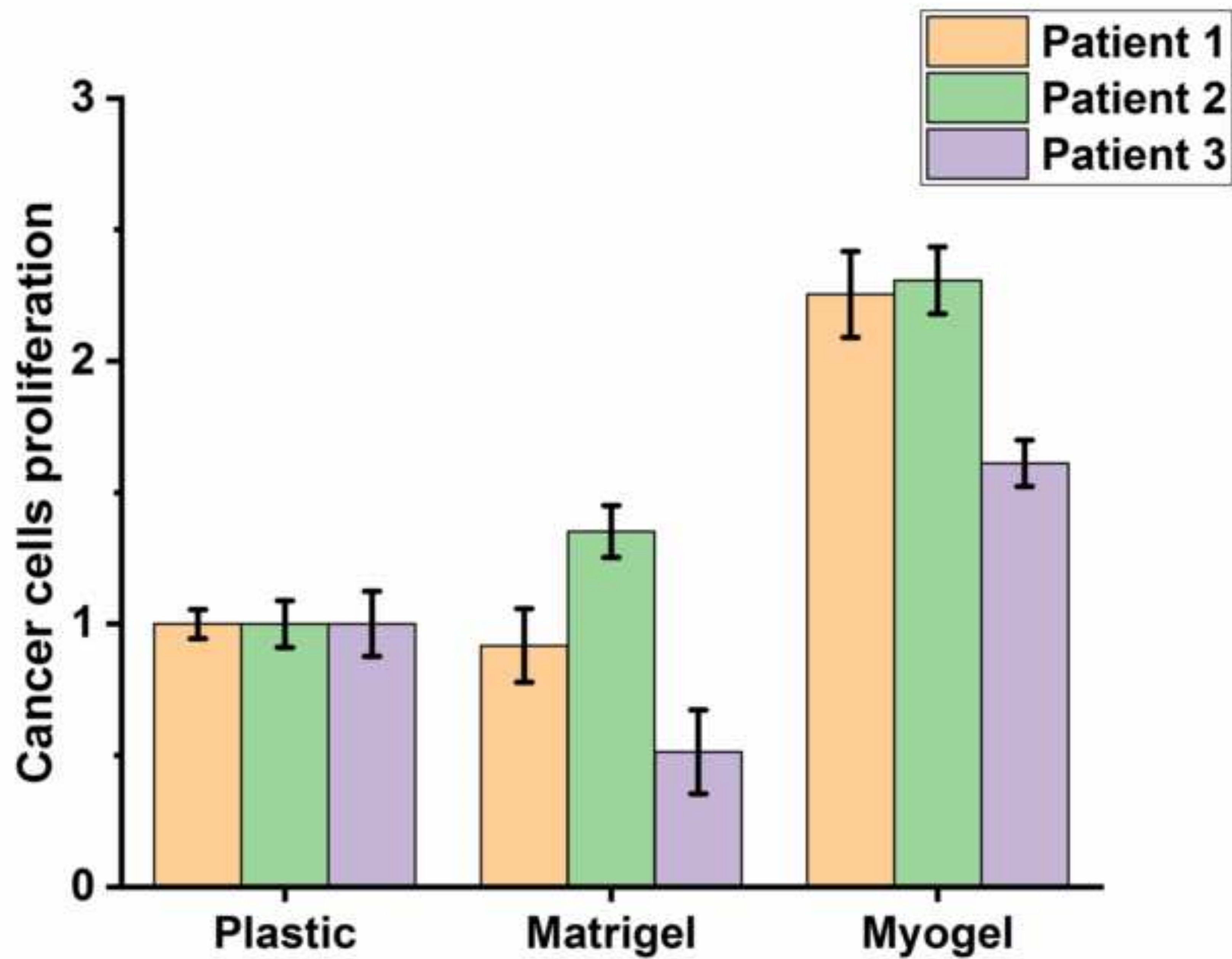

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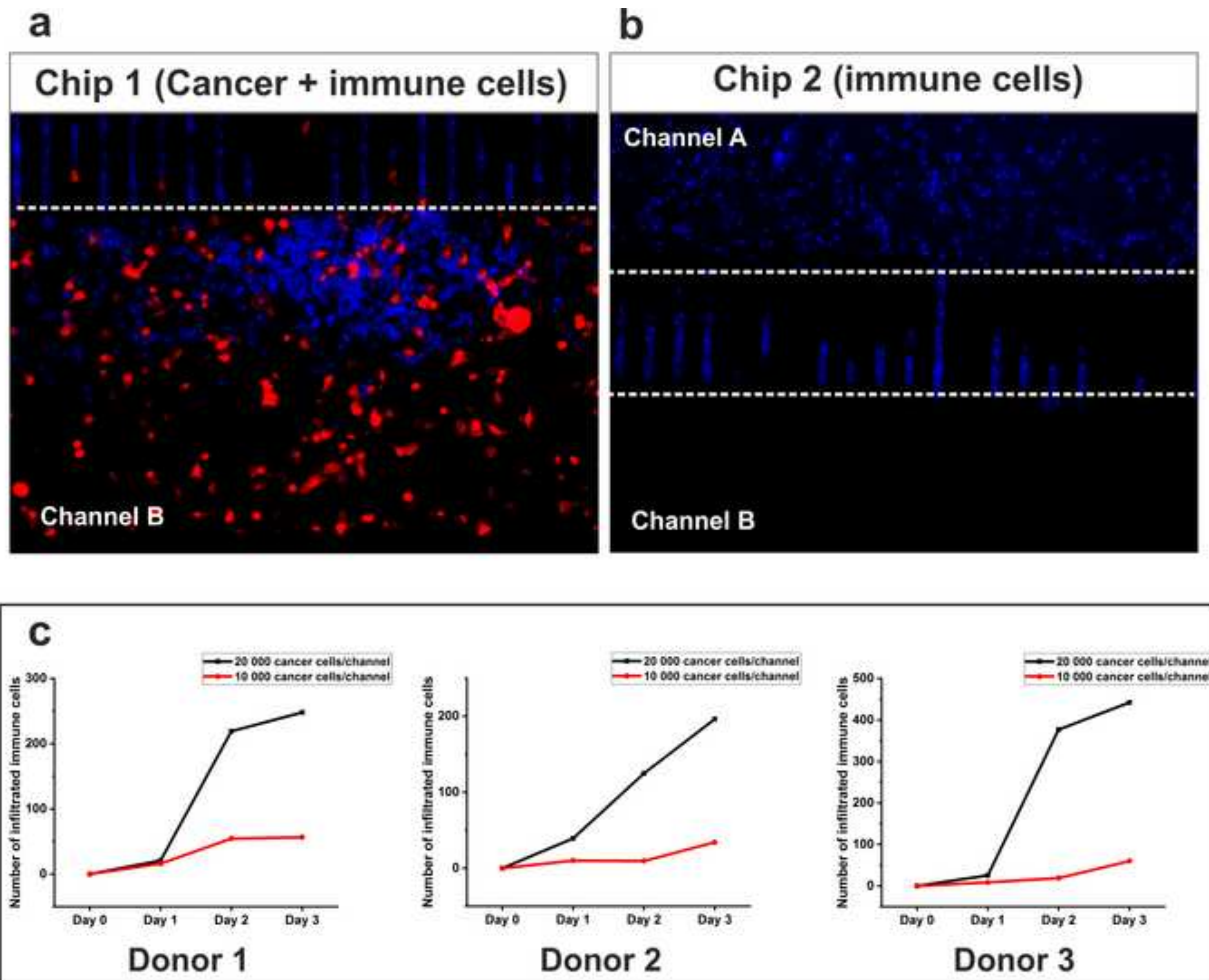

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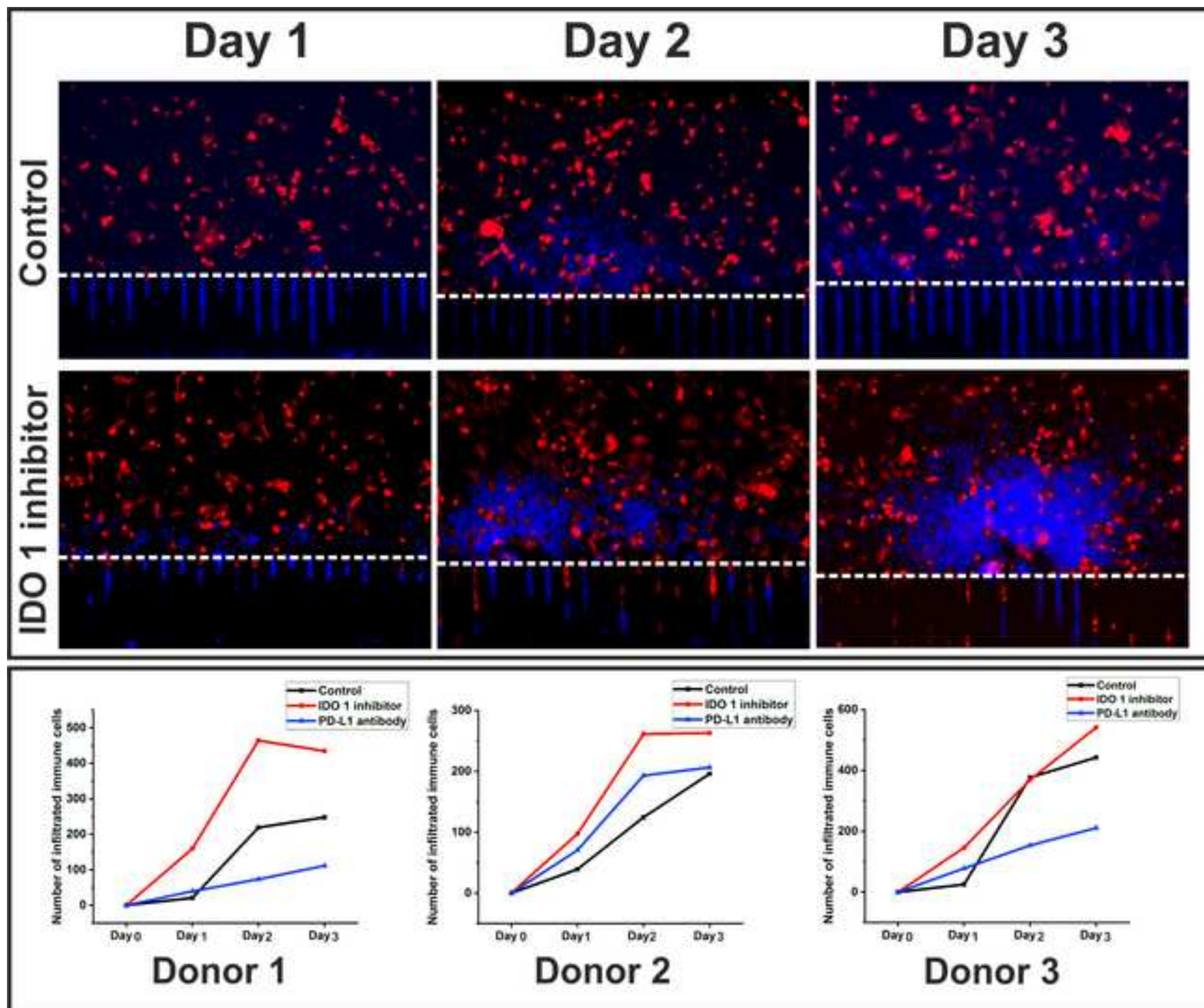

Figure

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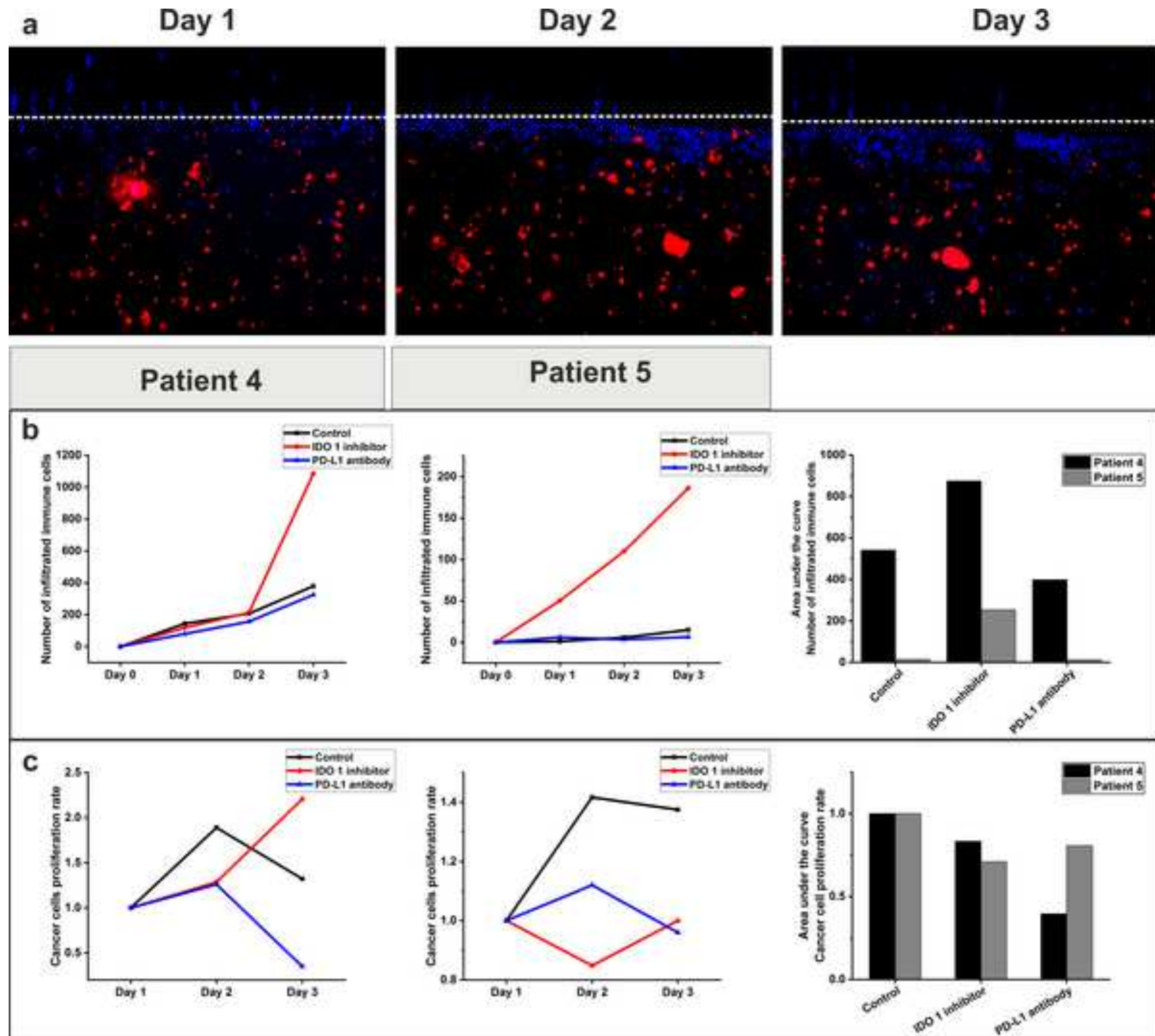

Table

Table 1: Clinical and pathological characteristics of the HNSCC patients.

Patient number	Sex [a]	Age [b]	TNM	Specimen site	Grade
1	F	73	T3N0M0	Gingiva	G3
2	F	80	T2N0M0	Buccal mucosa	G2
3	F	68	T4aN0M0	Overlapping lesion of other and unspecified parts of mouth	G1
4	M	61	T2N0M0	Mobile tongue	G3
5	M	53	T3N0M0	Mobile tongue	G2

[a] M=male , F=female, [b] Age in years

*Conflict of Interest Form

Conflict of interest disclosure statement. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.