Chapter 2

Computational systems biology approach for Permanent tumour elimination and normal tissue protection using negative biasing.

CHAPTER 2: COMPUTATIONAL SYSTEMS BIOLOGY APPROACH FOR PERMANENT TUMOUR ELIMINATION AND NORMAL TISSUE PROTECTION USING NEGATIVE BIASING

2. Outline

Complete spontaneous tumour regression (without treatment) is well documented to occur in animals and humans as epidemiological analysis show, whereby the malignancy is permanently eliminated. We have developed a novel computational systems biology model for this unique phenomenon to furnish insight into the possibility of therapeutically replicating such regression processes on tumours clinically, without toxic side effects. We have formulated oncological informatics approach using cell-kinetics coupled differential equations while protecting normal tissue. We investigated three main tumour-lysis components: (i) DNA blockade factors, (ii) Interleukin-2 (IL-2), and (iii) Cytotoxic T-cells ($CD8^+$ T). We found that permanent tumour regression can occur by: 1) Negative-Bias shift in population trajectory of tumour cells, eradicating them under first-order asymptotic kinetics, and 2) Temporal alteration in the three antitumour components (DNA replication-blockade, Antitumour T-lymphocyte, IL-2), which are respectively characterized by the following patterns: (a) Unimodal Inverted-U function, (b) Bimodal M-function, (c) Stationary-step function. These provide a time-wise orchestrated tri-phasic cytotoxic profile. Using the negative-biasing principle, we have furnished the dose-time profile of equivalent therapeutic agents (DNA-alkylator, IL-2, T-cell input) so that melanoma tumour may therapeutically undergo permanent extinction by replicating the spontaneous tumour regression dynamics.

2.1 Introduction

There are several significant drawbacks to cancer treatment by antitumour agents, as chemotherapy and immunotherapy. The first issue is an apparent "clinical cure" whereby the tumour cells are eliminated to a major extent, so that the tumour is clinically undetectable, even though microscopic amounts of cancer cells remain, which flare up much later after the initial therapy, thus producing tumour recurrence. The second is presence of cancer stem cells which, even though initially forming an miniscule cellular population, goes on multiplying as they have much less sensitivity to therapeutic agents, thus producing resistant tumour relapse [3]. Another issue is the inability of administering therapeutic agents intensively, as the latter produces appreciable normal tissue damage, producing intolerable side-effects that prevent the administration of further therapy. These disadvantages need to be well addressed, even though it is also known that occasionally there is permanent elimination of a tumour by exogenous therapeutic agents. Some examples of such exogenous regression of tumours are multimodal chemoimmunotherapy using drugs (like alkylators as dacarbazine or temozolomide), antitumour lymphocyte therapy, along with interleukin [5, 6].

Though the clinician usually encounters a malignant tumour in its progression phase, the reverse process of permanent spontaneous regression of malignant tumours is a well-documented phenomenon, occurring subclinically across human populations at 22–46% rate, as per the Scandinavian and Wisconsin Screening Registries which have tracked a population of 0.33 million and 2.95 million individuals respectively [10, 11]. It is evident in autopsy studies that about half the subjects have malignant focus in uterine cervix or prostate, with confirmation of permanent containment, and furthermore, malignant neuroblastoma fully regresses from larger-sized tumours [7, 10]. As per PubMed, there are about 14,000 titles of papers dealing with spontaneous cancer regression, covering virtually

all types of malignant diseases such as sarcomas, carcinomas, lymphomas, melanomas and so on [12]. Though the regression process eliminates malignant cells, it does not damage the normal tissue, i.e. normal cells are protected overall. Typically, the duration of a tumour's regression generally occurs across a period of months, generally 1 to 2 months. Such endogenously-initiated regression of malignancy also occurs in animals, including worms and molluscs and even took place in dinosaurs which are now extinct [13]. In fact, there are numerous species of animals known to completely regress malignant lesions, which are usually fatal in man, such as melanoma. We have earlier elucidated the energetics and biothermodynamic basis of spontaneous tumour regression [14, 15]. Indeed, the investigation of the spontaneous regression process may indicate incisive pointers on inducing the permanent regression process on human malignancies.

Spontaneous cancer regression (both endogenous or exogenous process) depends on various factors like the amount of the tumour load, the invasiveness of the disease, the intensity of the treatment, and the robustness of the patient's immune response. Mathematical modeling is seen to be a potentially vital tool for creating better treatment strategies for cancer patients to address the cancer regression process. Over the years, researchers have used various models to address the biological process of tumour growth and of anticancer treatment [16–19]. In this study we have considerably modified our previously developed model [20], in which tumour cell kinetics, chemotherapy dynamics, dynamics of immune system (NK cell, circulating lymphocyte and cytotoxic T-cell), and immunomodulation/immunotherapy dynamics, are represented by a system of six differential equations. Our novel mathematical model is incisively based on experimentally observed biological processes, namely the lethal effect on the tumour cell, as induced by the immune cells, namely cytotoxic T-cells ($CD8^+$), and natural killer (NK) cells.

In this paper, we attempt to quantitatively formulate the general methodology of complete tumour regression (whether endogenous or exogenous) and discern the unitary principles that enable this regression. Various differential equation-based quantitative models are available in the literature to replicate the dynamics of the biological process of cancer regression, and some of these models will be delineated now. For instance, Perry [14] has used the laws of mass action and first-order dynamics to characterize the reaction kinetics of tumour cell lysis during exogenous tumour regression brought on by therapeutic agents such as chemotherapy medications that cause DNA damage in the malignant lesion. As a result, the tumour cell population T declines exponentially [14]. Furthermore, when a lesion spontaneously regresses, the tumour cell population reduces exponentially with time [49, 50]. However, a residual tumour cell population asymptotically exists under the exponentially-decreasing trajectory and this population of residual cells is frequently a factor in tumour recurrence and incurability.

It is well-known that three complementary processes can reduce the tumour cell population:

(iv) Decrease of the proliferation of tumour cells: Here, chemical alkylation or chemomodulation of DNA are two methods for reducing cell proliferation that can lead to DNA damage [21, 22],

(v) Increase of tumour cell lysis: This occurs through the medium of antitumour lymphocytes [23–25],

(vi) Further enhancement of tumour cell lysis: The activation of the antitumour lymphocytes can be boosted by cytokines (for example, immunomodulation by interleukin-2) [26].

The mathematical framework of these three processes have been developed by de Pillis et al. [27], Kuznetsov et al. [28] , and Kirschner et al. [29] based on experimental

data, and the predictions of the modelling have also been empirically validated [30]. These models effectively describe the computational dynamics of antitumour activity by DNA damage and immunological action. However, in all these models, the tumour cell population follows first-order biochemical kinetics and exponential asymptotic decay of tumour population with some residual malignant cells remaining, and complete eradication of all tumour cell fails to occur, and thus future relapse of the cancer lesion happens. In this study we aim to improve the aforesaid models using our procedure. The methodology of this paper can remove the aforesaid asymptotic cancer cell population, and enable eradication of all malignant cells, with permanent elimination of the tumour without any future recurrence. Finally, the translational aspects and validated corroboration of our approach is furnished, which enables the formulation of a guided controller-based treatment planning system governing the infusion of chemotherapy, interleukin, and antitumour T-cell immunotherapy, for complete extinction of the malignant lesion, cancer stem cell elimination and normal tissue protection.

2.2 Materials and methods

2.2.1 Formulating the computational framework of spontaneous tumour regression:

During exogenous tumour regression induced by therapeutic agents such as chemotherapy drugs that induce DNA damage, the reaction kinetics of tumour cell lysis is described by laws of mass action and first-order dynamics, hence the tumour cell population M decreases exponentially [14]. Likewise, in endogenous or spontaneous regression of tissue lesion or malignancy, the tumour cell population exponentially decreases with time [49–51]. For instance, in the former experiment [14], the proliferative cell activity is estimated by metabolic phosphorylation intensity, namely by ATP dynamics. Thus, the trajectory is $\mathrm{M} = \mathrm{M}_0 exp(-\varepsilon t)$, where ε, the rate parameter, is the intensity of the

tumour regression effect (Figure 2.1(a)). Typically, a clinically just-detectable tumour has 10^7 malignant cells, and for regression in a reasonable time (say 1-2 months), this tumour cell population can decrease essentially to near zero (say 0.01 of cell population, or 10^{-4} of cell population), which can be enabled by using a suitable value of the rate parameter, induced by the regression process.

It may be noted here that there always persists asymptotically a residual tumour cell population under the trajectory and this can often be the factor of tumour recurrence. However, one can resolve the asymptotic issue and enable the tumour population trajectory to become zero at a definitive time point, using the principle of negative biasing by following a path of guided control [52, 53]. Here the tumour cell trajectory exponentially approaches a negative value M*, indicating that the tumour cell population trajectory becomes zero at time t_F (Figure 2.1(b)). This indicates that $M = [(M_0+M^*)exp(-\varepsilon t)] - M^*$, so that bias $M^* = -M_0\exp(-\varepsilon t_F)/(1-\exp(-\varepsilon t_F))$.

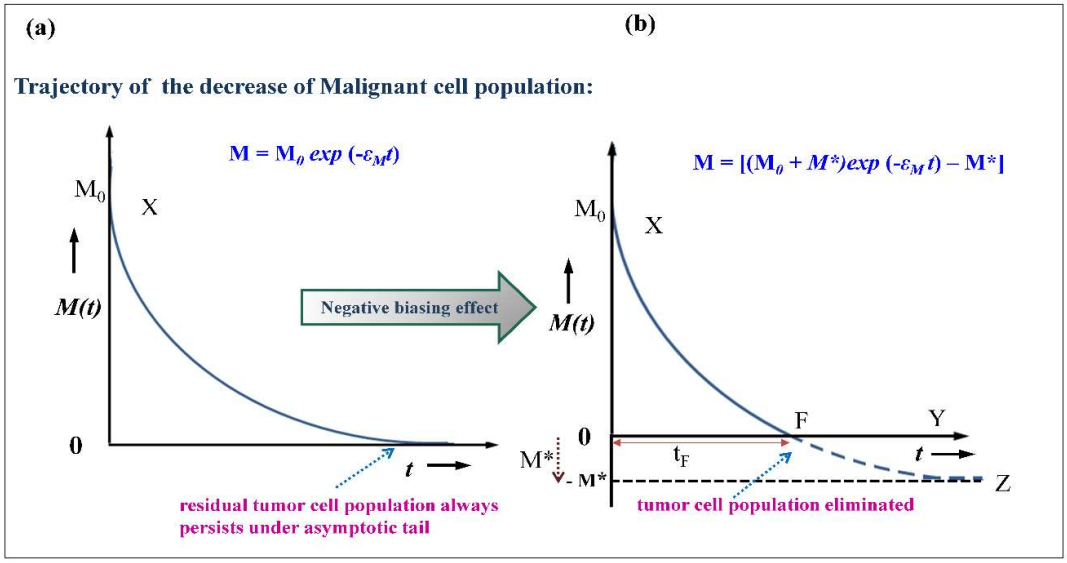

Figure 2.1 Complete tumour elimination process by principle of negative biasing. (a): In conventional therapy, the elimination of the tumour cell population M(t) follows an exponentially-decreasing trajectory, with tumour cells always persisting asymptotically under the curve, thereby leading to tumour relapse after therapy duration has ended. (b):

The Negative bias shift process enables the residual tumour cell population to become zero at a finite time t_F. This curve M(t) decreases exponentially by approaching the negative bias (−M*) value, so that, at time point F, it hits the horizontal x-axis, where tumour cell population is zero. Thus, at F the tumour cell population becomes extinct and there is no further tumour cell to replicate, i.e., complete and permanent tumour regression occurs, eliminating the malignant lesion

2.2.2 Systems analysis of tumour extinction:

Regarding exogenous regression (therapy-initiated regression) and endogenous regression (tissue-initiated regression), we have formulated a multimodal equivalence of the two regression processes from a systems biology perspective. Thereby, as Figure 2.2 elucidates, the three input terms to the tumour-host system (boxes on left side), respectively correspond to the entities of DNA damage or cell-proliferation blockage, tumour-infiltrating lymphocytes, and cytokine as interleukin. For a malignant tumour to undergo elimination, three complimentary processes for tumour cell reduction may need to occur:

(i) Decreasing the tumour cell proliferation: Proliferation of cells can be reduced by processes that enable DNA impairment, as by chemical alkylation or chemomodulation of DNA,

(ii) Increasing tumour cell lysis, for example, by antitumour lymphocyte formation,

(iii) Activation of these lymphocytes which can be enhanced by cytokines (for instance, immunomodulation by chemokines as interleukin-2).

Implementation of the aforesaid tumour regression processes can happen in two ways:

(a) Exogenous process, where the above three factors, DNA chemomodulation, antitumour lymphocyte formation and cytokine immunomodulation can be respectively induced by externally injecting alkylating chemotherapy drug, tumour infiltrating lymphocyte, and interleukin-2.

(b) Endogenous process, where the three factors are autogenously generated by the host tissue itself, such as, by DNA blockage, cytotoxic lymphocyte infiltration into the tumour, and interleukin-2 upregulation, respectively.

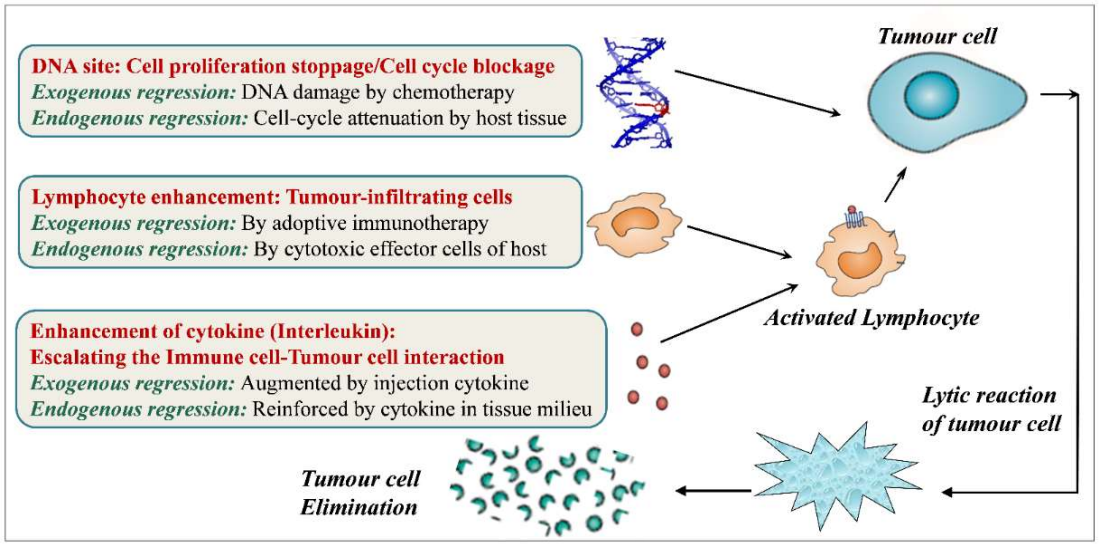

Figure 2.2 Multimodal equivalence between 1) Therapy-induced elimination of tumour, i.e., Exogenous tumour regression, and 2) Host tissue-induced elimination to tumour, i.e., Endogenous tumour regression. Endogenous regression of tumour is due to internally-generated factors, while Exogenous regression is due to externally-generated factors. Each of the three causative entities of Exogenous regression (therapy-initiated regression) and Endogenous regression (tissue-initiated regression) have similar factors, as detailed in the three boxed entities on the left side.

It should be mentioned here that the main route of DNA damage in both exogenous and endogenous tumour regression is by alkylation. For instance, in exogenous regression (e.g., by drugs), the DNA chemotherapy agents such as the most widely used pharmaceuticals as alkylators, function by alkylating the guanine moiety of DNA. Likewise, in endogenous regression (e.g., by spontaneous cancer regression), T-cell upregulation is a major factor, these cells secrete granzyme factor which activates Trex enzyme that induces DNA single-strand breaks at the 3'-portion [23]. It is known that such single-strand breaks at 3'-portion function onwards by alkylating the adjacent guanine

moiety of DNA [14]. We have worked out the quantitative equivalence between the dynamics of spontaneous tumour regression and of therapy-induced tumour regression in the following section:

2.2.3 Equivalence between endogenous and exogenous tumour regression

Regarding exogenous tumour regression by therapeutic agents, the upper bound and values of cell lysis parameters of a particular chemotherapy drug (e.g., DNA damage producing drug as dacarbazine or temozolomide) are available from the literature [14, 20], including k_M, k_A , k_K , k_B which respectively denote the DNA damage-based lysis rates of tumour cells, antitumour lymphocytes, natural-killer cells, and circulating lymphocytes. Figure 2.2 shows the correspondence between tumour cell elimination by endogenous regression and by exogenous regression, i.e., by spontaneous tumour remission and by therapy-induced tumour remission respectively.

We now proceed further to delineate the threshold values of DNA damage under endogenous as spontaneous tumour regression, taking a cue from cell lethality by DNA damage or alkylation under exogenous regression by chemotherapy alkylator drugs. For instance, it is known that the maximum physiologically tolerated limit for DNA damage in a human subject of 60 kg weight is 270 mg dose of alkylator drug temozolomide, $C_6H_6N_6O_2$ [14]. This amount corresponds to 8.35×10^{20} molecules of temozolomide, since 194 grams is its molar weight (containing Avogadro's number of temozolomide molecules). On the other hand, there are 3 billion nucleotide bases in a human cell [23]. Since such alkylating drugs do methylate the guanine base, there are 0.75 billion potential target guanine molecules in a human cell.

Furthermore, one also knows that the average human body volume is 60,000 cc., however the majority of tissues are finally differentiated non-dividing form of cells at G_0 or quiescent state of cell cycle. These quiescent tissues are the nervous system, bone,

muscle, skin and blood serum (with erythrocytes), totaling about 83% of body mass [54]. Indeed, such quiescent cells do not give scope of cell proliferation and DNA alkylation, hence only 17% of the body, i.e., 14,800 cc. of tissue is amenable to DNA interference or damage. Given the body's average cell density is 10^8 cells/cc [55], we find that there are 7.12×10^{20} guanine molecule targets potentially available for DNA alkylation damage.

This value of 7.12×10^{20} target guanine sites well corresponds to the aforesaid 8.35×10^{20} molecules of alkylator temozolomide molecules available for enabling the DNA damage events (this value of 8.35×10^{20} alkylator molecules well corresponds 7.12×10^{20} guanine sites targets, within about ±5% experimental error around mean value). Hence, it can be posited that the maximally- tolerated limit condition indicates mechanistically that the upper bound of DNA damage is 1 alkylator molecule per 1 alkylation target, i.e., 1 temozolomide methyl moiety acting univalently on 1 methylable C-6 vertex of guanine in DNA, this situation is physiologically the maximum tolerable bound in exogenous tumour regression (Figure 2.3). This same rationale will apply to other univalent alkylator chemotherapy drugs as dacarbazine.

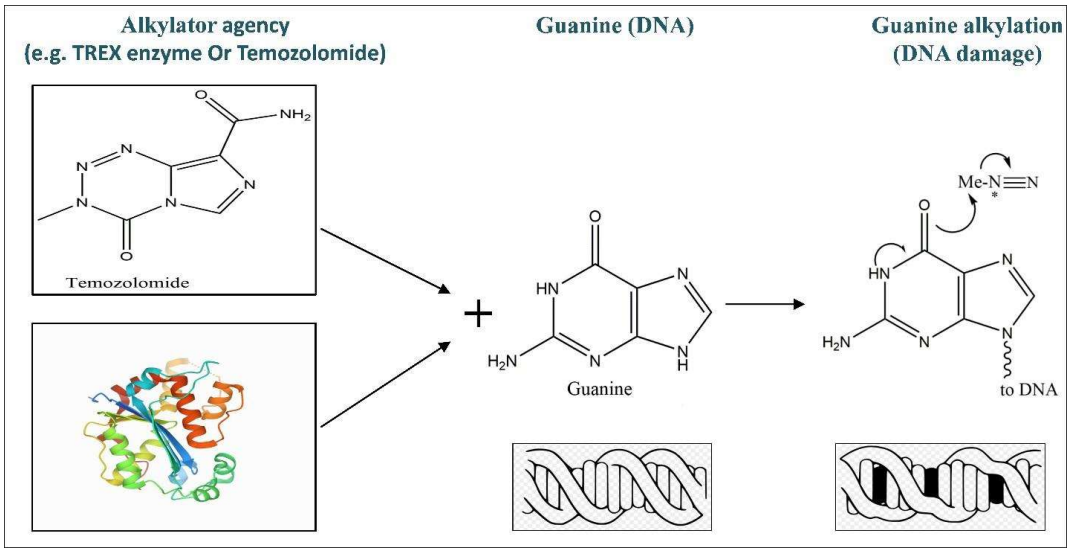

Figure 2.3 Estimation of DNA interference in endogenous spontaneous regression in terms of equivalent alkylation units. (the motifs are drawn in ChemDraw, the DNA helix is from Open Source, the Trex enzyme is from RCSB-PDB database).

We now endeavor to develop a quantitative analysis of endogenous or exogenous tumour regression. Consider the milieu of the tumour's interaction, namely the populations of malignant tumour cells, natural killer cells, and circulating white blood cells (lymphocytes in the blood), which can be denoted respectively by M, K, B. Let C, A, and D denote the intensity levels respectively of IL-2 (e.g., concentration), of Antitumour lymphocyte (e.g., T-lymphotyte infiltration population), and of DNA chemomodulation, namely, DNA interference in cells, e.g., concentration level of DNA-damaging moieties in tumour tissue (Figure 2.4), which can be gauged by the level of TNFα or by the level of DNA damage's checkpoint activation, or other procedures.

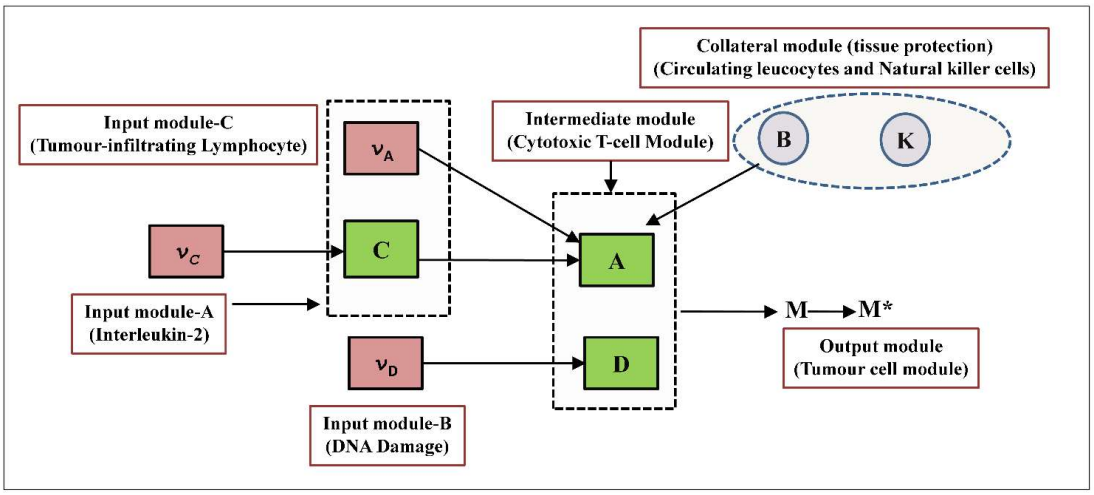

Figure 2.4 Computational systems analysis of activation of multimodal entities (Interleukin-2, Cytotoxic T-cells, and DNA damage) which enables tumour eradication, endogenously or exogenously.

2.2.4 Formulation of extinction of tumour cells

We here represent the interaction of the different cellular populations in terms of the flowsheet in Figure 2.5 The strategy is all-encompassing and adaptable to typical circumstances. In this interaction diagram, we have mainly focused on the relationships between Interleukin-2, Tumour-infiltrating lymphocytes, DNA blockage factor, cytotoxic T-cells, Circulating lymphocytes, Natural Killer cells, and tumour cells. The mathematical framework of these three processes have been developed by de Pillis et al. [27], Kuznetsov et al. [28] , and Kirschner et al. [29] based on experimental data, and the predictions of the modelling have also been empirically validated [30]. These models accurately capture the computational dynamics of DNA damage- and immune-mediated anticancer activity.

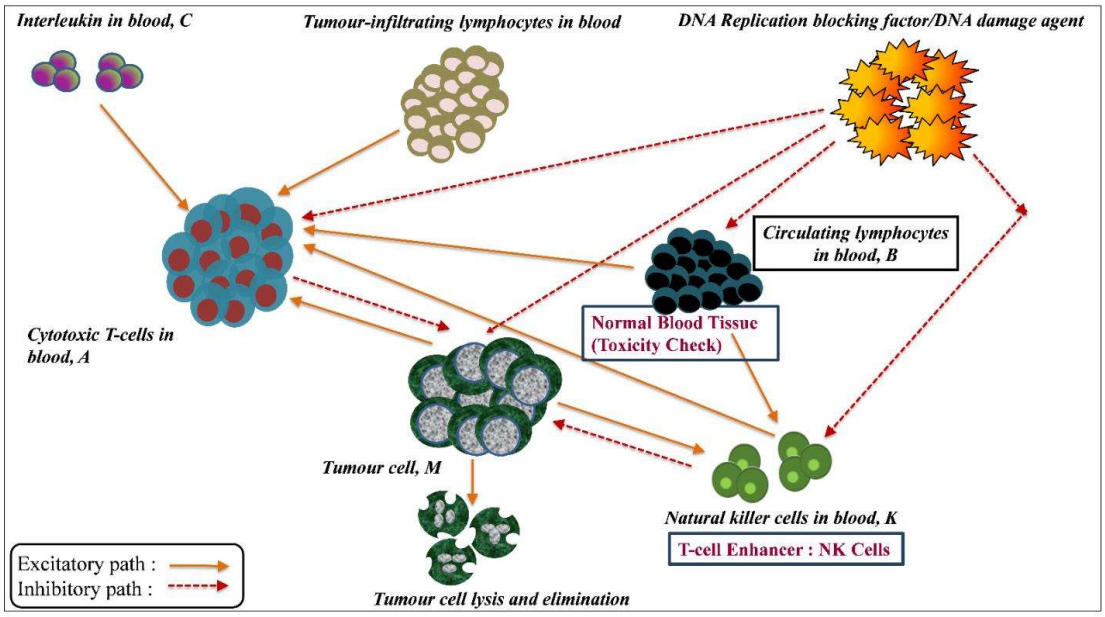

Figure 2.5 Interaction between the malignant lesion and the entities in its environment during endogenous or exogenous regression of the lesion (i.e. host-induced or therapy-induced regression).

Using primed symbols to denote temporal rates or time derivatives, we can formulate the temporal dynamical model of all cell-cell interactions, specifically the rate of change of concentration of IL-2 activation, as given in the equations below.

(i) ***Interleukin:***

$$C' = v_C(t) - \mu_C C \tag{1}$$

where $v_C(t)$ represents the interleukin-2 formation rate in the system, and the interleukin degradation or elimination being according to the rate principle, i.e., proportional to its concentration, with μ_C being the decay rate. Similarly, we have the formulation of the rate of change of level of DNA interference:

(ii) DNA damage:

$$D' = v_M(t) - \gamma D \quad (2)$$

where $v_M(t)$ is the DNA blockage factor generation rate and γ is the corresponding decay rate of the elimination or degradation of the DNA blockage factor. Likewise, we can formulate the dynamics of circulating white blood cells B:

(iii) Circulating leucocyte:

$$B' = \alpha - \beta B - k_B(1 - e^{-D})B \quad (3)$$

where the leucocytes are generated at rate α from marrow, and they age with a death rate β, while the last expression $(1 - e^{-D})$ is the saturation term denoting the fractional kill of these cells by the DNA blockage factor. After that, we formulate the natural killer cell dynamics:

(iv) Natural killer cells:

$$K' = eB - fK - pKM - k_K(1 - e^{-D})K + g\frac{M^n}{h+M^n}K \quad (4)$$

Here, the term eB represent the growth of NK cell by circulating leucocyte, fK is the NK cell death by senescence, pKM is NK cell deactivation by tumour cell debris, $k_K(1 - e^{-D})K$ terms explains the NK cell lysis associated with DNA damage factor , and the last expression $[KM^n/h + M^N]$ is a modified Michaelis-Menten type term, providing a saturation effect in cell-cell interactions by tumour cells, and g, h and n are three logistic constants ($n \approx 2$ here). Furthermore, we consider another saturation effect, the intensity Q of the interaction between tumour cells (M) and antitumour lymphocytes (A). Using another three logistic constants d, s, and l, we can elucidate Q as:

(v) Tumour cell–Cytotoxic T cell interaction:

$$Q = d\frac{\left({}^{A}/_{M}\right)^{l}}{s+\left({}^{A}/_{M}\right)^{l}} \tag{5}$$

The cytotoxic T-cell dynamics can now be delineated as:

(vi) Cytotoxic T-lymplocyte (CTL) dynamics: CD8^{+} T-cells:

$$A = -mA + j\frac{(QM)^2}{k+(QM)^2}A - qAM + (r_1K + r_2B)M - uKA^2 - k_A(1 - e^{-D})A$$

$$+ \frac{p_C\,AC}{g_C+C} + v_A(t) \tag{6}$$

where mA represents T-cell death (senescence), $j\frac{(QM)^2}{k+(QM)^2}A$ term represents the T-cell recruitment by tumor cell, qAM is T-cell deactivation by tumour cell debris, $(r_1K + r_2B)M$ term is T-cell activation by NK and circulating cells, uKA^2 is T-cell suppression by NK cells,and the last term $v_A(t)$ represents Tumour infiltrating lymphocyte formation rate. And second-last term $[(p_CAC)/(g_C + C)]$, are saturation effects in cell-cell interactions. Lastly, we formulate the tumour cell dynamics as:

(vii) Tumour cell dynamics:

$$M' = aM(1 - bM) - (cKM + QM) - k_M(1 - e^{-D})M \tag{7}$$

where $aM(1 - bM)$ represents a logistic tumour growth, here a is a growth rate and b is a decelerating rate, $(cKM + QM)$ term represents the tumour cells lysis bt NK-cells and T-cells, and the last term $k_M(1 - e^{-D})M$ represents the tumoe cell lysis by DNA dmage factor. It may be mentioned that the right-side of Eqs (1)–(7) has several cellular parameters (such as a, b…, α, β…), and their symbols, numerical values, significance and references, are given in Table 2.1.

Table 2.1 Values of the biological parameters of the tumour system

Parameter	Value of Parameter	Description of Parameter	Reference
μ_c	1×10^{1} (per day)	Rate of decay of IL-2 concentration	[29]
γ	9×10^{-1} (per day)	Temporal decay rate of DNA blockage factor	[56]
α	7.5×10^{8} (cells per day)	Birth rate of circulating lymphocytes	[27]
β	1.2×10^{-2} (per day)	Death rate of circulating lymphocytes	[27]
k_B	6×10^{-1} (per day)	Lysis of circulating lymphocytes by DNA blockage	[14]
e	2.08×10^{-3} (per day)	Fraction of circulating lymphocytes that become NK cells	[28]
f	4.12×10^{-2} (per day)	Death rate of NK cells	[28]
p	3.42×10^{-7} (per cell per day)	Inactivation of NK cells due to tumour cells	[57]
k_K	6×10^{-1} (per day)	Lysis of NK cells by DNA blockage	[14]
g	4.91×10^{-1} (per day)	Maximum rate of NK cells recruitment by ligand-transduced tumour cells.	[58]
h	2.02×10^{7} ($cell^2$)	Steepness index of NK cells recruitment by tumour cells	[28]
d	2.34 (per day)	Saturation level of fractional tumour cell kill by CD8+ T cells, priming by ligand-transduced cell.	[58]
l	2.09 (dimensionless)	CD8+ T cell-induced tumour cell lysis	[58]
s	8.39×10^{-2} (dimensionless)	Steepness coefficient (Q) of CD8+ T cell induced tumour cell lysis.	[58]
m	2.04×10^{-1} (per day)	Death rate of CD8+ T cell.	[59]
k	3.66×10^{7} (per cell per day)	Steepness index of CD8+ Tcell recruitment, primed with ligand-transduced tumour cells.	[57]
j	2.49×10^{-2} ($cell^2$)	Max. value of CD8+ Tcell recruitment, primed with ligand-transduced tumour cells.	[57]
q	1.42×10^{-6} (per cell per day)	Inactivation rate of CD8+ Tcell induced by tumour cell	[28]
r_1	1.1×10^{-7} (per cell per day)	Generation rate of CD8+ Tcell, induced by tumour cell lysis due to NK cell	[59, 60]
r_2	6.5×10^{-11} (per cell per day)	Generation rate of CD8+ Tcell, induced by interaction between tumour cell and circulating lymphocyte.	[27]
u	3×10^{-10} ($cell^2$ per day)	Regulation of CD8+ T cell by NK-cell	[27]
p_C	1.25×10^{-1} (per day)	Max. value of rate of CD8+ T cell recruitment by IL-2	[29]
g_C	2×10^{7} ($cell^2$)	Steepness index of rate of CD8+ T cell recruitment by IL-2	[29]
k_A	6×10^{-1} (per day)	Lysis of $CD8^+$ T-cells by DNA blockage	[14]
a	4.31×10^{-1} (per day)	Growth rate of tumour	[56]
b	2.17×10^{-8} (per cell)	Logistic growth of tumour (deceleration effect)	[56]
c	6.41×10^{-11} (per cell per day)	Non-ligand-transduced tumour cell lysis by NK cell.	[57]
k_M	9×10^{-1} (per day)	Lysis of tumour cells by DNA blockage	[14]

2.2.5 Protection of normal host tissue

Note that DNA damage, whether endogenous or exogenous, induces lysis of the host's cellular populations: antitumour lymphocytes, natural-killer cells, and circulating leucocytes, the lysis rate parameters being respectively k_M, k_A, k_K, k_B in Eqs (3), (4), (6) and (7). However, tumour cells are more sensitive to the DNA damage lysis than other cells ($k_M > k_A, k_K, k_B$ [27]). An important aspect here is that normal host cells should not be damaged appreciably and should be protected. This is taken care of by setting up limits that should not be crossed by the host system during the regression process since the host cell populations need to be maintained within a minimum and maximum value (upper and lower bounds). Too high a value of an entity can be toxic to the system, and the population of host cells that protect against infection (as circulating lymphocytes and natural killer cells) should be above a minimum value, whereby the normal tissue will be protected. The importance of the lysis parameters and limit thresholds for both endogenous and exogenous regression are described below and these values are displayed therein, in Table 2.2.

2.2.6 Bounds of parameters in endogenous and exogenous tumour regression

2.2.6.1 Bounds of DNA damage

In endogenous regression, the DNA impairment is occasioned by DNA interference, as alkylation, adduct-formation and intercalation. Accordingly, for endogenous or spontaneous tumour regression, one can take the parallel physiological approach, and delineate that the upper limit of DNA damage is one DNA alkylation event per one guanine base. That is, we can take DNA alkylation as an equivalent formulation for DNA damage in endogenous regression. Thereby, it can be construed that the maximum limit of DNA damage in endogenous tumour regression is equivalent to 7.12×10^{20} guanine molecule

targets. This can be expressed as alkylation activity corresponding to 270 mg amount of imidazole alkylator temozolomide equivalent units, or alternately to 1.412 mili- equivalent units (expressing the univalently-reacting temozolomide amount in mili-equivalents). For other univalent alkylator drugs, as dacarbazine, the maximum limit DNA damage will be similar, i.e., 1.412 mili-equivalent units. In parenthesis, one may mention that in cells, the biochemical interaction between targets, receptors, antibodies, immunomodulators etc, can be well described in equivalent amounts expressed as normalities or equivalent weights.

The aforesaid or correspondence enables one to quantitate the equivalence between (i) DNA damage due to endogenous spontaneous regression, i.e., by 7.12×10^{20} DNA molecular intercalation targets, and (ii) DNA damage due to exogenous drug-induced regression, i.e., by 1.412 mili-equivalent units or 270 mg alkylator. Thus, we have the following quantitative equivalence between endogenous and exogenous tumour regression, involving the same amount of damage of the malignant lesion.

Exogenous tumour regression (Therapy-induced tumour egression): Malignant lesion damage by 270 mg alkylator (chemotherapy temozolomide drug)	≡	Endogenous tumour regression (Spontaneous tumour regression): Malignant lesion damage by 7.12×10^{20} DNA molecular intercalation targets

From the above equivalence, one can obtain that 10^{10} DNA damage events produced by antitumour activity of endogenous spontaneous regression has the same number of damage events as produced by alkylation activity of 27 pico-gram temozolomide.

Hence, in our formulation, we can express the DNA damage of spontaneous tumour regression in terms of mg. of equivalent imidazole alkylator units, or in mili-equivalent alkylation units, and the latter parameter is taken as the units of parameter D in Eq (2). To

underscore, in endogenous spontaneous regression, the maximal bound of DNA damage in the adult person is thus elucidated as 1.412 mili-equivalent units of alkylation (say, temozolomide-equivalents or dacarbazine-equivalents). To put in proper perspective, units in mili-equivalents are not small amounts, for instance the total amount of cuprous and cupric ion moieties in the adult human body is 1.13 mili-equivalents [54], these ions take a crucial role in protein metabolism and enzyme reactions across the body.

2.2.6.2 Bounds of antitumour factors

We hereby work out below the values of the maximum and minimum bounds of all the other antitumour entities: interleukin-2, cytotoxic T-cells (tumour-infiltrating lymphocyte), DNA alkylation, circulating lymphocytes, and natural killer cells, respectively. The values of these entities are elucidated as follows (the values are also displayed in **Table 2.2** Limits of upper and lower boundsbelow):

Regarding Interleukin-2 in endogenous tumour regression, the upper limit of Interleukin-2 that can be physiologically tolerated, without harmful effects, is 7.2×10^4 i.u./kg body weight [14], which, in a therapy perspective, translates to 4.32×10^6 i.u. per average adult person (60 kg. weight). We take this value to likewise be the upper bound of interleukin-2 that can be physiologically tolerated or produced in the adult person during endogenous regression (spontaneous cancer regression). Furthermore, if there is no tumour, the immune reaction and its inflammatory cytokine (IL-2) concentration will be negligible, i.e. the lower bound of IL-2 can be taken to be 0.

On the other hand, with reference to cytotoxic T-cell population (CD8+), one knows that 20150 cells/mm3 is the subject's maximum tolerated CD8+ T cell population [58]. So, the total number of CD8+ T-cells in the 4.5 liters of blood (whole body) is 6.05 × 1010 cells in the adult (upper bound). Likewise, as a tumour completely regresses, one knows

that cytotoxic T-cell intensity reduces to negligible values [57]. The lower limit of these T-cells may be considered to be nil.

Now we come to the matter of cumulative bound of tumour infiltrating lymphocytes or cytotoxic T-cells. It transpires that in exogenous regression, the maximal bound of the injected tumour infiltrating lymphocyte or cytotoxic T-lymphocyte is 13.7×10^{10} cells cumulatively across the duration of immunotherapy [58]. Hence, we can here set this value to be the maximal bound of cytotoxic T-lymphocyte that can be physiologically tolerated or generated cumulatively in the human host during endogenous regression. Also, as per the discussion in the earlier paragraph, the minimum level of cytotoxic T-cells cumulatively can be delineated as zero.

Now we consider the limits of DNA alkylation. We have seen in the earlier sections that there is quantitative equivalence between DNA damage in endogenous spontaneous cancer regression and in exogenous therapy-induced cancer regression. The upper limit of DNA alkylation damage for both regression processes can be expressed with respect to activity of alkylator temozolomide, namely in terms of temozolomide alkylation equivalent units (tae units). It is known that the maximum tolerable input of temozolomide for a human subject is 4.45 mg/kg/day [14]. The minimum limit of DNA alkylation for tumour regression that can be tolerated by the human body is 0, which would occur if the tumour had not occurred, so that there was no need of endogenous nor exogenous tumour regression.

Thereafter, we consider the circulating lymphocyte bounds. Under active circulation, the typical blood volume ranges from 3.5 to 4.5 liters. Individuals may have lymphocyte concentrations as high as 100×10^9 cells per liter [61]. A subject thus can have an upper bound of 4.5×10^{11} lymphocytes, using the upper bound of the blood volume. Similarly,

the minimum lower bound tolerated may vary from 663–1160 lymphocyte per microliter for 6–12 months across a therapy for the patient [62]. To obtain the minimum bound, we take the lower value of cell count and lesser value of blood volume, so the minimum number of lymphocytes a subject can tolerate for up to six months is 2.32×10^9 cells.

Coming to the natural killer cells we consider as follows. The upper bound of these NK cell (with CD56/CD16 lymphocyte as a marker) is limited to 13% of the lymphocyte population [63]. So, the maximum value of the NK cell population in the individual is 5.85×10^{10} cells because the upper bound of the lymphocyte population in the preceding paragraph is 4.5×10^{11} cells. However, the lower limit of NK cells is zero in individuals with natural killer cell deficiency state, and one knows that an individual can tolerate this condition for 3.5 months before any significant infection can occur [64]. As mentioned earlier, **Table 2.2** summarizes the information of this section.

Table 2.2 Limits of upper and lower bounds

Parameters	Lower limit	Upper limit
Interleukin-2 input, per day (v_C)	0	7.2×10^4 I.U/kg/day
Cytotoxic T cells, total in person (*A*)	0	6.05×10^{10} cells
Tumour infiltrating lymphocytes, cumulative input over full duration (cumulative v_A)	0	13.7×10^{10} cells
DNA alkylation input (tae units), per day (v_D)	0	4.45 mg/kg/day
Circulating lymphocytes in blood, total in person (*B*)	2.32×10^{11} cells (max. duration: 6 months)	4.5×10^{11} cells
Natural killer cells, total in person (*K*)	0 (max. duration: 3.5 months)	5.85×10^{10} cells

A fundamental prerequisite is that the regression process should take place in such a way that all the tumour cells are eradicated, but there should be least damage and cellular

toxicity to the host. A measure of normal tissue damage due to the aforesaid antitumour entities (chemomodulation or immunomodulation) can be described by the standard toxicity cost functional J, wherein toxicity to the cell, a second-order mass-action effect [4], depends on second-power of the intensity-level of the antitumour entity:

$$J = \tfrac{1}{2} (r_1 U_1^2 + r_2 U_2^2 + \ldots.) \tag{8}$$

where U_1 , U_2, …. are the levels or effects of different antitumour entities, while r_1 , r_2 , …. are the weighting factors of each of the various entities. For instance, in the case of endogenous tumour regression, the effect level of immunomodulation U_1 can be taken to be the intensity of tumour-cell lysis by cytotoxic T-lymphocyte, i.e., term Q in Eq (7), while the effect level of chemomodulation U_2 can be gauged by the intensity of tumour-cells lysed by the DNA damage factor, i.e., the last term $k_M(1 - e^{-D})$ in Eq (7). We use this least damage principle to probe the conditions of tumour regression that would be optimal for the system, producing the minimal damage to normal tissue.

2.2.7 Path of complete tumour regression process

As the tumour undergoes permanent regression as per the formulation in Eq (7), we now endeavor to find out the temporally-varying activation level of the five associated factors: interleukin C, DNA damage D, circulating leucocytes B, natural-killer cells K, and antitumour lymphocytes A. In other words, we need to solve the Eqs (1)–(7) using the boundary conditions that these biological antitumour parameters (C, D, B, K and A) do not cross the physiological limits or bounds, see first paragraph of section 2.2.5 above and also the **Table 2.2**.

Through solution of the Eqs (1)–(7), we find the values of the five antitumour factors and their alteration with time, which are necessary for inducing the tumour to follow the temporal trajectory of Figure 2.1(b), so that there is elimination of full tumour cell

population at time t_F, of that figure. Solving the equations, we have following pattern of activation intensity of the factors that together produce the complete tumour regression. The description of the equations is first mentioned below, and then information is given on their derivation in Appendix-I. These equations are:

(i) Temporally-varying pattern of the activation level of DNA damage for tumour extinction:

$$D(t)^{\ddagger} = -ln\left[1-\left\{b_M g_{M_1}/r_{M_1}\mathrm{G}\right\}\right] \tag{9}$$

where $D(t)^{\ddagger}$ indicates the required DNA blockage factor concentration in blood which enables the tumour to undergo complete regression by the extinction time t_F, [the duration t_F, is shown in Figure 2.1(b)]. The term G is the combined normalized toxicity weighting factor for both DNA blockage agent and cytotoxic T cells together, namely

$$\mathrm{G} = [(g_{M1}{}^2/r_{M1}) + (g_{M2}{}^2/r_{M2})] \tag{10}$$

(ii) Temporal pattern of cytotoxic lymphocyte activation for tumour extinction:

$$A(t)^{\ddagger} = -\left[sM^{l}\left(b_M g_{M_2}/r_{M_2}\mathrm{G}\right)/\left\{d-\left(b_M g_{M_2}/r_{M_2}\mathrm{G}\right)\right\}\right]^{1/l} \tag{11}$$

where $A(t)^{\ddagger}$ signifies the desired cytotoxic T-cell population in the blood which enables the tumour to undergo complete regression by time t_F. In Eq (1), the terms s, l and d are the tumour cell lysis parameters due to action of cytotoxic T cell on the tumour (explained in Table 2.1).

(iii) Temporal pattern of interleukin-2 activation for tumour extinction:

$$C(t)^{\ddagger} = \mathrm{g_C} b_A/\left(p_C A\, r_{A1} H - b_A\right) \tag{12}$$

where $C(t)^{\ddagger}$ represents the Interleukin-2 concentration in the blood which enables the tumour to undergo complete regression by time t_F. The term H is related to the above-mentioned toxicity weighing factors r_{A1} and r_{A2}, namely

$$H = [(1/r_{A1}) + (1/r_{A2})] \quad (13)$$

The details of the aforesaid derivations have been explained in Appendix-I.

The parameters b_M , b_A , g_{M1} , g_{M2}, G and H depend on the value of the tumour characteristics (M, k_M ,k_A) and on the toxicity-minimizing indices (r_{M1}, r_{M2}, r_{A1} , r_{A2} ,) (Appendix-II); the other parameters in the equations (s, l, d, g_C, p_C) depend on the tumour growth and tumour lysis characteristics, having the values as in Table 2.1. The temporal pattern of the circulating leucocyte population B and natural-killer cell population K, can be found by respectively solving Eqs (3) and (4), where we use the values of the relevant parameters which are also available in Table 2.1. The tumour cell population can be obtained as follows. We substitute these values of *C(t), D(t),* and *A(t)* into Eqs (1), (2) and (6) and thus arrive at the values of Circulating leucocyte, NK-cells, and Cytotoxic T-cells. Then we put the latter three values in Eq (7) and thus obtain the value of the tumour cell population *M(t)*.

2.2.8 Computational modelling of Endogenous or Exogenous regression

The quantitative formulation of our simulation model of endogenous or exogenous tumour regression is explained in Figure 2.6. We have used a Matlab platform to compute the profiles of the three entities (DNA damage, cytotoxic T-cell, and Interleukin-2), using tuning parameters values (r_{M2} and r_{A2}) described in Appendix-II. [Note that DNA damage events of endogenous regression are estimated in terms of equivalent alkylation activity of an alkylator agent (units of dacarbazine equivalents), as per section 2.2.3 For the initialization of the model, we have to provide initial intensity value of all the different

cellular populations, i.e., their values at time point t_0 (at the beginning of tumour regression). After that, we need to provide all the biological parameters used in Eqs (1)–(7) [33]. Thence, we need to calculate the negative bias value (M*) and set the tuning parameters (r_{M2} and r_{A2}). The tuning parameter r_{M2} is used to calculate the control parameter v_A and v_D, and the tuning parameter r_{A2} is used to calculate v_C (Appendix-II).

Here we have divided the profile of the three entities (DNA damage, T-cell, and IL-2) in 4 loops (M - Loop, A- Loop, D - Loop and C - Loop) as elucidated in Figure 2.4. M-Loop is again divided into 3 loops M1- Loop used to calculate value of U_A, U_D, A*, and D*, M2- loop is for the calculation of U_A and A*, and M3-loop is for U_D and D* (note that here U_A and U_D indicate the anti-tumour behavior of cytotoxic T-cell and DNA damage respectively). Similarly, A-loop is again divided in 3 loops; A1- Loop is used to calculate value of U_C, v_A, and C*, A2- loop is for the calculation of U_C and C*, and A3-loop is for v_A calculation alone (here U_C denotes the anti-tumour behavior of IL-2). At last, the D-loop and C - loop is used to calculate v_D and v_C dose rates according to Figure 2.4. In other words, initially, the curve of tumour decreases and extinction is formulated by allotting a desired suitable extinction time t_F (this gives the negative bias M*, Figure 2.1(b)). Then the modelling is performed, whereby the aforesaid methodology and algorithm above will simulate the tumour regression process, so that the tumour cell population follows the extinction process of Figure 2.1(b). The moment the simulation gives the tumour cell population as a (small) negative value, the procedure will convert the negative value to zero (the tumour cells have become extinct before attaining a negative value). From this time point onwards, the simulation procedure will maintain the tumour cell population at 0 henceforth, and so there will be no tumour relapse. Thus, the simulation process will give (for each time step) the levels of DNA damage, cytotoxic T-cell, and Interleukin-2,

whereby these three entities jointly gradually eradicate the tumour cell population by time t_F.

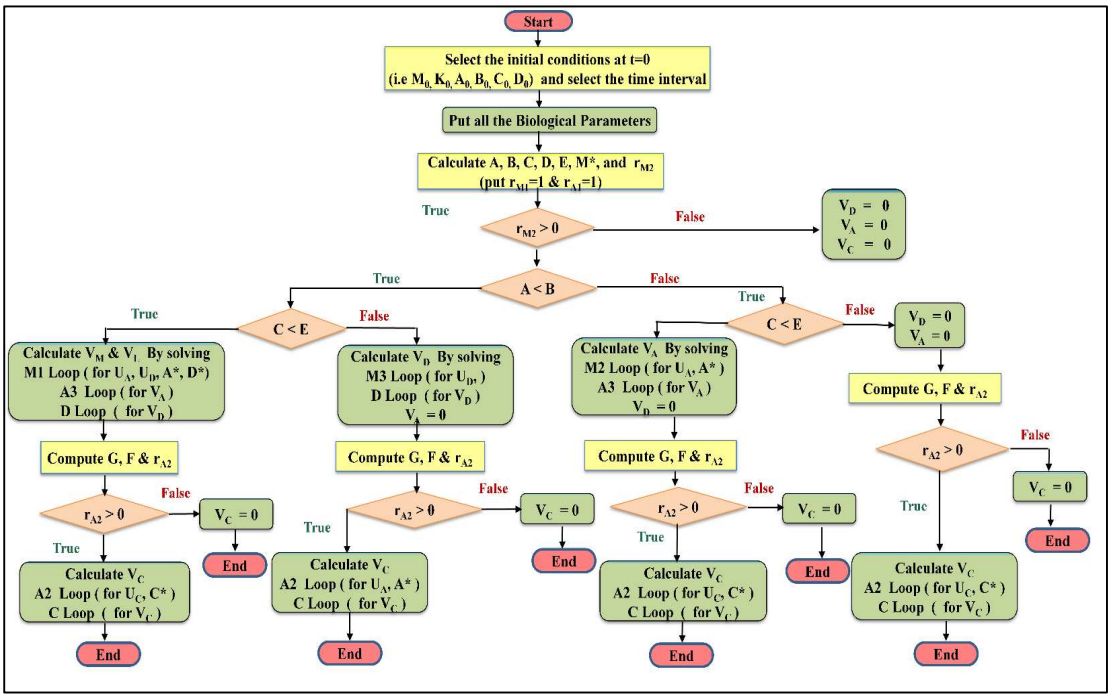

Figure 2.6 Flow chart for computational modelling of Endogenous or Exogenous regression of malignant lesion (i.e., Spontaneous tumour regression or Treatment-induced tumour regression respectively).

2.2.9 Model corroboration by collateral experimental findings: Complete elimination of malignant cell population by first-order kinetics

Now, we furnish the observations of permanent spontaneous regression of malignant fibrosarcoma tumours in mammalian systems (rodents). Here, the tumour is induced by injecting rats with a malignant cell culture of AK-5 fibrosarcoma tumour. After injection, some of the animals (36%, group-A) have massive malignant growth that rapidly turns fatal, while the other animals (64%, group-B) show decline and permanent disappearance of the tumour[65]. In the latter group of tumour-regressed animals (group-B), one observes that

by 8-10 hours of tumour cells inoculation, a high activation of infiltrating leucocyte cell occurs, of which lymphocytes form a major portion (such as T cells, natural killer cells, etc.)[65]**.** Therein, in another experiment, fibrosarcoma cells were injected at the dorsal back of wild-type rats (type-WT); the cells grew to become significant lesions of 300-800 mg by day-6. Then, leucocyte transfer from Type-B rats to these tumour-bearing wild-type host rats (type-WT) was done. Thereby, the tumour lesion in wild-type rats gradually underwent complete regression and extinction, without any sign of recurrence even at and after 300 days.

2.3 Results

We first obtained the inferences from the computational modelling analysis developed above. Then we furnished experimental findings from the preclinical studies, to validate the theoretical quantitative formulation.

2.3.1 Mathematical modelling and computational simulation for tumour regression model

We have used the MATLAB platform to numerically solve the Eqs (1)–(7), and find the values of these parameters and their alteration with time, while the tumour follows the temporal trajectory of Figure 2.1(b); the complete flow-chart is shown in Figure 2.6 and the simulation has already been described in the "Materials and methods" Section, item 2.2.8.

2.3.2 Tumour system behavior

Here we deal with an indicative case of melanoma. Melanoma is a neural crest cell tumour and shares many similarities with common neural system tumour as glioma. We can take the initial realistic situation as starting malignant cell population (T_0) of 2×10^7 cells, natural killer cell population of 10^5, cytotoxic T-cell population of 5×10^4, and

circulating lymphocytes of 10^9. These realistic values are adapted from an earlier analysis of tumour dynamics [27]. The number of tumour cells corresponds to a realistic tumour which has just been radiologically detected, say a metastatic melanoma mass in the liver (radiological detection threshold ≈ 1 cc. tumour, having 10^7 malignant cells [55]). All the other constants used in the model are given in [27], with the tumour cell growth rate, a = 0.301 per day, and the deceleration rate of logistic tumour growth $b = 1.01 \times 10^{-8}$. Here, we have considered a more aggressive melanoma tumour, so the tumour cell growth rates a can be taken to be 50% higher of the aforesaid a value (i.e., new value of a is now 0.43).

To be able to compare endogenous and exogenous regression in a common platform, we need to estimate the DNA blockage in both endogenous and exogenous tumour regression using a common unit, we have formulated this common unit as an equivalent unit of alkylation blockage activity (i.e., in terms of alkylation activity of equivalent amount of chemotherapy dacarbazine which will produce the same number of DNA blockage events either in exogenous or endogenous regression of tumour) (Section 2.2.6.1).

2.3.2.1 Tumour regression under conventional therapy (without Negative Bias): Tumour Relapse

We now formulate the case of a conventional protocol of chemotherapy and immunotherapy of melanoma, utilizing (i) Chemotherapy: Six pulses of chemotherapy, one every 10 days (dacarbazine 5 mg/kg/day pulse), (ii) Interleukin-2: Six pulses of 500,000 i.u/kg/day, from day-8 to day-11. (iii) Cytotoxic T-cells: Total 10^9 cells during an infusion at day-7 through day-8. We have applied these values to the initial Eqs (1)–(7) which are solved to obtain tumour cell population M as time elapses. Figure 2.7 shows that the tumour cell population initially decreases to about 100,000 malignant cells, but later manifests as

cancer relapse and increases to high values ($\approx 10^9$ malignant cells) that corresponds to a large tumour of diameter ≈6 cm., such a tumour will have penetrated the blood vessels, producing wide dissemination and lethality.

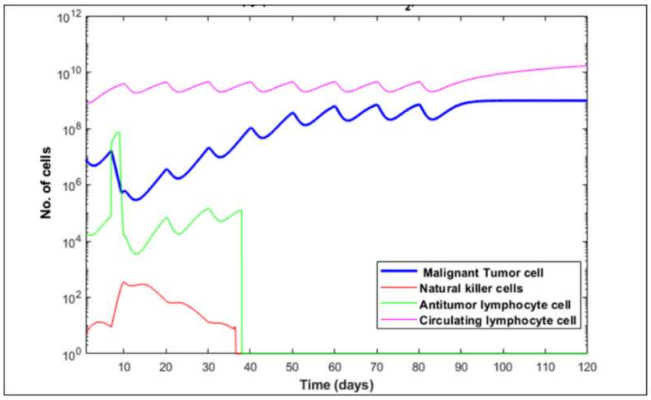

Figure 2.7 Conventional treatment protocol using chemotherapy and immunotherapy. This uses DNA blocking alkylator drug dacarbazine, and immunotherapy (Cytotoxic T-lymphocyte and Interleukin-2). The protocol fails to eliminate malignant melanoma tumour cells, and after treatment duration there is relapse of the malignancy.

2.3.2.2 Tumour regression by negative bias formulation: Permanent tumour elimination

Here we have used the different sets of Eqs (9), (11) and (12), which give the levels of the three entities (DNA damage, cytotoxic T cells, interleukin-2) that are required to enforce the cell population of the melanoma tumour to follow the exponentially declining trajectory to zero cell at a specific desired duration (46 days). We now simulated the equations at a time-step of a 0.01 second, and obtain the values of the levels of DNA damage, Interleukin-2, and Cytotoxic T-cells, which would enable the tumour population to follow the targeted diminishing curve trajectory of Figure 2.1(b), aimed at tumour extinction in 46 days. These levels of the above three entities are then used to obtain the

tumour cell population (using the other set of Eqs (1)–(7)). Therein, the populations of the circulating leucocytes and natural killer cells are also calculated. Note that the actual tumour cell population obtained (Figure 2.8 a)) follows the principle of desired exponentially decreasing trajectory that we planned, whereby all tumour cells undergo eradication. Note that none of the system parameters (DNA damage level, circulating lymphocytes, NK cell, cytotoxic T-cells) crosses the respective upper and lower physiological bounds or thresholds of Table 2.2 thereby ensuring normal tissue protection. Here, we found the negative bias to be $M^* = 1.8986 \times 10^5$ cells. Since the total number of initial tumour cells $M_0 = 2 \times 10^7$ cells, we observed that the relative value of M^* is small, at about 1% of the initial tumour load.

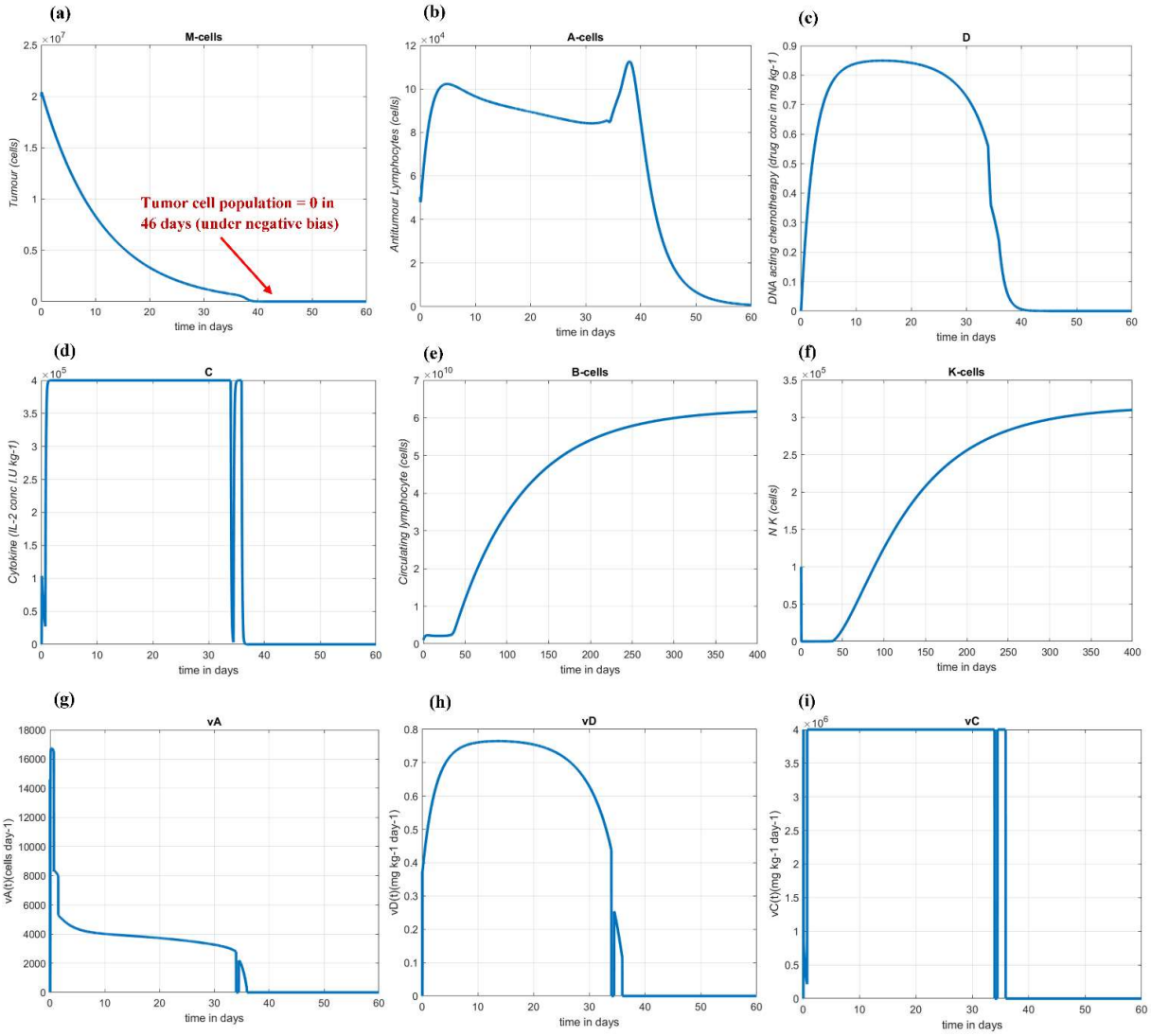

Figure 2.8 Complete elimination of melanoma tumour under negative bias. (a) Consistent decline of tumour cell population with time: Complete elimination of tumour at 46 days by following negative biasing behavior of fig. 1(b) scheme. (b) Bimodal temporal profile of cytotoxic T-cell required for eliminating Tumour cells. (c) Unimodal profile of level of DNA damage required for eliminating Tumour cells (DNA damage is estimated in terms of equivalent amount of alkylator substance dacarbazine that produces similar amount of DNA damage, see text). (d) Concentration profile of Interleukin-2 required for tumour elimination (the curve displays a stationary level). (e) Temporal profile of circulating lymphocyte level required for tumour elimination (the curve levels off at a saturating value). (f) Temporal profile of natural killer cell level required for tumour elimination (the curve levels off at a saturating value). (g) Interleukin-2 input rate that would enable tumour elimination. (h) DNA blockade input rate that would enable tumour

2.3.2.3 Behaviour of cancer stem cells, natural killer cells and circulating lymphocytes

From Figure 2.8(e,f), we see that the population of circulating lymphocytes and natural killer cells in the body attain the values respectively of 6×10^{10} cells and 3×10^{5} cells, which are very much below their corresponding upper bounds, and are only about 10 and 0.001% of the respective upper bounds in Table 2.2. Furthermore, it is known that cancer stem cells become much resistant to anti-tumour drugs as they have drug efflux channels which ejects out the drugs from the tumour cells. For instance, the sensitivity of the cancer stem cells to a drug can become 8–23% of the sensitivity of general cancer cells to that drug [66], this drug sensitivity of tumour cell is the parameter D in Eq (2). Hence, to formulate cancer stem cells in the model, we decrease the drug sensitivity D to 1% of the value that we have used in the earlier paragraph, this very low value of D has been put as a precautionary measure. We performed the simulation of the earlier paragraph again, and we observed that there is also complete tumour eradication (Figure 2.9), though a somewhat longer therapy duration is needed (59 days). Thus, our negative bias procedure can successfully eliminate the cancer stem cell base in the tumour, though the time taken is a little longer.

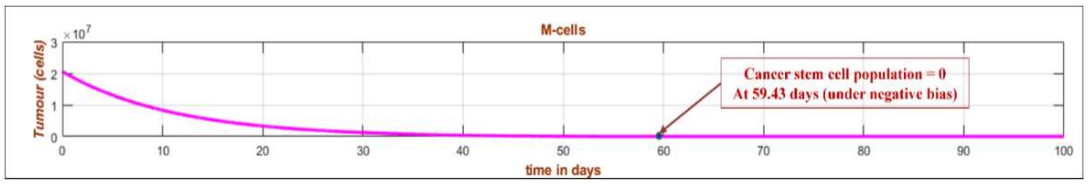

Figure 2.9 Complete elimination of cancer stem cells. The tumour will need more time for the cancer stem cells to be extinct (in 59 days) with chemotherapy sensitivity at 1% of usual cancer cells.

2.3.2.4 Adaptability and robustness of tumour elimination process

In real-life conditions, different patients can have different initial conditions and constitutions. Hence, different patient-specific initial situations are now considered, for example, we examined the different initial condition of immunological system (like populations of the different effector cells) and carried out 500 arbitrarily simulations for complete tumour elimination for melanoma tumour with different biological parameters or characteristics (Figure 2.10).

We found that tumour extinction occurred in 100% cases if the coefficient of variation in effector cell population was 0%, while extinction happened in 98% of the cases if the coefficient of variation was increased to 10%. It has been known that the physiological parameters are generally kept constant homeostatically by organisms, with a 10% variation around the mean level [67]. Thus, within the considered range of physiological variation, it transpires that our proposed approach may be able to induce tumour elimination in the majority of the cases (98% of cases).

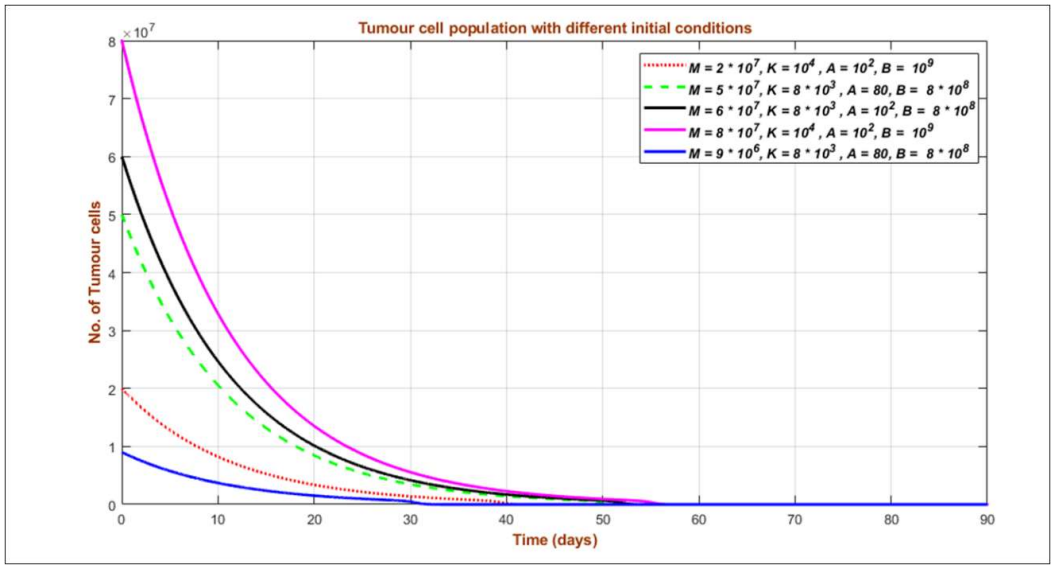

Figure 2.10 Decrease of tumour cells population with different initial conditions. Extinction of the tumour occurs regardless of the initial conditions.

2.3.2.5 General characteristics of tumour regression process

We have simulated the permanent tumour regression process using different values of the tumour parameters, and we have always observed the similar type of patterns as in Figure 2.8. This general pattern is exhibited in Figure 2.11, whereby we note that for inducing permanent tumour regression, the three antitumour entities should have three distinct temporal profiles (tri-phasic activation):

1) Bimodal intensity for lymphocyte activation, showing two temporal peaks (Figure 2.11(a)),

2) Unimodal intensity for activation of DNA damage (such as strand blockade or alkylation), displaying one peak temporally (Figure 2.11(b)).

3) Stationary intensity for cytokine activation (interleukin-2) exhibiting uniform level (Figure 2.11(c)).

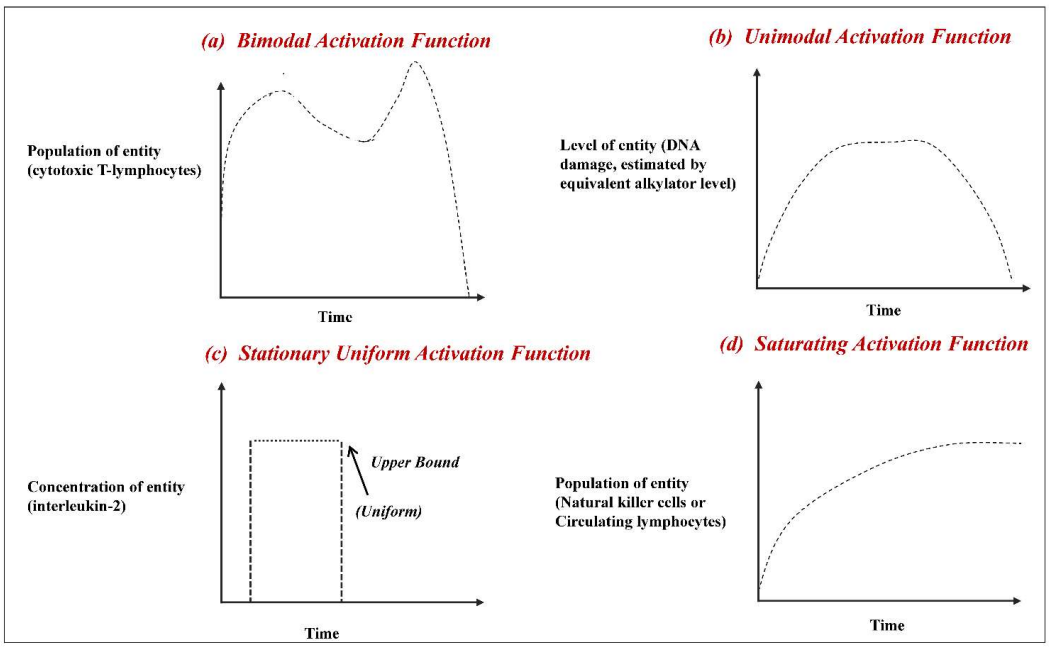

Figure 2.11 For complete regression of tumours with different initial conditions, the time-wise alteration of the tumour-affecting entities involved does follow the common pattern template: (a) Bimodal intensity of Cytotoxic T-cell, (b) Unimodal intensity of DNA blockade factor, (c) Uniform stationary intensity of Interleukin-2, (d) Saturating intensity of Natural killer cells and Circulating lymphocytes.

2.3.2.6 Basis of the tri-phasic activation

The three aforesaid activational phases can be accounted for from a dynamic perspective of Figure 2.12. Initially, in the tumour cell surroundings, there occurs tumour cell antigen binding to T-lymphocyte receptor, stimulating the secretion of IL-2 (event-1). Thereby, T-cell is activated to cytotoxic T-cell (event-2). This T-lymphocyte then counters a tumour cell, secretes granzyme protease, which binds to tumour cell DNA, cleaving the nucleic acid binding protein, blockading the DNA and damaging tumour cell DNA replication (event-3). Note that in event-1, since there are no second-order deceleration terms nor Michelis-Menten terms in Eq (1), the interleukin level can rise rapidly or steeply,

nevertheless the increase of interleukin halts or saturates as soon as the interleukin toxicity level or upper bound is reached.

The aforesaid three sequences of events are clearly reflected in the three temporal profiles above. The first event corresponds to Interleukin-2, the earliest agent to increase (Figure 2.11(c)). Then, the second event correlates with cytotoxic T-cell increase, but sometime later (Figure 2.11(a)). Thereafter, the third event relates to the increase of tumour cell's DNA damage intensity, which happens still later Figure 2.11(b). Here, tumour cells are increasingly lyzed, and their debris thus produced deactivates the cytotoxic T-cells whose activated population hence decreases, this corresponds to the negative term "-*qAM*" in Eq (6). As tumour cell population continues to decrease, its debris production falls, so that the negative term becomes smaller, whereby cytotoxic T lymphocyte population increases again (second temporal peak) (Figure 2.11(a)).

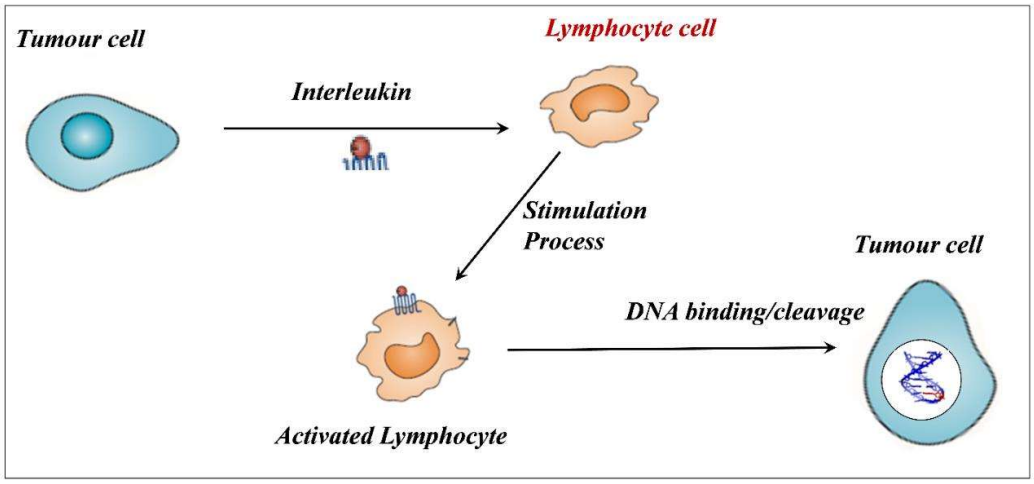

Figure 2.12 Basis of the tri-phasic activation process in tumour regression as illustrated by the three temporal transitions.

We may recollect that the step-like shape and stationarity in the interleukin-2 concentration is due to its level being initiated rapidly at the requisite intensity (Figure

2.11(c)). Such a brisk interleukin response has also been independently observed in the immunological system, where requisite stimulation can enable interleukin-2 receptor activation to reach substantial intensity within 2-4 days [68]. The result is that a stationary level of interleukin activation is rapidly attained, and tumour regression occurs. Indeed, it is well known from clinical experience [26], that interleukin-2 administered at significantly augmented dose, induces long-lasting immunomodulation to act against those residual malignant cells that bypass usual therapeutic intervention.

2.3.3 Model corroboration by collateral experimental findings: Complete tumour eradication by First-order kinetics

We now furnish experimental preclinical findings of complete permanent tumour regression that provide empirical corroboration of two kinetic conditions for regression: first-order kinetics and negative bias. We show that the experimental findings of permanent tumour regression follow the tumour extinction dynamics of Figure 2.1b, that is:

$$\mathrm{M} = (\mathrm{M}_0 + M^*)exp(-\varepsilon t) - \mathrm{M}^*$$

where the negative bias is $\mathrm{M}^* = [\mathrm{M}_0 \exp(-\varepsilon t_F)]/(1 - \exp(-\varepsilon t_F))$

We show in Figure 2.13(a) the data points that represent the average value of 10 such regressing experimental cases, the findings being obtained from the above-cited investigation [65], the first day of the time axis in Figure 2.13 (a) starts from day-6 of tumour implantation, when the tumour actually starts to regress and decline. Actually, the microscopic appearance of the regressing tumour shows increasing decline and sparseness of tumour cells, together with the formation of fibrotic scar healing due to generation of fibroblasts, leaving the necrotic core of tumour to atrophy and degenerate. We will now infer

the values of the bias shift M* and the first-order exponentially decrementing trajectory of tumour cell population, by utilizing the experimental findings. As mentioned, the baseline tumour lesions induced by the injection in the control animals are 300-800 mg (average 550 mg, i.e., 0.55 g, or 0.55 cc. of tissue). Details of the tumour growth and regression information are available [65]. After tumour implantation of the control animals, the immunomodulation by leucocyte transfer from group B rats to a subset of the control animals were done on day 4 post-implantation. At day-6 post-implantation, the tumour size in the latter subset of animals peaked to 174% of the average baseline of 550 mg. tumour tissue (i.e., peaked to 957 mg.), and then the tumour gradually underwent complete permanent regression. Since 1 cc. or 1 g. of tumour tissue is taken to have 10^7 malignant cells[55], their initial population in this lesion can be estimated as 9.5935×10^6 malignant cells (this is thus the estimated value of M_0).

The complete elimination of the tumour occurs at 21 days after leucocyte immunomodulation, i.e. at day-25 post-implantation [this corresponds to point P of tumour extinction in Figure 2.13 (a)]. Thus, the actual tumour elimination time duration t_F is the difference between the day-6 and day-25, i.e., t_F = 19 days. The experimental data points of Figure 2.13 (a) are shown in Table 2.3 (first and second columns). We now analyse the experimental data-points with respect to the two situations of Figure 2.13 (a), that is (i) plain exponential decline case without negative bias (Figure 1a), vis-a-vis (ii) exponential decline with negative bias (Figure 2.1b), for both conditions the exponential decline occurs for 19 days.

Table 2.3 Tumour cell population decline with time during the spontaneous regression process (Experimental findings compared with the two theoretical models of Figure 2.1a and 2.2b).

Time duration since start of tumour regression process (Days)	**Tumour load: Experimental data (No. of tumour cells)**	**Tumour Load: First-order exponential model (No. of tumour cells)**	**Tumour Load: First-order exponential model with small Negative Bias (No. of tumour cells)**
0.0372/0	9,477,500	9,531,693	9,363,552
0.8748/1	8,663,800	7,815,517	7,763,503
2.1274/2	4,564,200	5,808,051	5,899,998
5.1057/5	2,185,100	2,867,349	3,025,948
6.0702/6	2,037,900	2,281,428	2,420,882
7.0768/7	2,890,300	1,797,213	1,907,887
8.0329/8	2,293,600	1,432,816	1,511,836
9.099/9	1,417,900	1,112,907	1,154,914
10.1058/10	945,200	876,660	884,118
11.0196/11	658,400	705,953	683,482
11.9925/12	379,400	560,580	508,389
13.0416/13	193,400	437,176	355,808
14.023/14	38,400	346,452	240,626
19.2087/19	0 (tumour fully eliminated)	101,365	0 (tumour fully eliminated)
30	0 (tumour fully eliminated)	≈28,600,000 [tumour increase and relapse]	0 (tumour fully eliminated)

Note: Regarding the tumour elimination process, observe the close correspondence between column 2 (experimental data) and column 4 (theoretical computational model with first order kinetics and mild negative bias). The goodness-of-fit criterion (statistical χ^2 test) is satisfied between these two columns.

2.3.3.1 Exponential decline without negative bias:

Using the experimental data-points of Figure 2.13 (a), we obtained the trajectory equation [$M=M_0\exp(-\varepsilon t)$] using the least squares method, and the trajectory is found to be:

$$M \textit{ (in million cells)} = [9.59\times10^6] \; \textit{exp}\,(-0.2367\, t) \qquad (14)$$

where time t is in days. At point P (t = 19 days) (Figure 2.13 (a)), equation 14 gives 101,365 malignant cells remaining, which is 0.94% of the initial tumour cell population, implying that the vast majority (over 99%) of tumour cells have been eliminated. Nevertheless, since the 19-day duration of the tumour regression process is over, this modest amount of residual malignant cells (101,365 cells) will start to increase again at the aforesaid tumour cell growth rate a = +0.301/day (Figure 2.13 (b)). Thus, from day 19 onwards, the tumour will grow as per the following equation, where τ denotes the number of days after the 19th day, so that there is malignant recurrence:

$$\textit{Tumour relapse:} \qquad M = [0.1014\times10^6] \; \textit{exp}\,(-0.301\,\tau) \qquad (15)$$

In a short time of a month (30th day, Figure 2.13 (b)), the tumour will have

M = 28.6 million malignant cells.

Table 2.3 (third column) shows the values of the tumour cell population as per the theoretical graph of Figure 2.13 (b). We note the increasing divergence between the experimental data column (column 2) and the theoretical exponential model column (column 3) as time progresses;

indeed, column 2 shows tumour extinction, while column 3 shows tumour increase and recurrence.

2.3.3.2 Exponential decline with negative bias:

Here we used the data-points of Figure (a), and obtained the negative bias trajectory decline [$M = (M_0 + M^*)exp(-\varepsilon t) - M^*$] by method of least squares, arriving at the equation:

$$M\ (in\ million\ cells) = [(9.39 + 0.262\ )e^{-0.21}]\ -\ 0.262 \qquad (16)$$

which gives the bias value M* = 262,000 cells. Equation 16 is represented as the graph in Figure 2.13 (c), showing that the curve definitively meets the time-axis at 19 days, showing that the tumour cell population becomes nil. As all tumour cells have become extinct, the tumour cell population remains zero for the rest of the time, whether 30 days or 300 days. Column 4 of Table 2.3 gives the values of the tumour cell population according to the graph of Figure 2.13 (c). We observe the close agreement between the experimental data (column-2) and our theoretical negative bias model (column-4), especially notable is the concurrence in the later time points when the tumour undergoes complete extinction. Indeed, we find is a strong goodness-of-fit characteristic between columns 2 and 4, with the statistical χ^2 test being well satisfied.

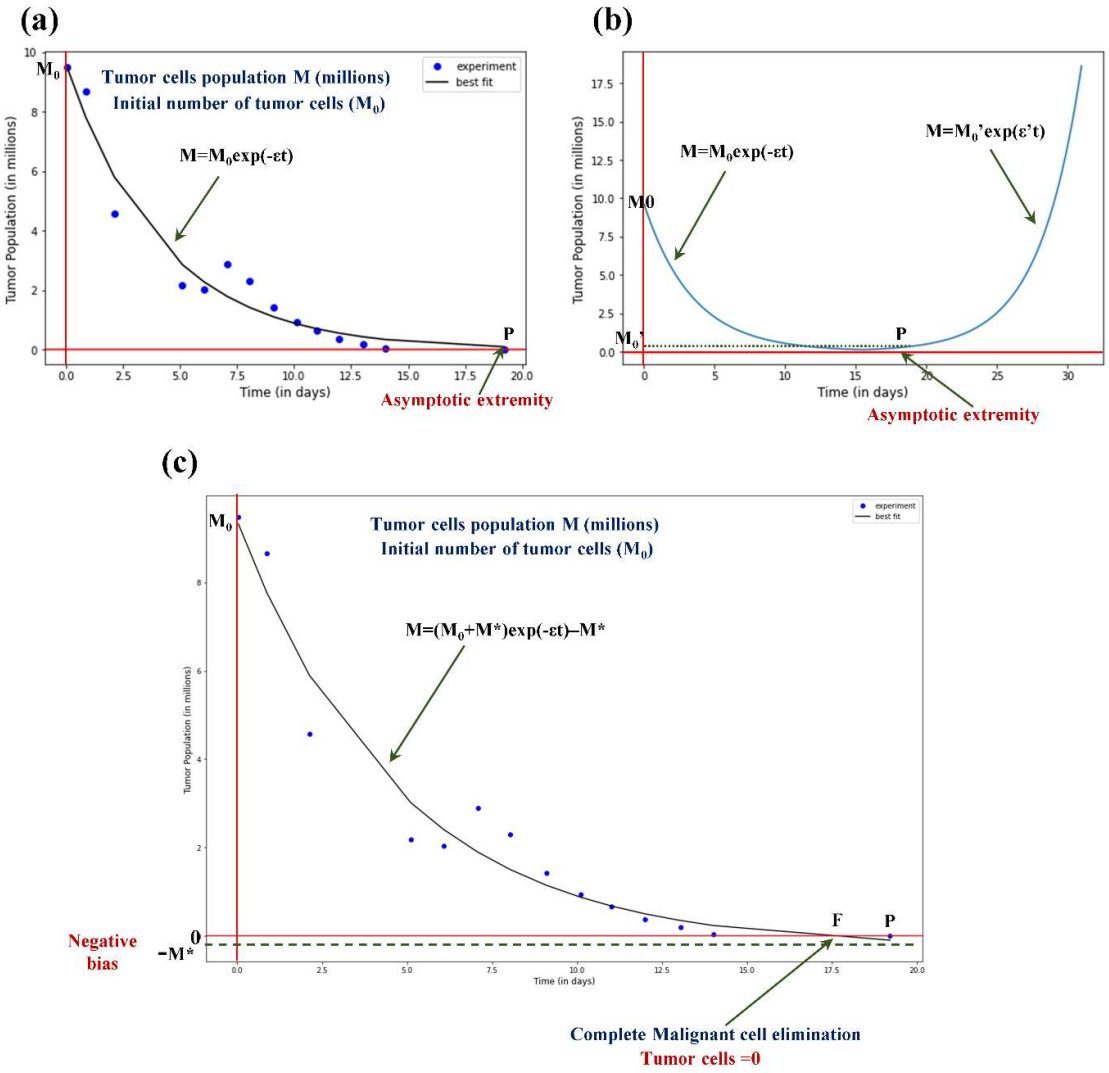

Figure 2.13 Model corroboration by collateral experimental findings: Feasibility of complete elimination of malignant cell population by means of first-order kinetics and mild negative bias.

(a) The data points are experimental values of the tumour cells population as mammalian malignant fibrosarcoma tumour undergoes permanent spontaneous regression, and tumour extinction occurs at day 19 in the rodent study. The first-order kinetics-based declining curve that fits the data is shown; note that there are appreciable tumour cells under the asymptotic tail of the graph, and this curve cannot account for zero tumour cell population at day 19.

(b) Tumour recurrence after a month due to replication of the small residual cell population under the asymptotic tail of the mathematical exponential curve at day 19.

(c) Tumour cell population extinction on 19th day accounted for by a first-order kinetic-based declining curve with a small negative bias. This new curve fits the experimental data, and definitively meets the time-axis (red horizontal line) around 18 days, whereby the tumour cell population becomes zero, this population becomes extinct as there are no tumour cells to replicate later. Here, the theoretically formulated mathematical curve is well validated by the experimental data points (blue circles) [goodness-of-fit criterion is satisfied using statistical χ^2 test].

2.4 Discussion

We have developed a quantitative mechanistic formulation and analysis of the permanent spontaneous regression of the malignant tumour, with experimental validation and its clinically-relevant implications. This extinction of tumour cells is possible due to a negative biasing process. We have delineated the temporal profile of the causative factors that enable the complete extinction of the tumour, namely:

(i) the three separate activation characteristics (unimodal, bimodal and uniform stationary activation function) respectively of DNA damage or mitosis blockage, cytotoxic lymphocyte and cytokine IL-2

(ii) the two separate saturation activation profiles of both the natural killer cells and circulating lymphocytes.

This multiphasic temporal orchestration of the antitumour entities minimizes toxicity to normal tissue, without damaging the usual host tissue. We now elaborate the clinically-pertinent implications of the research.

2.4.1 Normal tissue protection

An important aspect of the methodology developed is survival and safeguarding of normal tissue, while at the same time the tumour lesion is being eliminated. Taking care of these two contradictory objectives, our procedure performs the following two contrasting functions:

(i) Minimization of toxicity to the normal tissue due to the antitumour entities: This is done by developing a toxicity cost parameter which is kept minimal using standard Lagrangian method.

(ii) Keeping the concentration of antitumour entities within tolerated ranges: This is done by using physiological limits to the level of DNA damage, cytotoxic T-cells, IL-2, circulating lymphocytes and NK cells.

A major handicap in oncology is that often treatment (chemotherapy and radiotherapy) is started vigorously [69, 70], but soon the intervention has to be stopped or downscaled due to the toxicity to normal tissue. Thus, in the latter stage, the tumour proliferates and spreads out through vascular metastasis. In the clinical scenario, an important aspect is that the therapeutic agents may cause considerable damage to normal tissue, such as tissue inflammation and infection, since the normal immunological balance and homeostasis has been impaired due to the drug-induced lysis of the circulating lymphocytes and natural killer cells whereby these cell populations fall below normal protective range. This hazardous condition does not occur in our formulation, since the antitumour entities (or therapeutic agents) and their toxicity functions are always kept within biological bounds, such that the immunological cells (circulating leucocytes and NK cell levels) are always maintained within normal limits, thus protecting against infection and inflammation.

2.4.2 Robustness of cancer stem cell abolition with negative bias

As is well-known, a critical factor responsible for the failure of tumour containment is the presence of a small side-population of cancer stem cells, which are distinct from the usual cancer cells. Conventional antitumour intervention may act well on the usual cancer cells (the majority part of tumour tissue), the tumour may shrink and become clinically undetectable. However, the small population of cancer stem cells are far more resistant to antitumour drugs since they have drug ejecting efflux-channels, so that these cells survive the drug effect and proliferate continuously, and the tumour soon recurs and disseminates [71]. For instance, the sensitivity of these cancer stem cells to antitumour drugs are only 8–23% of the sensitivity of usual cancer cells to the drug [66].

On the other hand, our negative biasing procedure can successfully eliminate cancer stem cell based tumours, though the time taken is longer than eliminating usual cancer cells. This indicates that more DNA damage induction (i.e., DNA-damaging drug agent) would be needed for lysing the cancer stem cells which are more chemotherapy resistant. However, it may be noted, that there are no normal tissue toxicity issues in our cancer stem cell elimination process, as the physiological bounds are always being maintained. Furthermore, our procedure is robust enough (Figure 2.10), that is all the malignant stem cells are eliminated even if there is appreciable patient-specific variation in the tumour cell population.

2.4.3 Double pulsed lymphocyte activation for final tumour eradication

A critical element enabling complete tumour regression is the bimodal activation of antitumour lymphocytes. In conventional multimodal therapy (Figure 2.7) where often temporary but not permanent regression occurs, here the T-cell immunotherapy is administered usually at a week's time (day 7–8), and the second peak in T-cell population is absent. We can construe that the second T-cell peak is essential to completely eradicate the residual malignant cells in the late stage of the regression process. Just before the occurrence of the second peak, the tumour cell population is below 5% of the initial tumour population (Figure 2.8(a)), i.e., the vast majority of the tumour cells has already been eliminated by that time, nevertheless a second T-cell peak is needed to eliminate the residual tumour cells to prevent relapse. The lack of the second T-cell dosage peak might be a factor why in the well-known clinical trial of the T-cell therapy [72], one observes that the majority of patients (54%) showed no response, while the others showed only a partial response with tumour somewhat shrinking yet remaining extant, a portent of future relapse.

Our cytotoxic T-cells (CTL) immuno-modulation used is of tactical utility, as these cells can exhibit [73] a range of unique behaviors, that chemotherapeutic drugs cannot, such as:

(a) the T-cells can migrate to the primary and secondary growths of the tumour, even in hidden tissue depths,

(b) CTLs can continue to automatically multiply in response to immunogenic proteins of malignant cells, until all those tumour cells become extinct,

(c) T-cells enable immune memory to be stored, allowing further elimination of the tumour, if there is recurrence.

Though our proposal of doublet pulse therapy has not been earlier used in oncology, a doublet pulse approach to cytotoxic therapy has been satisfactorily used in other cell proliferation disorders, as Wegener's granulomatosis [74]. Hence, our proposed double pulse lymphocyte activation procedure may hold appreciable potentiality in the clinical oncology scenario.

2.4.4 Therapeutic implications of the natural process of spontaneous regression of tumour

Utilizing a systems analysis methodology, our investigation endeavors to elucidate a general unitary basis of tumour regression, which can be applied to both processes: (a) endogenous or spontaneous regression (b) exogenous or therapy-induced regression. Our investigation has formulated that the basic dynamics of both regression processes are comparable and equivalent, and consists of three aspects: (i) DNA interference in malignant cell, (ii) cytokine-based activation of tumour tissue environment (IL-2), and (iii) actuation of antitumour white blood cells (lymphocytes). The paradoxical phenomenon of spontaneous tumour regression has intrigued physicians since the time of St. Peregrines,

the patron saint of cancer, these inquirers pondered how the process could be therapeutically replicated on patients, though now the process is known to be much ubiquitous, as the Wisconsin [5] and Scandinavian [4] cancer population registries show.

To analyze the spontaneous cancer regression phenomenon more precisely, we have quantified the regression behaviour in terms of numerically-based DNA interference sites across the tissue (section 2.2.6.1). This same DNA interference formulation was shown to apply to drug-induced tumour regression, delineating the unitariness of the two processes. To paraphrase, our investigation of the spontaneous tumour regression process can help design newer modalities of therapeutic regression or treatment. Actually, our approach using three antitumour agents does notably satisfy the requirements needed for mimicking the occurrence of spontaneous tumour regression, as analyzed from the immunoediting perspective. From this immunoediting analysis, the requirements for spontaneous regression are (i) lymphocyte activation (ii) cytokine/interleukin-based activation of antitumour cells (ii) tumour DNA interference using microbial metabolites [75].

2.5 Conclusions

In retrospect, our approach, shows that a robust quantitative systems biology formulation can be developed to obtain incisive mechanistic insights into the process of complete permanent tumour regression, which often occurs naturally in the form of endogenous spontaneous regression as noted in cancer registries of general populations. Alternatively, the tumour regression process can be replicated therapeutically by antitumour agents, as chemotherapy and immunotherapy. The salient feature of our formulation is that the regression is enabled by very specific but universal characteristics of the antitumour entities, namely single peak level of DNA damaging factor, double

pulse level of white blood cell activation (T-lymphocyte), and uniform activation level of the immunomodulator cytokine (IL-2). Indeed, the second pulse feature of lymphocyte activation is an unexpected finding and accounts for the complete extinction of all the residual tumour cells which is in the order of 1% of the initial tumour load. The absence of this second pulse of lymphocytes in customary multimodal therapies may be a factor that prevents these therapies to induce lasting tumour eradication, and one often observes tumour relapse in these cases. Our proposed formulation does not have high-intensity levels of any of the therapeutic agents for a prolonged time, their levels can become much less at intervening times, and there is no appreciable drug-induced toxicity as the immune system (circulating lymphocytes and natural killer cells) are kept always protected. Thus, it can be suggested that combinational multi-pulsed multimodal therapy, using systems biology based analysis, can offer a principled approach to permanent tumour elimination, with germane implications for the clinical oncology scenario.